Factors affecting chromosome copy number and nuclear organisation in human sperm and embryos

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K Gothami Lakshika Fonseka

Department of Biosciences

Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of learning.

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Research activities including publications arising from this thesis

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D. Ioannou, **K.G.L. Fonseka,** E.J. Meershoek, A.R. Thornhill, A. Abogrein, M. Ellis, D.K. Griffin. Twenty four chromosome FISH in human IVF embryos reveals patterns of post-zygotic chromosome segregation and nuclear organisation *Chromosome Res* 20(4):447-60.

Published abstracts

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K.G.L. Fonseka, Griffin DK, Thornhill AR. FISH and PGS – the Bridge Experience of 5 years of treatment

KGL Fonseka, D.Ioannou, EJ. Meershoek, AR Thornhill,. M Ellis, DK. Griffin. Twenty four chromosome FISH in human IVF embryos revisited. Chromosome copy number counts differ with differing fixation methods

KGL Fonseka, D.Ioannou, AR Thornhill. M Ellis, DK. Griffin. Nuclear organisation of human IVF embryos suggests specific centromeres occupy different addresses in relation to specific parameters

Oral presentations

K.G.L. Fonseka, Tempest H, Ioannou D, Ellis, M, Handyside A, Thornhill A, Griffin DK. The organisation of the genome in sperm heads of men undergoing chemotherapy for testicular cancer and Hodgkin's lymphoma. Human Reproduction. **(ESHRE-2010)**

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K.G.L.Fonseka Nuclear organisation in sperm : related to cancer and chemotherapy. **('New treatments for Cancer; hope or hoax' school symposium, The Royal Society of Chemistry Kent Local Section 2011)**

Poster presentations

Dimitris Ioannou, **K. Gothami L. Fonseka**, Collins Omugar, Eric J Meershoek, Dimitra Christopikou Michael Ellis, Alan R Thornhill, Darren K Griffin. Nuclear reorganisation of sperm heads remains remarkably constant despite severely compromised spermatogenesis upstream **(International Chromosome Conference Manchester 2011)**

K. Gothami L. Fonseka , Collins Omugar, Dimitris Ioannou, Alan Thornhill, Helen Tempest, Darren K Griffin The organisation of the nucleus in sperm heads of men undergoing chemotherapy for testicular cancer and Hodgkin's Lymphoma **(ICC Manchester 2011)**

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Abbreviations

ABL	Abelson murine leukemia
aCGH	Array comparative genomic hybridisation
AMA	Advance maternal age
AR	Androgen receptor
ART	Assisted reproduction technology
ASRM	American society for reproductive medicine
AZF	Azoospermia Factor
BAC	Bacterial artificial chromosome
BCR	Breakpoint cluster region
BSA	Bovine serum albumin
CGH	Comparative genetic hybridisation
CENP- A	Centromeric protein A
CML	Chronic myeloid leukemia
CNV	Copy number variation
CT	Chromosome territories
CVS	Chorionic villus sampling
DAPI	4',6-diamidino-2-phenylindole
HD	Hodgkin's disease (Hodgking's lymphoma)
ddH_2O	Double distilled water
df	Degrees of freedom
DTT	Dithiothreitol
EBV	Epstein barr virus
EDTA	Ethylene diamine tetraacetic acid
ESHRE	European Society of Human Reproduction
EtOH	Ethanol
FA	Formamide
FSH	Follicle stimulating hormone
FISH	Fluorescent in situ hybridisation
FITC	Fluorescein isothiocyanate
HLA	Human leukocyte antigen
HSA	Homo sapiens
IC	Interchromatin compartment
ICD	Inter chromosomal domain
ICM	Inner cell mass
ICN	Inter chromatin network
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilisation
KS	Klinefelter syndrome
LH	Luteinizing hormone

LMNA	Lamin A
m-CGH	Metaphase comparative genomic hybridisation
MI	Meiosis I
MII	Meiosis II
N	Number
NDRDP	Not discernable from random distribution pattern
NOR	Nucleolus organiser region
OAT	Oligoasthenoteratozoospermia
PB	Polar body
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFA	*Paraformaldehyde*
PGD	Preimplantation genetic diagnosis
PGS	Preimplantation genetic screening
RCT	Randomised control trial
RIF	Repeated implantation failure
RM	Repeated miscarriage
RS	Reed sternberg
RT	Room temperature
SGD	Single gene disorder
SNP	Single nucleotide polymorphism
SRY	Sex determining Region Y
SSC	Saline sodium citrate
STR	Short tandem repeats
TBE	Tris borate EDTA
TC	Testicular cancer
TE	Trophectoderm
UV	Ultraviolet
WGA	Whole genome amplification
WHO	World health organisation
X-EDMD dystrophy	X-linked Emery-Dreifuss muscular
ZP	Zona pellucida

Abstract

Chromosome copy number aberrations are a leading cause of birth defects, stillbirths, pregnancy loss and infertility. Every human male has a proportion of chromosomally abnormal sperm however conditions such as infertility, cancer, cancer treatments, and environmental factors can increase this. Chromosome abnormality is commonplace in human embryos and one reason for the development of the controversial preimplantation genetic screening (PGS). Factors such as embryo quality and maternal age are common correlates. Appropriate nucleus positioning of chromosome territories is also though to be indicative of a "healthy" nucleus with aberrations in such nuclear organisation associated with disease. The purpose of this study was to provide insight into the relationship between chromosome copy number, nuclear organisation and various aetiological factors in human sperm and early stage embryos. Specifically.

- To investigate the nuclear positioning of telomeric and sub telomeric region in sperm cells and test the hypothesis that such organisation is altered in infertile males.
- To investigate the nuclear positioning of centromeric and locus specific regions of 5 chromosomes in sperm cells from males undergoing chemotherapeutic treatment for testicular cancer and Hodgkin's lymphoma and test the hypothesis that either the cancer, or its treatment significantly alters patterns of nuclear organisation.
- To analyse FISH based PGS and "follow up" in 250 treatment cycles to investigate levels of aneuploidy, false negative and positive results, also well as effects of different indications such as maternal age.

- To investigate the levels of aneuploidy for all 24 chromosomes using a newly developed multicolour FISH technique. To test hypotheses that factors e.g. maternal age and embryo morphology significantly effect levels, and that day 3 and day 5 results are concordant.
- To assess levels nuclear organisation of human embryos for loci on all 24 chromosomes and their relationship to maternal age, day 3 and day 5 embryo morphology.

Overall, results provide some evidence for differences in nuclear organisation in infertile males compared to controls for telomeric but not sub-telomeric loci. Effects of cancer (testicular cancer and Hodgkin's lymphoma) and chemotherapy were subtle at best with one testicular cancer patient showing a significant difference compared to controls. In embryos, monosomy appeared more common that trisomy and effects of maternal age and embryo quality were apparent when a small subset of chromosomes were analysed. Similar analysis with a 24 FISH assay confirmed monosomy/trisomy ratios however failed to show significant relationship with maternal age and embryo morphology, thereby raising questions about the reliability of the technique. Finally comparison of various parameters and nuclear organisation revealed consistent alterations of the position of specific centromeres (e.g. for chromosomes 3 and 4). In conclusion, FISH is now clearly old technology for PGS but has great potential for the analysis of mosaicism and nuclear organisation.

1. Introduction

1.1. Human reproduction

Sexual reproduction allows random mixing of parental genomes to produce offspring that are genetically similar to their parents. It involves the formation of haploid gametes, fusion of gametes during fertilisation to generate diploid embryos followed by cell growth, division and development to become a living individual. Gametes are produced via meiotic cell division of diploid cells in the testes and ovaries resulting in haploid daughter cells i.e. sperm or oocytes. Fertilisation is the union of a haploid sperm and oocyte to generate a new living individual and begins when sperm comes into contact with the egg and ends with the fusion of the two haploid pronuclei in the egg cytoplasm (Mengerink and Vacquier, 2001). Embryonic cell growth, division via mitosis and development involve a series of biochemical processes.

1.1.1. Mitosis

Mitosis is the process of cellular division which produces two daughter cells that are genetically identical to the parent cell. It is the most common form of cell division and occurs during cell growth and repair.

In mitosis, during the S-phase, chromosomes are copied to create identical sister chromatids. During the prophase of mitosis, chromosomes start to condense. This is followed by metaphase where chromosomes align at the equator of a cell and are held

by microtubules attached to centromeres. Anaphase facilitates centromere division and condensing of sister chromatids as well astheirseparation and transfer toward opposite poles. This leads to telophase where daughter chromosomes arrive at the poles followed by cytokenesis (Haber, 1998a, 1998b).

1.1.2. Meiosis

Meiosis is the process of cell division that produces haploid gamete cells containing half the genetic complement from diploid mother cells (Thomas and Hassold, 2003). Meiosis is a key step in the process of sexual reproduction and plays an important role in mixing maternal and paternal genetic information by facilitating various mechanisms such as random segregation of parental chromosomes, programmed DNA recombination and chromosomal crossover producing germ cells with higher genetic viability and higher genetic variation (Kleckner, 1996; Terasawa *et al.*, 2007).

During meiosis the diploid genome of the mother cell replicates and undergoes two rounds of division resulting in four haploid cells. The first meiotic division involves the migration of homologous chromosomes to opposite poles of the cell producing two haploid nuclei. This is followed by the second meiotic division which is similar to mitosis; sister chromatids migrate to opposite poles resulting in four daughter cells with haploid genomes differing from the maternal cells as well as the other individual daughter cells (Chen *et al.*, 2008; Egozcue *et al.*, 2000; Jones, 2008; Kleckner, 1996; Lynn *et al.*, 2004).

Human meiosis prophase 1 involves paring of homologous chromosomes facilitating the occurrence of crossing over between homologous chromosomes. During metaphase 1, homologous pairs become aligned in the centre of the cell. In anaphase 1 homologous chromosomes separate and at the end of the 1st meiosis, two cells with half of the genome are produced. This is followed by meiosis 2 division which is identical to mitotic cell division.

Any disturbance in meiosis can result in gametes with chromosomal abnormalities that may continue to the next generation. The meiosis error rate in humans is significantly high and causes abnormalities such as monosomies and trisomies in embryos (Hassold and Chiu, 1985; Hassold and Jacobs, 1984).

1.1.2.1. Gametogenesis

1.1.2.1.1. Spermatogenesis

Sperm cells are generated through spermatogenesis in which male spermatogonia proliferate to develop into mature sperm cells. It is characterised by well defined mitotic and meiotic divisions (Ehmcke *et al.*, 2006) followed by morphological differentiations of spermatozoa. Spermatogenesis can be divided in to three phases namely proliferative, meiotic and spermiogenic. During the proliferative phase, diploid spermatogonia undergo mitotic division to form spermatogonia and primary spermatocytes. Each primary spermatocyte undergoes meiotic division to produce four haploid secondary spermatids (Sun *et al.*, 2008). They are then differentiated to from elongated spermatids and finally formmature spermatozoa. Differentiation

involves acquiring the capacity for motility during the spermatogenetic process (Chakrabarti *et al.*, 2007).

1.1.2.1.2. Oogenesis

Oogenesis is the proliferation of oogonium to produce primary and secondary oocytes (Hunt and Hassold, 2008). Female meiosis initiates prenatally; primary oocytes reach their maximum level (about 7 million) after 20 weeks of gestation. The next step is the meiotic division of primary oocytes to form ootids. This process begins prenatally and arrests at prophase I until ovulation. In this way, post-meiosis I oocytes are arrested at least until puberty and most up to menopause. After puberty, mature oocytes continue to develop in each menstrual cycle initiate meiosis II and arrest in metaphase II which is believed to be associated with female factor infertility and aneuploidy (Hawley, 2003; Lamb *et al.*, 1996; Orr-Weaver, 1996; Warren and Gorringe, 2006). Meiosis is completed after fertilisation, creating an ootid and one polar body (Daphnis *et al.*, 2005; Delhanty, 2005; Harper *et al.*, 2004; Warren and Gorringe, 2006).

1.1.2.2. Fertilisation

Fertilisation is defined as the union of two gametes; eggs and sperm (Wassarman *et al.*, 2001). Fertilisation starts when a sperm comes into contact with an oocyte resulting in the fusion of the two haploid pronuclei in the oocyte cytoplasm (Mengerink and Vacquier, 2001). This involves firstly, the interaction and attachment of a sperm with an intact acrosome to the zona pellucida surrounding the egg. The

sperm must then undergo the acrosome reaction which allows it to penetrate the extracellular coat and bind to the plasma membrane. This is followed by fusion of the sperm with the plasma membrane of the oocyte which prevents other free swimming sperm fusing with the oocyte (Mengerink and Vacquier, 2001).

1.1.2.3. Embryogenesis

The formation and development process of the embryo is defined as embryogenesis. It starts with fertilisation which triggers the completion of meiosis II of the oocyte (Wassarman *et al.*, 2001). This is followed by the formation of a zygote which undergoes rapid mitotic division to produce identical replicas of the original zygote. Mitotic division results in the cleavage of the zygote into 2 cells called blastomeres followed by the 4 and 8 cell stages within three days. By the fourth day the embryo is developed into a morula with approximately 32 cells. Following the formation of the morula, the embryo starts to differentiate into the blastocyst with a trophectoderm and inner cell mass (Ambartsumyan and Clark, 2008).

1.2. Human chromosomes and aneuploidy

1.2.1. Chromosomes

Chromosomes are structures found in eukaryotic cells and are formed of tightly coiled DNA and histone proteins. Human cells have 23 pairs of chromosomes; 22 pairs of autosomes and one pair of sex chromosomes either XX in female or XY in male.

Human chromosomes consist of coding and non coding sequences (International human genome sequencing consortium: 2004; Lander *et al.*, 2001; Levy *et al.*, 2007; Moreno *et al.*, 2011; Venter *et al.*, 2001). Coding sequences are DNA that is translated into proteinss responsible for a variety of functions such as gene regulation, RNA transcription, RNA splicing, and DNA methylation and represent about 2% of the whole genome (Lander *et al.*, 2001; Venter *et al.*, 2001). Non coding sequences make up around 98% of the genome that is characterised by many kinds of repetitive DNA sequences (International human genome sequencing consortium: 2004). Studies have reported genetic variation of non coding sequences in different human groups, and their impact on human diseases and life characterising properties (Altshuler D, 2010) . These variants include single nucleotide polymorphisms (SNPs), block substitutions, heterozygous or homozygous insertion or deletion events, inversions, numerous segmental duplications and copy number variation regions (Levy *et al.*, 2007).

Chromosome structures include centromeres and telomeres. Centromeres divide the chromosomes into two arms. The location of the centromere is used as a parameter to divide chromosomes into sub classes of metacentric, submetacentric, acrocentric and telocentric. Telomeres are at the end of chromosomes and contain repetitive DNA sequences.

1.2.1.1. Centromere region

The centromere is a chromosomal locus that contains of very large part of satellite DNA and is responsible for accurate chromosomal segregation in meiotisis and

mitotsis (Rocchi *et al.*, 2012). During cell division, a kinetochore is formed within the centromere region and therefore it is an important structure for spindle attachment. Centromere functions are associated with both genomic and epigenetic mechanisms (Stimpson and Sullivan, 2010). Human centromeres are characterised by the presence of a histone H3 variant known as centromeric protein A (CENP-A) located on AT-rich repeats and α satellite DNA (Gonzalez-Barrios *et al.*, 2012). This is believed to be important for the assembly of the kinetochore on the centromere.

It has been found that the formation of neocentromeres at new sites on the chromosome which is an unusual epigenetic change in the human genome (Chan and Wong, 2011). This was first discovered in 1993 (Voullaire *et al.*, 1993) and since then, over 90 cases of human neocentromeres have been reported on 20 different chromosomes (Chan and Wong, 2011; Marshall *et al.*, 2008; Warburton, 2004) and detected in other model organisms (Ishii *et al.*, 2008). In nanocentromeres no accumulation of α-satellite DNA or rearrangements to the linear gene order at the neocentromere domain has been detected (du Sart *et al.*, 1997), suggesting that development of neocentromeres is an exclusively epigenetic event.

1.2.1.2. Telomere regions

A telomere is a region of repetitive DNA with a TTAGGG sequence at the end of the chromosome and plays a vital role in chromosome pairing during meiosis, genome stability and nuclear architecture. Telomeres shorten when cells divide and this is repaired by the enzyme telomerase which is absent in most somatic cells resulting in age related telomere shortening.

It has been reported that in vitro telomere length has a role in preventing uncontrolled cell growth as shortened telomeres are the main source of genome instability in cells that have lost proliferative control and acquire tumour phenotypes (Londono-Vallejo, 2008). Jones and colleagues reported that telomere shortening causes fusions and dysfunction of the genome causing instability and large scale chromosomal rearrangements in haematological cancers, therefore suggesting that telomeres could be both clinically useful as a prognostic tool and as a potential target for drug intervention (Jones *et al.*, 2012). A study by Lin and colleagues reported that telomere dysfunction and fusion is associated with the progression of chronic lymphocytic leukemia (Lin *et al.*, 2010). Similarly, shortened telomere lengths in the oral epithelia are associated with carcinoma has been reported (Aida *et al.*, 2010).

Telomere deficiencies are also known to be associated with fertility related issues. In a recent study Treff and colleagues tested the hypothesis that telomere DNA deficiency plays a role in the development of aneuploidy in human polar bodies and embryos. They discovered that aneuploid human polar bodies have significantly less telomeric DNA than normal polar bodies, suggesting that oocytes with telomeric DNA deficiency are prone to aneuploidy development during meiosis. In addition, aneuploid embryos in the cleavage stage also had significantly less telomeric DNA than normal embryonic cells (Treff *et al.*, 2011b). Another study reported that both telomere shortening and reduced telomerase activity is associated with women with ovarian insufficiency compared to controls (Butts *et al.*, 2009). Abnormal telomere shortening has also been associated with male factor infertility (Zalenskaya and Zalensky, 2002). A recent study reported that the swim up procedure selects

spermatozoa with longer telomere length (Santiso *et al.*, 2010) and disruption of telomere-telomere interactions are related to DNA damage in human sperm cells (Moskovtsev *et al.*, 2010) suggesting that the telomere plays an important role in male fertility.

1.2.2. Aneuploidy

Aneuploidy is defined as having an abnormal number of chromosomes in a given nucleus. It is the most common chromosome abnormality in humans and is the leading cause of pregnancy loss resulting in low fecundity. Aneuploidy can also lead to various congenital birth defects such as mental retardation, abnormal birth weight and imprinting syndromes like Down syndrome (Hassold *et al.*, 2007). Aneuploidies can be derived from sperm, eggs or the early embryo during the period of gemetogenesis or embryogenesis due to errors in both mitotic and meiotic divisions.

1.2.2.1. Sperm aneuploidy

Most human males have a certain level of sperm aneuploidy. Traditionally, sperm aneuploidy was studied by using a human sperm-hamster oocyte fusion method which allows karyotyping of condensed sperm chromosomes (Martin *et al.*, 1991). Using this method aneuploidy levels seen for human sperm from healthy controls were 1-2% (Hassold *et al.*, 1996). With the arrival of FISH, using chromosome specific probes, a number of other groups found out the sperm aneuploidy levels in human sperm (Bischoff *et al.*, 1994; Griffin *et al.*, 1995; Spriggs *et al.*, 1995; Williams *et al.*, 1993;

Wyrobek, 1993). Results from FISH studies suggest that, for individual autosomes, the likelihood of aneuploidy is about 0.1%, suggesting a total frequency of autosomal disomy of approximately 2% (Hassold *et al.*, 1996). Martin 2006 reports sex chromosome disomy was 0.43% suggesting that sex chromosomes may be more susceptible to nondisjunction in male meiosis (Martin, 2006).

It has also been reported that absent or reduced levels of meiotic recombination or sub optimally positioned recombination events have been associated with chromosomal non disjunctions causing sperm disomy (Sun *et al.*, 2008). For example, studies by Martin(Martin, 2005 , 2006 , 2008a) suggest that chromosomes 21 and 22 could be more susceptible to aneuploidy due to their small size resulting in reduced recombination. Also the same studies suggest that sex chromosomes are also more likely to be involved in aneuploidy due to the restricted recombination in the pseudoautosomal region (Martin, 2005 , 2006,, 2008a). In addition, two other studies also report that reduced XY recombination was seen in paternally derived XXY patients (Hassold *et al.*, 1991; Lorda-Sanchez *et al.*, 1992).

1.2.2.2. Aneuploidy and fertility

Several studies have established that increased chromosomal aneuploidy is related to male infertility (Harton and Tempest, 2012; Tempest *et al.*, 2004). Moosani and colleagues were the first to report the association between increased levels of sperm chromosomal abnormalities and impaired fertility (Moosani *et al.*, 1995). A study from Tempest and collegues reported that increased chromosomal aneuploidy relates to male infertility with certain men having a 10-30 fold increase in levels of sperm

disomy (Tempest *et al.*, 2004). Another study regarding aneuploidy in males with different types of infertility (e.g. oligo, astheno, terato, zoosperia) also reported that infertile groups have an increased frequency of chromosome abnormalities varying from 2 to 10 times higher compared to control males (Martin, 2005). For example, a study from Mehdi and collegues have reported that severe teratozoospermia patients have an increased sperm aneuploidy rate (Mehdi *et al.*, 2011).

1.2.2.3. Aneuploidy and its relation to cancer and chemotherapy

To date, several studies have reported the high incidence of sex chromosome and autosomal aneuploidy associated with sperm in cancer patients compared with healthy donors (Fait *et al.*, 2001; Frias *et al.*, 2003; Martin *et al.*, 1997; Martinez-Pasarell *et al.*, 1999; McInnes *et al.*, 1998; Paulasova *et al.*, 2011; Robbins *et al.*, 1997a). For example, Tempest and colleagues reported a high incidence of chromosomal aneuploidy associated with testicular cancer and Hodgkin's lymphoma. A more recent study on Li-Fraumeni syndrome (autosomal dominant cancer predisposition syndrome) reported an increase in sperm aneuploidy compared to normal male controls. The authors postulated the involvement of the mutated p53 gene in spermatogenesis, due to its role in aneuploidy in cancer (Paulasova *et al.*, 2011). The effect of various mutagenic and non mutagenic drugs and therapeutic regimes on sperm cells during (Frias *et al.*, 2003; Martinez-Pasarell *et al.*, 1999; Robbins *et al.*, 1997a) and after treatment has also been well documented (De Mas *et al.*, 2001b; Frias *et al.*, 2003; Martin *et al.*, 1995a; Martin *et al.*, 1997; Martinez-Pasarell *et al.*, 1999; McInnes *et al.*, 1998; Robbins *et al.*, 1997a; Thomas *et al.*, 2004). For example, studies by De Mas and colleagues have reported that in sperm, there is a significant

increase in the frequency of diploidy and disomy for both autosomal and sex chromosomes, 6 and 18 months after chemotherapy (De Mas *et al.*, 2001a). However, similar studies by Martin and colleagues (1995, 1997) suggested that the frequency of chromosomal abnormalities was not significantly increased compared to control donors three years after the chemotherapy treatments (Martin *et al.*, 1995a; Martin *et al.*, 1997), suggesting that perhaps a chemotherapeutic regimen does not have long term effects on stem cells. More recently, Tempest and colleagues (2008) have assessed sperm aneuploidy for chromosomes 13, 21, X and Y in TC and HD patients before and 6, 12 and/or 18-24 months after chemotherapy compared to control samples of the same age. Results suggested that sperm aneuploidy increased six months after initiation of treatments, followed by a decline in aneuploidy frequency up to pre-treatment level approximately 18 months after treatment (Tempest *et al.*, 2008).

1.2.2.4. Aneuploidy and paternal age

Numerous epidemiological studies and sperm studies have been implemented to investigate the effect of paternal age on sperm aneuploidy. Some of these studies observed effects while others did not. Therefore the effect of paternal age on sperm aneuploidy still remains controversial. Tables 1.1, 1.2 and 1.3 below demonstrate the outcome of various studies investigating the paternal age effect on sperm aneuploidy.

Table 1.1: Paternal age effect on autosomes

Chromosome number	Sample size	Age range	Sperm scored / donor /chromosome	Total sperm scored	Result	Reference
1	10	21-52	10000	115000	↑ p 0.01	(Martin *et al.*, 1995b)
	24	20-49	10000	240000	NS	(Lahdetie *et al.*, 1996)
	6	23-57	2000	12000	NS	(Guttenbach *et al.*, 1997)
	18	23-58	10000	180000	NS	(McInnes *et al.*, 1998)
	3	>80	1500	6940	NS	(Guttenbach *et al.*, 2000)
3	7	23-57	1500	12000	NS	(Guttenbach *et al.*, 1994)
6	12	22-55	10000	120000	NS	(Martinez-Pasarell *et al.*, 1999)
	18	24-74	10000	194024	NS	(Bosch *et al.*, 2000)
7	7	23-57	1500	12000	NS	(Guttenbach *et al.*, 1994)
	24	20-49	10000	240000	NS	(Lahdetie *et al.*, 1996)
	6	23-57	2000	12000	NS	(Guttenbach *et al.*, 1997)
8	14	22-59	10000	205218	NS	(Robbins *et al.*, 1995)
9	18	24-74	10000	190117	↑p<0.0001	(Bosch *et al.*, 2003)
	23	>60, <30	8000	335665	NS	(Luetjens *et al.*, 2002)
10	7	23-57	1500	12000	NS	(Guttenbach *et al.*, 1994)
	6	23-57	2000	12000	NS	(Guttenbach *et al.*, 1997)
11	7	23-57	1500	12000	NS	(Guttenbach *et al.*, 1994)
12	10	21-52	10000	115000	NS	(Martin *et al.*, 1995b)
	25	>39, <25	2000	50000	NS	(Asada *et al.*, 2000)
13	18	23-58	10000	180000	NS	(McInnes *et al.*, 1998)
	10	22-37	10000	100281	NS	(Shi and Martin, 2000)
14	11	>60, <30	10000	110000	NS	(Rousseaux *et al.*, 1998)
17	7	23-57	1500	12000	NS	(Guttenbach *et al.*, 1994)
	6	23-57	2000	12000	NS	(Guttenbach *et al.*, 1997)
	3	>80	1500	6940	NS	(Guttenbach *et al.*, 2000)
18	45	19-35	10000	450000	↓p 0.009	(Robbins *et al.*, 1997b)
	13	18-35	12000	390096	NS	(Griffin *et al.*, 1995)
	3	>80	1500	5646	NS	(Guttenbach *et al.*, 2000)
	25	>39, <25	2000	50000	NS	(Asada *et al.*, 2000)
	23	>60, <30	8000	335665	NS	(Luetjens *et al.*, 2002)
21	11	>60, <30	10000	110000	↑ p 0.001	(Rousseaux *et al.*, 1998)
	18	23-58	10000	180000	NS	(McInnes *et al.*, 1998)
	10	22-37	10000	100281	NS	(Shi and Martin, 2000)
	18	24-74	10000	194024	NS	(Bosch *et al.*, 2000)
	38	24-57	10000	398681	NS	(Lowe *et al.*, 2001)
	36	24-57	10000	360000	NS	(Eskenazi *et al.*, 2002)

Table 1.1 shows the paternal age effect on different autosomal chromosomes by various studies. For each study, table shows the chromosome analysed, sample size, age range, probes used, number of patients and number of sperm cells scored for each patint and whether results are significant or not at p=0.05. Adapted from (Fonseka and Griffin, 2010)

Table 1.2: Paternal age effect on sex chromosomes

Aneuploidy	Sample size	Age range	Sperm scored / donor /chromosome	Total sperm scored	Result	Reference
XY	24	18-60	12000	390096	↑p 0.007	(Griffin *et al.*, 1995)
	25	<25, >39	50000	2000	↑p 0.01	(Asada *et al.*, 2000)
	38	24-57	10000	398681	↑p 0.006	(Lowe *et al.*, 2001)
	18	24-74	10000	194024	NS↑	(Bosch *et al.*, 2000)
	3	>80	1500	5646	NS ↑	(Guttenbach *et al.*, 2000)
	36	24-57	10000	360000	NS↑	(Eskenazi *et al.*, 2002)
	10	21-52	10000	115000	NS	(Martin *et al.*, 1995b)
	14	22-59	10000	205218	NS	(Robbins *et al.*, 1995)
	45	19-35	10000	450000	NS	(Robbins *et al.*, 1997b)
	6	23-57	2000	12000	NS	(Guttenbach *et al.*, 1997)
	12	22-55	10000	120000	NS	(Martinez-Pasarell *et al.*, 1999)
	10	22-37	10000	100281	NS	(Shi and Martin, 2000)
	23	>60, <30	8000	335665	NS	(Luetjens *et al.*, 2002)
XX	24	18-60	12000	390096	↑ p 0.02	(Griffin *et al.*, 1995)
	14	22-59	10000	205218	↑p 0.005	(Robbins *et al.*, 1995)
	45	19-35	10000	450000	↑p 0.002	(Robbins *et al.*, 1997b)
	18	24-74	10000	194024	↑NS	(Bosch *et al.*, 2000)
	7	23-57	1500	12000	NS	(Guttenbach *et al.*, 1994)
	10	21-52	10000	115000	NS	(Martin *et al.*, 1995b)
	6	23-57	2000	12000	NS	(Guttenbach *et al.*, 1997)
	12	22-55	10000	120000	NS	(Martinez-Pasarell *et al.*, 1999)
	25	<25, >39	50000	2000	NS	(Asada *et al.*, 2000)
	36	24-57	10000	360000	NS	(Eskenazi *et al.*, 2002)
	3	>80	1500	5646	NS	(Guttenbach *et al.*, 2000)
	10	22-37	10000	100281	NS	(Shi and Martin, 2000)
	38	24-57	10000	398681	NS	(Lowe *et al.*, 2001)
	23	>60, <30	8000	335665	NS	(Luetjens *et al.*, 2002)
YY	24	18-60	12000	390096	↑p 0.06	(Griffin *et al.*, 1995)
	14	22-59	10000	205218	↑p 0.0001	(Robbins *et al.*, 1995)
	10	21-52	10000	115000	↑p 0.04	(Martin *et al.*, 1995b)
	18	24-74	10000	194024	↑NS	(Bosch *et al.*, 2000)
	8	18-40	1000	8061	NS	(Guttenbach and Schmid, 1990)
	6	23-57	2000	12000	NS	(Guttenbach *et al.*, 1997)
	45	19-35	10000	450000	NS	(Robbins *et al.*, 1997b)
	12	22-55	10000	120000	NS	(Martinez-Pasarell *et al.*, 1999)
	25	<25, >39	50000	2000	NS	(Asada *et al.*, 2000)
	3	>80	1500	5646	NS	(Guttenbach *et al.*, 2000)
	10	22-37	10000	100281	NS	(Shi and Martin, 2000)
	38	24-57	10000	398681	NS	(Lowe *et al.*, 2001)
	36	24-57	10000	360000	NS	(Eskenazi *et al.*, 2002)
	23	>60, <30	8000	335665	NS	(Luetjens *et al.*, 2002)
	18	24-74	10000	190117	NS	(Bosch *et al.*, 2003)

Table 1.2 shows the paternal age effect on different sex chromosomes by various studies. For each study, table shows the chromosome analysed, sample size, age range, probes used, number of patients and number of sperm cells scored for each patint and whether results are significant or not at p=0.05. Adapted from (Fonseka and Griffin, 2010)

Table 1.3: Paternal age effect on sperm diploidy

Sample size	Age range	Sperm scored / doner /chromosome	Total sperm scored	Result	Reference
14	22-59	10000	205218	Inverse p 0.006↓	(Robbins *et al.*, 1995)
45	19-35	10000	450000	Inverse p 0.001↓	(Robbins *et al.*, 1997b)
11	>60, <30	10000	110000	Increase p 0.001↑	(Rousseaux *et al.*, 1998)
18	24-74	10000	194024	Increase p 0.002↑	(Bosch *et al.*, 2000)
24	18-60	12000	390096	NS	(Griffin *et al.*, 1995)
10	21-52	10000	115000	NS	(Martin *et al.*, 1995b)
24	20-49	10000	240000	NS	(Lahdetie *et al.*, 1996)
12	24-55	10000	120000	NS	(Martinez-Pasarell *et al.*, 1999)
25	<25, >39	50000	2000	NS	(Asada *et al.*, 2000)
3	>80	1500	12586	NS	(Guttenbach *et al.*, 2000)
10	22-37	10000	100281	NS	(Shi and Martin, 2000)
38	24-57	10000	398681	NS	(Lowe *et al.*, 2001)
23	>60, <30	8000	335665	NS	(Luetjens *et al.*, 2002)

Table 1.3 shows the paternal age effect on sperm diploidy by various studies. For each study, table shows the sample size, age range, probes used, number of patients and number of sperm cells scored for each patint and whether results are significant or not at p=0.05. Adapted from (Fonseka and Griffin, 2010)

1.2.2.5. Aneuploidy and life style

Various factors such as smoking, alcohol, mutagens and life style have implications in causing increased sperm aneuploidy (Brown *et al.*, 2008). Only a handful of studies have tried to establish the relationship between the disomy frequency related to lifestyle habits such as smoking and caffeine consumption which did not find any consistent association (Martin, 2003, 2006; Shi and Martin, 2000; Templado *et al.*, 2011). In a recent study, the effects of smoking on sperm chromosome number were analysed and an increase in sperm disomy among smokers compared to non-smokers was revealed (Robbins *et al.*, 2005).

1.2.2.6. Aneuploidy in oocytes

Aneuploidy in unfertilised oocytes ranges from 2 to 14.5% and is increased significantly with maternal age (Harper *et al.*, 2004; Hunt, 1998 ; Hunt and Hassold,

2008). Unlike spermatogenesis, oocyte production in human females is restricted to early fetal life. As a result, there are several stages in oogenesis which impose the risks of aneuploidy (Delhanty, 2005; Hassold and Hunt, 2009). Firstly, oocytes undergo many mitotic divisions before entering meiosis which is implicated in aneuploidy. Secondly, the fact that prophase I of meiosis is arrested until puberty, where one oocyte per month completes meiosis II (only if fertilisation occurs) also poses a risk. During meiosis II, the errors that may occur include chromosome non disjunction due to a failure of pairing, loss of one homologous chromosome due to it lagging behind at anaphase and premature separation of the constituent chromatids. Several recent studies have reported oocyte aneuploidy in humans and mice (Geraedts *et al.*, 2011; Yakut *et al.*, 2011; Zhou *et al.*, 2011). A study by Geraedts and colleagues reported that 72% of oocytes were aneuploid based on array CGH analysis. Authors also reported 76.3 % of the aneuploidies were in chromosome 13, 16, 18, 21 and 22 which could have been detected using five colour FISH (Geraedts *et al.*, 2011). Another large study with over 20,000 oocytes reported that 46.8% of oocytes were abnormal predominantly with extra chromatid errors. Abnormalities detected in oocytes included 40% complex abnormalities and more prominent aneuploidy in chromosomes 21 and 22 (Kuliev *et al.*, 2011). A recent study on mice has reported the effect of psychological stress on diminished oocyte developmental potential and increased levels of aneuploidy during meiosis I although the mechanism is still unknown (Zhou *et al.*, 2011).

1.2.2.7. Oocyte aneuploidy and advanced maternal age

It has been well established that aneuploidy of meiotic origin increases dramatically with a woman's age, and evidence suggests that most errors occur in meiosis I. There are several hypotheses regarding the mechanisms of maternal age related to aneuploidy including an increase in meiotic non disjunction, decline of folliculogenesis, recombination errors, defective spindle assembly checkpoints, and deterioration of sister chromatid cohesion with age resulting in reduced oocyte (Chiang *et al.*, 2012).

Advanced maternal age related to chromosomal aneuploidy was first published in 1933 by Panrose (Hassold *et al.*, 1996). Since then, various studies have confirmed that one third of pregnancies in women over the age of 40 are trisomies compared to 2% in women under 25 (Hunt, 2006). Most studies suggest that in trisomies, the extra chromosome is a result of maternal meiosis I (Hassold *et al.*, 2007; Hunt, 2006). For example, a study by Sherman and colleagues report that for trisomy 21, 95% of cases were due to errors in maternal MI (Sherman *et al.*, 2005; Sherman *et al.*, 2006; Sherman *et al.*, 2007). In contrast to that, a recent study by Hulten and colleagues proposed that trisomy 21 oocytes have a delay in their development in the pool of growing follicles and they could be ovulated later in life than normal oocytes. According to this hypothesis, age effects occur as a result of events taking place before oocytes enter meiosis at foetal stage (Hulten *et al.*, 2008). This is consistent with the study which investigated the in foetal oocytes which revealed that unusual ("vulnerable") crossover configurations give chromosome specific routes to non-disjunction due to events occurring in foetal oogenesis (Cheng *et al.*, 2009). In

addition, it has been reported that smaller size chromosomes are highly likely to be involved in aneuploidy as they form fewer chiasmata during meiosis I, and undergo reduced recombination which causes chromosomal non disjunction (Gutierrez-Mateo *et al.*, 2004).

1.2.2.8. Aneuploidy in embryos

Chromosome aneuploidy in early embryonic development has implications in pregnancy loss and congenital birth defects. It has been reported that between 50-70% of human cleavage stage embryos contain chromosomally abnormal cells (Delhanty *et al.*, 1997; Munne and Cohen, 1998) due to higher aneuploidy in human oocytes and sperm. The incidence of aneuploidy in humans is approximately 0.6% in newborns, 6% in stillbirths and 60% in spontaneous abortions (Martin, 2008b). Most abnormalities involving monosomies are lethal; however certain chromosomal abnormalities such as trisomies and sex chromosomal aneuploidies can survive.

Human embryo chromosomes were first analysed in the late 1980s with banding techniques available at the time, which was technically challenging due to insufficient sample size, the slow dividing nature of embryo cells and often contracted chromosomes limiting the accuracy of the test (Harper *et al.*, 2004). The arrival of the FISH technique generated new information about the genetics of human embryos and opened a new area of study.

FISH studies revealed that most human embryos are mosaic for chromosomes analysed (Daphnis *et al.*, 2005; Delhanty *et al.*, 1993; Delhanty *et al.*, 1997; Munne *et*

al., 1994; Munne and Cohen, 1998; Munne *et al.*, 1998). FISH also revealed that certain chromosomes are highly likely to be associated with aneuploidy in human embryos. These include chromosome 22, 16, 21 and 15 Munne et al. (2004). However, the least involved chromosomes include 14, X and Y. It has also been shown that higher rates of monosomy occur in embryos compared to trisomy. With regards to the mechanism leading to aneuploidy in human embryos, post zygotic chromosome loss was the most common mechanism (Munne *et al.*, 2004a; Munne *et al.*, 2004b) however it is possible that this could be as a result of a FISH probe hybridisation failure or overlapping signals. This was followed by chromosome gain, and the least common mitotic non disjunction which are related to maternal age (Daphnis *et al.*, 2005; Daphnis *et al.*, 2008; Delhanty and Handyside, 1995; Delhanty *et al.*, 1997). In a more recent study by Daphnis et al. (2008), embryos were investigated for the levels of chromosomal abnormalities at two different development stages i.e. at cleavage and blastocyst stages, it was found that a normal embryo on day 3 is more likely to develop as a blastomere with the correct chromosome complement on day 5. However, abnormal embryos on day 3 result in a poor outcome on day 5.

1.2.3. Mosaicism

Mosaicism can be defined as two or more cells with different chromosomal constitution existing in a single embryo. This could be cells with different abnormalities or normal and abnormal cells coexisting in the same embryo (Frumkin *et al.*, 2008; Lebedev, 2011).

The incidence of mosaicism was well known from prenatal diagnosis where mosaicism was seen in about 2% of chorionic villi samples (CVS) (Grati *et al.*, 2006). It is a major issue which makes clinical results difficult to interpret (Allan *et al.*, 2004; Delhanty and Handyside, 1995; Munne *et al.*, 1994; Munne *et al.*, 1995b)

The main cause for mosaicism is believed to be mitotic errors. Mosaicism can be seen in both placental and fetal tissues if post zygotic mitotic errors occur before the differentiation of the trophoblast and inner cell mass. However if errors occur after the differentiation, mosaicism can only be seen in either the foetus or the placenta (Grati *et al.*, 2006; Simoni and Fraccaro, 1992).

Interphase cytogenetic studies on human embryos have shown that chromosomal mosaicism is a common incidence in early development having been observed in all stages of early human development (Fragouli *et al.*, 2011; Fragouli and Wells, 2011; Harper *et al.*, 1995; Harper *et al.*, 2004; Johnson *et al.*, 2010; Lebedev, 2011; Mantzouratou and Delhanty, 2011; Santos *et al.*, 2010; Scott *et al.*, 2010; Wapner, 2010). It has also been found that mosaicism is largely independent of maternal age (Delhanty *et al.*, 1997; Wells and Delhanty, 2000).

Over last two decades various studies looked at the levels of mosaicism in human embryos and the rate of mosaicism observed varied from 15% (Harper *et al.*, 1995) to more than 90% (Daphnis *et al.*, 2005). Mosaicism in preimplantation embryos were initially identified for sex chromosome (Delhanty *et al.*, 1993). Several other studies at a similar time also investigated mosaicism in preimplantation embryos in autosomal chromosomes (Daphnis *et al.*, 2005; Delhanty *et al.*, 1997; Harper *et al.*,

1995; Munne *et al.*, 1993a). Using more advanced techniques such as comparative genomic hybridisation some other studies also demonstrated the presence of high level of mosaicism in good quality human embryos (Voullaire *et al.*, 2000; Wells and Delhanty, 2000). These studies also reported that approximately quarter of embryos are chromosomally normal and these embryos have higher chances to implant. A study by (Daphnis *et al.*, 2008) also looked at chromosome mosaicism in day 3 and day 5 and reported that embryos diagnosed as abnormal in day 3 have a higher chance to be mosaic or completely chaotic. However embryos diagnosed as normal in day 3 could be normal or mosaic. A more recent study by van Echten-Arends and colleagues performed a meta analysis of studies on the chromosomal constitution of human preimplantation embryos (van Echten-Arends *et al.*, 2011). Here the authors analyse 36 different studies and total of 815 embryos and reported that the presence of diploids occurred in 177 (22%) of cases, mosaic in 599 (73%) and 39 (5%) of these contained other chromosomal abnormalities. From mosaic embryos, 480 (59% of the total) were diploid aneuploid mosaicism and 119 (14% of the total) were aneuploidy mosaicism.

It has been suggested that in mosaic embryos at least 50% or more cells have to be normal for an embryo to achieve implantation (Baart *et al.*, 2006; Daphnis *et al.*, 2005; Kuliev and Verlinsky, 2004). On the other hand, a mosaic embryo can develop into a morphologically normal blastocyst or in some cases to a live birth if at least two thirds of the cells are normal and overpower the minority of abnormal cells in the embryo (Frumkin *et al.*, 2008; Li *et al.*, 2005).

1.3.Nuclear architecture

The nucleus is an organelle which contains DNA, a variety of proteins such as histones and RNA in the interior. Structures within the nucleus include chromosomes, chromosome binding proteins, the nucleolus, nuclear lamina, nuclear envelope and pores and other subnuclear bodies. Therefore, the term "nuclear organisation" widely describes the organisation of the cell nucleus in terms of a number of different levels but for the purpose of this thesis we define it as spatial and temporal location of chromosomes in the interphase nucleus.

Chromosomes in interphase nuclei are highly organised. In humans, approximately two metres of DNA and DNA binding proteins are confined within the 10 μm nucleus (Ridgway *et al.*, 2002). Within the nuclei, chromosomes are compartmentalised into their own regions known as chromosome territories (Cremer and Cremer, 2001; Parada and Misteli, 2002). In between these chromosome territories, there are regions called inter chromatin compartments that contain macromolecular complexes needed for chromosome replication, transcription, gene splicing and repair (Cremer and Cremer, 2001). Nuclear architecture is therefore based on these chromosome territories and inter-chromosome compartments. The location of a chromosome in this architecture is related to how easily accessible it is to various nuclear machinery and therefore is believed to play a vital role in the regulation of gene expression, DNA replication, damage, and repair, controlling all cellular functions and development (Cremer and Cremer, 2001; Dundr and Misteli, 2011; Foster and Bridger, 2005; Fraser and Bickmore, 2007; Lanctot *et al.*, 2007; Miguel and Pombox, 2006;

Pederson, 2004, 2011; Rajapakse and Groudine, 2011; Rouquette *et al.*, 2010; Schoenfelder *et al.*, 2010; Spector and Lamond, 2011).

The position of a chromosome in the interphase nuclei is non random (Cremer and Cremer, 2001; Foster *et al.*, 2005; Foster and Bridger, 2005; Khalil *et al.*, 2007; Manuelidis, 1985, , 1990; Meaburn *et al.*, 2005; Meaburn and Misteli, 2007; Oliver and Misteli, 2005; Parada and Misteli, 2002; Tanabe *et al.*, 2001). Several studies have reported a non random chromosome organisation associated with various cell lines including fibroblasts, lymphocytes (Croft *et al.*, 1999), sperm (Finch *et al.*, 2008b; Foster *et al.*, 2005), and embryos (Finch *et al.*, 2008a; McKenzie *et al.*, 2004). In many cell types, the positions of chromosomes 18 and 19 are conserved (Tanabe *et al.*, 2002). On the other hand, localisation of some chromosomes such as chromosomes 6, 8, 21 appear to be different depending on the tissue type (Parada *et al.*, 2004). Two models that have been established to describe the chromosome localisation in the interphase nuclei depending on chromosome size and gene density are known as size related and gene density models.

1.3.1. Size model

This model explains size related positioning of chromosomes with large chromosomes being located at the periphery and small chromosomes towards the interior of the nucleus (Sun *et al.*, 2000). This was postulated due to the observation of altered spatial positioning of small chromosomes (13, 18) in quiescent and senescent cells

towards the interior, while large chromosomes (4, X) remain unchanged at the nuclear periphery (Foster and Bridger, 2005).

Sun and colleagues (2000) revealed size dependent chromosome positioning can be observed in chromosomes 4, 5, 7, 8, 14, 17, 19, 20 and X in human lymphoblast and fibroblast cells. In this study it was observed that q arms of large chromosomes such as chromosomes 1 and 2 were positioned towards the nuclear periphery while q arms of smaller chromosomes such as chromosome 19 and 21were located at the interior (Sun *et al.*, 2000).

A study by Bolzer et al. (2005) further supported the size model of chromosome positioning (Bolzer *et al.*, 2005). Bolzer and colleagues tested the chromosome position for all chromosomes in human fibroblasts and amniotic fluid cell nuclei and found a highly non random correlation with chromosome size. Small chromosomes were distributed significantly closer to the centre independent of their gene density, however large chromosome were located towards the periphery (Bolzer *et al.*, 2005).

In addition, nuclear positioning studies of chicken chromosomes and porcine genome seem to fit with the size related model of chromosome organisation (Foster and Bridger, 2005).

1.3.2. Gene density model

The gene density model describes the positioning of chromosomes in the nucleus according to gene density. This model was first proposed by Croft and colleagues

(1999) considering disparate positioning of two similar sized chromosomes (Croft *et al.*, 1999). These chromosomes represent different levels of gene density with chromosome 18 being gene poor and 19 being gene rich. The study was carried out by assessment of the positioning of chromosomes 18 and 19 in lymphoblasts and proliferating human dermal fibroblasts cell lines. Their results suggested that chromosome 18 was mainly localised in the nuclear periphery and chromosome 19 in the interior of the nucleus (Foster and Bridger, 2005). These observations were also confirmed in a later study using 3D-FISH by (Cremer *et al.*, 2003b).

A study by Boyle and colleagues investigates the chromosome position in lymphoblast cells from normal and X-linked Emery-Dreifuss muscular dystrophy (X-EDMD) males that have cells lacking the emerin protein (Boyle *et al.*, 2001). This study found out that chromosomes 1, 16, 17, 19, 22 were positioned in the centre of the nucleus whereas chromosomes 2, 4, 13, 18 were more peripherally located and this preference was not altered in mutant cells.

A study by Lukasova et al. (2002) further supports this model. This study investigated the position of chromosome 8,9,13 and 17 and found out that gene rich chromosomes 9 and 17 were located towards the centre of the nuclei with chromosomes 8 and 13 located closer to the nuclear membrane (Lukasova *et al.*, 2002).

A more recent study by Federico et al. (2008) looked at the positions of gene rich and gene poor regions of chromosome 7 in human lymphocytes and found out that gene dense GC rich areas were located towards the nuclear interior while gene poor regions were exposed to the nuclear periphery (Federico *et al.*, 2008).

Gene density relative to genome organisation has also been observed in primates where sequences orthologous to human chromosomes 18 and 19 were used and found to occupy positions similar to humans (Tanabe *et al.*, 2002), old world monkeys (Tanabe *et al.*, 2005), rodents cited in (Cremer and Cremer), cattle (Koehler *et al.*, 2009) and birds (Habermann *et al.*, 2001); suggesting that gene density related genome organisation is evolutionarily conserved over a period of 30 million years among species (Foster and Bridger, 2005).

Gene density models are associated with the transcriptional machinery and the separation of the nucleus into transcriptionaly active and inactive regions in order to enhance gene expression or inhibition (Foster and Bridger, 2005; Meaburn and Misteli, 2007). The nuclear periphery is believed to be the area in which transcriptional repression occurs and that may be the reason why chromosomes with low gene density are preferentially located in this area. On the other hand, the nuclear interior is believed to be the region with enhanced transcriptional activities and is the area occupied by chromosomes with high gene concentration. Similarly, Sadoni and colleagues (1999) described the observation of hyperacetylated GC rich and early replicated genome fractions in the interior of the nuclei (Sadoni *et al.*, 1999). There is evidence to support this hypothesis; Takizawa and colleagues suggest that specific genes such as β-globin move from the periphery to the interior upon activation during the differentiation of the mouse erythroid cell (Takizawa *et al.*, 2008). However this topic is still under debate. It has also been reported that transcriptionally activated genes are also located in the periphery close to the nuclear pore complex (Casolari *et al.*, 2004).

1.3.3. Size vs. gene density

Although, controversy still exisits regarding whether the positioning of chromosomes in the nucleidepends on size or gene density, it is possible for both models to be limited depending on cell type, species or status of a specific chromosome (Foster and Bridger, 2005). Some systems fit into both models. For example, the nuclear positioning of chicken chromosomes fit both modes; large and gene poor macrochromsomes have preferential peripheral localisation compared to small and gene rich microchromosomes located in the interior parts of the nuclei (Habermann *et al.*, 2001). A more recent study which analysed 1600 nuclei for 10 chromosomes suggested that radial arrangement of chromosomes correlates equally well with gene density and chromosome size for human epithelial cells from a bladder carcinoma (Heride *et al.*, 2010). In addition, New World monkey (Mora *et al.*, 2006) and porcine genomes also fit into both models (Foster and Bridger, 2005) while other systems such as murine did not seem to fit into either model (Meaburn *et al.*, 2008).

1.3.4. Other models

There are several other models that have been proposed to describe chromosome positions in the interphase nuclei. The first is the chromosome territory interchromatin compartment (CT-IC) model which describes the existence of two domains in the nuclei called chromosome territories (CT) and interchromatin compartments (IC). This concept was originally reported by (Lichter *et al.*, 1993) as an inter chromosomal

domain which is described as the space around the chromosome that has penetrated into the territory (Branco and Pombo, 2007; Pombo and Branco, 2007). This model suggests that regulatory and coding sequences of active genes are located in the periphery of the chromosome territory so they can interact with the transcription machinery which contains macromolecular complexes for gene transcription (Cremer *et al.*, 2004). However inactive genes located at the interior of the chromosome territory have limited access to the transcription factors (Branco and Pombo, 2007; Foster and Bridger, 2005; Heard and Bickmore, 2007; Pombo and Branco, 2007).

Another model proposed by Dehghani for chromosome architecture is the lattice model which suggests that fibres from different chromosomes are able to intermingle to a certain extent at the edges of CTs (Dehghani *et al.*, 2005). This model also proposes the presence of large chromatin-free channels within the lattice of chromatin fibres and the absence of interchromatin compartments in the nucleus (Branco and Pombo, 2007; Heard and Bickmore, 2007).

In addition, the interchromatin network (ICN) model has been proposed and reviewed in Branco and Pombo (2007). This model suggests that a high degree of intermingling of chromatin i.e 19% of the nuclear volume is involved in intermingling regions (Branco and Pombo, 2006). Intermingling regions include the interior of individual chromosome territories and the area between neighbouring territories (Cremer and Cremer, 2010).

1.3.5. Nuclear organisation and cell differentiation

There are several studies that have reported differential chromosomal positioning occurs during the cell differentiation. Kuroda et al. (2004) have studies the relative and radial positioning of the chromosome territories 12 and 16 during adipocyte differentiation, and found out a the close proximity of chromosomes 12 and 16 in differentiated adipocytes (Kuroda *et al.*, 2004). A similar study by Marella et al. (2009) examined the position of chromosome territory 18 and 19 during keratinocyte differentiation and found out that the position of chromosome 19 is relatively peripheral in differentiated cells (Marella *et al.*, 2009b). Also Foster et al. (2005) reported alterations of sex chromosome position occur during spermatogenesis i.e. from spermatocytes to mature sperm position change from prripohery to the interior.

Some studies have reported alterations in the position of genes during cellular differentiation (Cremer and Cremer, 2010; Foster and Bridger, 2005; Kosak and Groudine, 2004; Schneider and Grosschedl, 2007). For example, a study by Szczerbal et al. (2009) found that during adipogenesis. certain genes reposition from the nuclear periphery to the interior (Szczerbal *et al.*, 2009). The authors also reported a correlation between chromosome repositioning and the up regulation of gene expression. Another study has reported that the immunoglobulin gene cluster repositions to the centre of the nuclei in pre-B cells compared to the peripheral localisation in non lymphoid cells. Also, it has been reported that during mouse embryogenesis, gene relocation occurs towards the interior of the nucleus (Takizawa *et al.*, 2008). These examples also suggest that active genes tend to localise in the interior of the nucleus compared to the periphearal localisation of their inactive state.

1.3.6. Nuclear organisation and diseases

As chromosome localisation is vital in regulation of transcription and mediation of cellular function, it can be hypothesised that cells undergo diseases at the alteration of genome organisation and there is evidence in the literature that nuclear architecture is altered in disease (Dauer and Worman, 2009; Lever and Sheer, 2010; Misteli, 2010; Rajapakse and Groudine, 2011; Stein *et al.*, 2010). The most common example for this is laminopathies which are known to be associated with altered nuclear organisation (Boyle *et al.*, 2001; Bridger and Kill, 2004; Elcock and Bridger, 2010; Foster and Bridger, 2005; Misteli, 2005). Laminopathies are characterised by the expression of disease symptoms in particular tissues derived from mesenchymal origins such as muscle, adipose tissue and neurons (Hutchison and Worman, 2004). Diseases associated with the LMNA gene include muscular dystrophy, lipodystrophies, neuropathies and progeroid disorders (Misteli, 2005). As reviewed in Foster & Bridger 2005 and Misteli 2005, mutations in the LMNA gene can either weaken the structural integrity of the nucleus by exposing the nucleus, making it fragile and prone to physical stress (Hutchison *et al.*, 2001; Raharjo *et al.*, 2001) or perturb genome organisation and signal pathways that affect the correct nuclear functioning of the cell (Burke *et al.*, 2001; Cohen *et al.*, 2001; Mounkes *et al.*, 2001).

Another recent study by Petrova et al. (2007) tested the radial positions of the centromeric regions of chromosomes 1 and X from a patient with XXXXY polysomy. Results suggest that the radial position of chromosome 1 was changed in XXXXY cells compared to normal XY cells (Petrova *et al.*, 2007).

Changes in chromosome 17 position were seen in a study by Li and colleagues in 2009 due to infection with Epstein-Barr virus. Authors suggest that this implies the effect of viral infection on genome instability (Li *et al.*, 2010).

1.3.6.1. Nuclear organisation and cancer

Current studies suggest that chromosomal structure, position and genome instability play a critical role in cancer initiation and progression (Cremer *et al.*, 2003b; Heng *et al.*, 2004; Marella *et al.*, 2009a; Meaburn and Misteli, 2008). Alteration of nuclear organisation relates to change in the proximity of chromosomal arms which can ultimately result in chromosomal translocations which have consistently been associated with cancers (Elliott and M Jasin, 2002).

One of the well studied cancers caused by a translocation is chronic myeloid leukaemia (CML). It has been linked to translocation involving chromosome 9 and 22 in most CML patients which gives the fusion of BCR gene in chromosome 22 with the ABL gene in chromosome 9 resulting in the Philadelphia chromosome (Lukasova, 1997). Lukasova and colleagues (1997) also reported that in bone marrows in CML patients ABL and BCR genes shift towards the central region of the nucleus facilitating translocation between these genes.

Recent studies by Taslerova and colleagues (2003, 2006) have reported that chromosome position in Ewing sarcoma cell lines show an alteration in radial position of chromosome 11 and 22. 85% of Ewing sarcomas involve a balanced translocation

(11;22)(q24;q12). This study was carried out by analysing 2D radial positions of EWSR1, BCR, FLI1, BCL1 genes and fluorescence weight centres of chromosome territories and comparing these to intact and derivative chromosomes 11 and 22 in nuclei of Ewing sarcoma samples. Their results suggested that significant radial migration was obtained for the derivative EWSR1, FLI1 and BCL1 genes and for the derivative chromosome 11 compared with the intact ones facilitating fusion of EWSR1 and FLI1 genes which have implications in malignancies (Taslerova *et al.*, 2006; Taslerová *et al.*, 2003).

Cremer and colleagues (2003) reported a relocation of chromosome 18 from the nuclear periphery to the central region in tumour cell lines (Cremer *et al.*, 2003b). This study involved assessment of chromosome 18 and 19 positions in different tumour cell lines. Authors reported that compared with nuclei of normal cells, in tumour cell lines chromosome 18 was located more interior than 19, resulting in smaller average radial distances between these territories that might have implications in cancer initiation and progression.

A recent study by Marella et al. (2009) reported a difference in the position of chromosomes 4 and 16 in breast cancer cell lines giving more evidence to altered nuclear organisation involved in cancer cells compared to normal cells. This study tests the position of human chromosomes 1, 4, 11, 12, 16, and 18 in normal and cancerous tissues. Results suggested nearly a twofold increase of chromosome 4 and 16 associations in a malignant breast cancer cell line compared to the related normal epithelial cell line demonstrating cancer related changes in chromosome arrangements (Marella *et al.*, 2009a).

More recently a study by Guffei and colleagues has revealed nuclear remodelling has been associated with a Hodgkin's lymphoma cell line proposing relevance of nuclear architecture in cancer (Guffei *et al.*, 2010). This study looked at inter nuclear DNA bridges, i.e. consisted of chromatids and chromosomes in mono nucleated Hodgkin (H) cells and multi-nucleated Reed-Sternberg (RS) cells. The study revealed the presence of inter-nuclear DNA bridges in RS cells but not in H cells indicating that the complexity of chromosomal rearrangements increased with tumour progression.

1.3.7. Nuclear orgaanisation in sperm

DNA in human sperm is tightly compacted and at least six fold more highly condensed than the DNA in mitotic chromosomes (Ward and Coffey, 1991). Several studies have reported the nuclear organisation and architecture in human sperm cells (Finch *et al.*, 2008b; Haaf and Ward, 1995; Hazzouri *et al.*, 2000; Kramer and Krawetz, 1996; Mudrak *et al.*, 2005; Tilgen *et al.*, 2001; Zalensky *et al.*, 1995).

In sperm, positions of chromosomes are non random. Centromeres of chromosomes cluster to form the chromocentre in the centre of the nuclei while 2 telomeres are located towards the periphery, where they interact to form dimers (Luetjens *et al.*, 1999; Mudrak *et al.*, 2005; Solov'eva *et al.*, 2004; Zalenskaya and Zalensky, 2004; Zalensky and Zalenskaya, 2007; Zalensky *et al.*, 1993; Zalensky *et al.*, 1995). This organisation has been conserved in other mammals such as mice, rats, cattle, pigs, and horses (Haaf and Ward, 1995; Meyer-Ficca *et al.*, 1998; Meyer-Ficca *et al.*, 2005; Zalenskaya and Zalensky, 2004).

Martin (2006b) suggested that the sperm chromocentre is formed of pericentric heterochromatin from different chromosomes (centromeres), which tend to aggregate together (Martin *et al.*, 2006b). Telomeres are however from dimers between the p and q arm adopting a hairpin loop structure which is located towards the periphery of the nuclei (Mudrak *et al.*, 2005; Solov'eva *et al.*, 2004). Several studies suggest that the advantage of such unique nuclear architecture is to coordinate unpacking and activation of the male genome during fertilization (Greaves *et al.*, 2003; Mudrak *et al.*, 2005; Zalensky and Zalenskaya, 2007). Also sperm chromosome positioning is believed to be vital in its inactivation and genomic imprinting. Further chromosome positioning in sperm nuclei heads is believed to play an important role in spatial chromatin differentiation and development and gene expression regulation of the fertilised egg as well as early embryonic stage (Greaves *et al.*, 2003). Greaves has also underlined the importance of evolutionary conserved similarities of the positions of sex chromosomes relative to the acrosome in mammals implicating the functional significance with regard to paternal X inactivation (Greaves *et al.*, 2003).

Nuclear organisation in sperm can be measured both radially and longitudinally. There are several studies that have tested the radial chromosome positions in the sperm head (Finch *et al.*, 2008b). A study from Finch and colleagues examined the radial position of centromeres of chromosome X, Y, and 18 in normal and infertile males (Finch *et al.*, 2008b). This study found that all centromeres occupied central positions in normal males but the sex chromosomes showed altered nuclear address in some of the infertile patients. Another study was performed to test the chromosome position across all chromosomes in the human karyotype by Ioannou and colleagues

(Ioannou and Griffin, 2010). In this study the position of 18 chromosomes were assayed in 10 infertile male and compared with 10 normal controls. Results suggested that all loci examined in the control group adopted defined, interior positions providing evidence for the presence of a chromocentre. In the infertile group however there were subtle alterations in the nuclear address for certain centromeres in individual patients. When all patient results were pooled, some different nuclear addresses were observed for chromosomes 3, 6, 12 and 18. To the best of my knowledge, the position of telomeres from normal and infertile groups has not been assessed so far to see the situation on other side of the chromo centre.

Several studies have also assessed the longitudinal position of chromosomes in human sperm (Hazzouri *et al.*, 2000; Luetjens *et al.*, 1999; Mudrak *et al.*, 2005; Zalenskaya and Zalensky, 2004). Data from above studies arrange chromosomes into the following order: X, 7, [6, 15, 16, 17], 1, [Y, 18] 2, 5, starting from the acrosome towards the tail (Zalensky and Zalenskaya, 2007). The functional importance of this is that during fertilisation most peripheral chromosomes are first exposed to ooplasm and undergo earlier remodelling from others (Zalensky and Zalenskaya, 2007). Another study that investigated the longitudinal chromosome position was Sbracia et al. (2002) which assessed the sex chromosomes from normal and oligospermic males going through ICSI (Sbracia *et al.*, 2002). This study did not find a significant difference between the two groups (Sbracia *et al.*, 2002). A study by Wiland et al. (2008) has analysed the longitudinal localisation of centromeres of certain chromosomes in the sperm nuclei of six reciprocal translocations carriers compared to four control males with normal karyotypes and found out that chromosomes with translocations had shifted positions (Wiland *et al.*, 2008). This study also suggested

that the chromocentre in sperm nuclei of translocation carriers was widened toward the apical side compared to chromocenters in control males, thus authors postulate that translocations influence the localisation of other chromosomes in sperm nuclei (Wiland *et al.*, 2008). Another study by Olszewska et al. (2008) compared the longitudinal positions of chromosomes 15, 18, X and Y in infertile patients to healthy controls and found out that there was no difference between the two groups (Olszewska *et al.*, 2008).

There are a number of studies that have investigated the positions of centromere regions of chromosomes in the sperm head; however the positions of telomeres and sub telomere regions remain under explored. One of the chapters of this thesis attempts to investigate the positions of telomere and sub telomere regions in sperm from fertile and healthy control gropes.

1.3.8. Nuclear organisation in oocytes

In many countries, obtaining human oocytes from natural cycles is very difficult due to ethical issues, (Delhanty, 2005) thus most studies have been performed in animal models. Studies by Zuccotti and colleagues have reported that in mouse during oocyte growth, chromosome centromeres are initially found to be well spread within the nucleus but with time, they cluster around the periphery of the nucleolus. Based on chromatin morphology, two types of oocytes exist known as surrounded nucleolus oocytes and unsurrounded nucleolus oocytes. Authors suggest that a similar nuclear structure exists in human oocytes as well (Zuccotti *et al.*, 2005). Authors also postulate that in oocytes, nuclear architecture is developmentally regulated, and

represents the role of nuclear organisation in the regulation of genome functioning during differentiation and development (Zuccotti *et al.*, 2005).

1.3.9. Nuclear organisation in embryos

Only a few studies have attempted to address the nuclear organisation in human embryos due to limitations in finding materials. The first to do this was Mackenzie (2004) who studied the position of seven chromosomes including chromosome 13, 16, 18, 21, 22, X & Y in normal and abnormal human blastomeres using centromeric and locus specific probes (McKenzie *et al.*, 2004). They found out that in normal blastomeres chromosome 13, 18, 21 and X were central and chromosome 16, 22 and Y were more peripherally located. However this pattern was altered in aneuploid blastomeres with more peripheral localisations (McKenzie *et al.*, 2004). The localisation of specific chromosomes (13, 18, X) in the interior part of the nuclei during the embryonic development, whereas they are normally found to be in the periphery of committed cell line indicates a different nuclear organisation pattern associated with totipotent cells at the cleavage stage of human development (McKenzie *et al.*, 2004).

A similar study by Diblik et al. (2007) reported that the chromosomes 13, 16, 21, 22, X and Y in were not significantly different from random distribution in both normal and abnormal embryos. The only difference they could observe was that arrangement in chromosome 18 was significantly different to random distribution and shifted towards the peripheray in aneuploidy blastomeres (Diblik *et al.*, 2007).

More recently, Finch and colleagues (2008) attempted to establish a correlation between chromosomal abnormalities and nuclear organisation in human embryos and then compare this to a range of committed cell lines (Finch *et al.*, 2008a). This study reported a significant alteration of nuclear organisation associated with chromosomally abnormal embryos compared to control committed cell lines. For example, chromosome 15 was localised in the periphery of nuclei in committed cells, however in aneuploidy blastomeres chromosome 15 had a central localisation. This study also reported that embryos with no detected abnormalities adopt a less distinct pattern in genome organisation due to the existence of mixed populations of cells, each with a different nuclear organisation (Finch *et al.*, 2008a).

The above studies propose that examining nuclear architecture during early embryogenesis could provide insight into the mechanisms of aneuploidy and improve the possibility of embryo selection in pre implantation diagnosis.

1.4. Infertility in humans

1.4.1. Male factor infertility

Infertility is defined as the failure to conceive following a year of regular unprotected sexual intercourse, and accounts for one in six childless couples wishing to start a family in the western world (Shah *et al.*, 2003). The World Health Organisation (WHO) reported that in infertile couples, a male factor is implicated in more than 50% of cases. This is followed by 38% of female factor infertility and then 27% ofcases of both partners contributing (Seli and Sakkas, 2005). Causes for infertility

include genetic causes, hormonal related issues, age and lifestyle, medical conditions such as cancers, surgeries and other medical procedures such as chemotherapy and radiotherapy, abnormal semen parameters, and finally unexplained (idiopathic) causes.

1.4.1.1. Genetic causes of infertility

Genetic causes account for about 15% of male infertility (Ferlin *et al.*, 2007; Seli and Sakkas, 2005). Genetic causes can be divided to four categories such as Y chromosome deletions, single gene disorders, multifactorial causes and structural and numerical chromosome abnormalities (Griffin *et al.*, 2005).

1.4.1.1.1. Y chromosome micro deletion

An important genetic cause of infertility is associated with deletion in the long arm of the Y chromosome. Chromosome Y is a small largely heterochromatic gene poor chromosome and consists of genes for testis development and spermatogenesis (Ellis and Affara, 2006).

Y chromosome microdeletion has been seen in 10-15% non obstructive azoospermic patients and 5-10% in severe oligospermic patients (Ferlin *et al.*, 2007; O'Flynn O'Brien *et al.*, 2010). The regions of the Y chromosome involved in microdeletions are known as the azoospermia factor or ''AZF'' interval. The AZF interval is subdivided it into the AZFa, AZFb and AZFc smaller regions and deletions within

these regions cause various spermatogenic and infertility phenotypes (Affara and Mitchell, 2000; Ferlin *et al.*, 2007; O'Flynn O'Brien *et al.*, 2010; Shah *et al.*, 2003; Wong *et al.*, 1999). A recent study comparing the genotype and sperm phenotype in infertile patients with various Y chromosome aberrations revealed meiotic pairing defects related to azoospermic factor microdeletions (Antonelli *et al.*, 2011). Mateu and colleagues have reported that patients with Y chromosome microdeletions had a high percentage of numeric chromosome abnormalities in sperm. They have also observed a higher percentage of chromosomal abnormalities in embryos with a significant increase in X monosomy (Mateu *et al.*, 2010). It has also been reported that the significantly increased hormone profiles (FSH, LH and testosterone) (Pandey *et al.*, 2010), reduced sperm quality (Khan *et al.*, 2010) and increased apoptosis levels in germ cells (Yamada *et al.*, 2010) are associated with various deletions in the Y chromosome.

1.4.1.1.2. Single gene disorders

Many genes have been reported to have links to male infertility. These include cystic fibrosis trans membrane conductance regulator gene (CFTR), the androgen receptor gene (AR), the insulin like factor 3 gene (INSL3), leucine-rich repeat containing G-protein couple receptor 8 gene (LGR8), orphan nuclear receptor Dax-1(dax1) and Kallmann syndrome 1 sequence (KAL1), the importance of which have been reviewed in (O'Flynn O'Brien *et al.*, 2010; Shah *et al.*, 2003). Recent reviews by Massart and colleagues have presented a large number of genes that may be associated with male infertility phenotypes (Massart *et al.*, 2012).

1.4.1.1.3. Multifactorial causes

Multifactorial causes of infertility may include defects in one or more genes combined with environmental mechanical factors. Each of these factors can be considered as a 'risk factor' on its own (Massart *et al.*, 2012). One example of this is sertoli cell-only syndrome which is the absence of germ cells in testicular tissue and isinvolved in the accumulation of risk factors. In these patients, various single gene defects can be expected, for example genes required to maintain the stem cell pool of spermatogonia (Massart *et al.*, 2012). Recent studies have also reported that SNPs (Miyakawa *et al.*, 2011), copy number variance (Niederberger, 2012; Tuttelmann *et al.*, 2011) as well as injuries to the venous drainage of the male reproductive system causing hypoxia in the sperm production site (Gat *et al.*, 2010) are associated with sertoli cell only syndrome.

Having identified over 200 genes involved in fertility, most cases of male infertility have a genetic component (Matzuk and Lamb, 2002). However, numerous other factors such as mechanical problems e.g. vas deferens blockage, physical trauma, infection, lifestyle e.g. obesity, psychological problems, age, exercise, diet and smoking can have influences on fertility. In order to understand the multifactorial nature of infertility, more studies in human model systems will be required.

1.4.1.1.4. Aneuploidy

There are a large number of studies suggesting that humans have the highest level of chromosomal abnormalities of any known animal species (Daphnis *et al.*, 2005; Daphnis *et al.*, 2008; Delhanty *et al.*, 1997; Handyside and Delhanty, 1997; Thomas and Hassold, 2003) and aneuploidy is believed to be a leading cause for infertility in humans. As discussed earlier, more autosomal aneuploidies originate in human female meiosis I.

Only a few aneuploidies can survive until puberty and the reproductive age. Trisomy 21; Down syndrome is one example. Most Down syndrome affected males are sterile, with the phenotype including arrested spermatogenesis, azoospermia or severe oligospermia. The mechanism by which trisomy 21 affects male infertility is still unclear, however there are physical and psychosocial limitations that could be due to numerous factors including hormone imbalance (Egozcue *et al.*, 2000).

Klinefelter Syndrome is usually associated with the karyotype 47, XXY, either in all cells or in ''mosaic'' form (O'Flynn O'Brien *et al.*, 2010) and is present in 5% of severe oligospermic and in 10% of azoospermic males (Ferlin *et al.*, 2007; Selice *et al.*, 2010). In more than 50% of cases, the extra X chromosome has paternal meiosis I origin with non disjunction of the XY bivalent. In 40% of cases the extra X chromosome has maternal origin due to errors in meiosis I or II. The remaining 10% are believed to be of post zygotic origin (Griffin *et al.*, 2005). With new technology such as ICSI, Klinefelter Syndrome patients can father their own children, however (Plotton *et al.*, 2011) there is still an elevated risk of passing chromosomal

aneuploidies into the offspring due to the increased number of sperm disomy (Ferlin *et al.*, 2007).

Turner syndrome is a genetic disorder associated with 45, X karyotype, some cases in mosaic form (Reindollar, 2011). It has been suggested that about 5% of Turner syndrome patients may have abbreviated menstrual function and 1 to 2% of all patients may become pregnant (Onalan *et al.*, 2011; Reindollar, 2011). Pregnancy could be either spontaneous or from a donor oocyte, however 100 fold or more increase in maternal mortality rate is estimated (Reindollar, 2011).

Hyper Y syndrome is another sex chromosome aneuploidy found in 1/1000 males. The extra Y chromosome is due to paternal meiosis II. Similar to Klinefelter Syndrome patients, patients with XYY can have fertility ranging from normal to azoospermia conditions with issues in hormonal balance (Shah *et al.*, 2003).

1.4.1.1.5. Structural abnormalities

A study by Kim and colleagues report that complex chromosomal rearrangements are associated with male infertility and that they may affect the severity of spermatogenetic impairment (Kim *et al.*, 2011).

Reciprocal translocations can lead to reduced fertility depending on the chromosomes involved and the nature of the translocation. As reviewed in Griffin 2005, mechanisms involved in reduced fertility by translocations include affecting the

pairing of chromosomes, reducing the crossing over, producing unbalanced gametes, mediating non homologous pairing with the X and Y chromosome interfering with X inactivation resulting in lethal gene dosage, and finally producing meiotic errors by interaction with other chromosomes (Griffin and Finch, 2005). Translocations are found 4-10 times more in infertile men compared to in fertile men (O'Flynn O'Brien *et al.*, 2010). For example, a recent study by Mikelsaar and colleagues reported balanced reciprocal translocation t(5;13)(q33;q12.1) and a microduplication in the region of 9q31.1, in a man suffering from infertility (Mikelsaar *et al.*, 2011). Authors postulated that the the TUBA3C gene is located in the region 13q12.1 is , known to be important in the motility of flagella;disruptions in the gene could be the cause of poor motility of sperm (Mikelsaar *et al.*, 2011).

Another structural chromosome abnormality is an inversion event, which is known to be the rearrangement of a chromosome segment, resulting in a change of the gene sequence. Several studies have reported various inversions associated with infertility (Belangero *et al.*, 2009; Chantot-Bastaraud *et al.*, 2007; Morel *et al.*, 2007; Vialard *et al.*, 2007). A study by Belangero reported that an inversion of chromosome 9 had been seen in infertile patients and morphological differences between homologous chromosomes 9 might be the reason for errors in crossing over. This can therefore lead to abnormalities in sperm cells subsequently causing infertility (Belangero *et al.*, 2009). The mechanism involved in inversions that cause infertility include the formation of a pairing loop, disrupting meiosis and reduced recombination or abnormal recombination in the pairing loop resulting in abnormal gametes (Griffin and Finch, 2005).

1.4.1.1.6. Sperm DNA damage/fragmentation

Sperm DNA fragmentation is a relatively common feature of human spermatozoa and one of the main causes of male infertility and repeated assisted reproduction failures (Aitken and De Iuliis, 2007a, 2007b; Tarozzi *et al.*, 2007; Venkatesh *et al.*, 2011; Zini and Libman, 2006a, 2006b).Other consequences of DNA damage include impaired fertilisation, poor embryonic development, high levels of miscarriage and health related issues in offspring (Venkatesh *et al.*, 2011). Three major mechanisms seem to be involved in DNA damage including poor chromatin protamination, oxidative stress due to generation of high levels of oxygen free radicals and abortive apoptosis (Aitken and De Iuliis, 2009; Tarozzi *et al.*, 2007). These could be due to various factors such as age (Horta *et al.*, 2011), obesity (Chavarro *et al.*, 2010; Du Plessis *et al.*, 2010; Kort *et al.*, 2006; Rybar *et al.*, 2011), cancer and cancer treatment (Romerius *et al.*, 2010; Said *et al.*, 2009; Smit *et al.*, 2010), smoking (Tawadrous *et al.*, 2012), use of a laptop (Avendano *et al.*, 2012) and environmental pollutants (Firestone *et al.*, 2011; Huang *et al.*, 2011) . It has been suggested that infertile men can possess significantly more spermatozoa with DNA damage; therefore with ICSI there is a risk of transmitting a detrimental genetic or epigenetic effect to the offspring as a consequence (Aitken and De Iuliis, 2007a).

1.4.2. Female factor infertility

According to the WHOs female factor infertility accounts for 38% of infertility cases (Seli and Sakkas, 2005) and of this, genetic causes account for 10% of female infertility. Other factors associated with female factor infertility include women's age,

chromosomal aberrations, monogenic diseases, endocrine dysfunctions, sexually transmitted diseases, and immune system dysfunctions amongst others (Haller-Kikkatalo *et al.*, 2012).

1.4.2.1. Advanced maternal age

It is a well known fact that female fertility decreases with advanced maternal age. The age related decrease in fertility is mainly due to a decreasing number of oocytes with age. The total number of oocytes declines bi exponentially with age and this process speeds up around the age of 37–38 years (Balasch and Gratacos, 2011; Ng and Ho, 2007). Advanced maternal age also leads to infertility as older women are more likely to produce embryos with chromosome abnormalities which would not fully develop as a live birth (Munne *et al.*, 1999). Evidence has been reported that most autosomal trisomies and monosomies occur due to errors arising during the first maternal meiotic division and this error rate increases with maternal age (Hall *et al.*, 2006; Hassold *et al.*, 1996; MacDonald *et al.*, 1994; Pellestor *et al.*, 2005; Penrose, 1933; Savage *et al.*, 1998; Sherman *et al.*, 2005; Sherman *et al.*, 2006). Similarly, Hassold *et al.* (2000) reported that the likelihood of AMA patients at the risk of trisomy pregnancy is 15 times greater than females in their twenties. The clinical data suggests that chromosomal abnormalities become more obvious in IVF for mothers above the age of thirty five years even though the exact mechanism of the effect of AMA on fertility is still controversial (Hassold and Sherman, 2000). High level of aneuploidy is a major cause of increased spontaneous abortion and decreased live birth rates in women in their advanced age; it has been reported that more than 40% of spontaneous abortions occur in clinically detected pregnancies and more than 60% of these

spontaneous abortions are also related to trisomies due to AMA (Robinson *et al.*, 2001). Also in advanced age, declining rates of implantation, clinical pregnancy, and delivery were observed. In addition, higher maternal mortality rates related to advanced maternal age were also reported (Balasch and Gratacos, 2011; Temmerman *et al.*, 2004).

1.4.3. Cancer and infertility

Cancers have frequently been linked with male infertility in three different ways (Meirow and Schenker, 1995). Firstly, cancers can cause infertility. Secondly, improved survival rate can raise concern about the effects of anti-cancer treatments on germ cell lines and therefore pose a potential risk to the future offspring. Thirdly, an unrelated or independent factor may cause both cancer and infertility in certain individuals.

Cancers can influence gonadal dysfunction through hormonal alterations. In cancer patients, reproductive hormones might be low due to high levels of stress. Some tumours produce endocrine substances such as b-human chorionic gonadotrophins which can down regulate the reproductive hormone levels in the blood stream. It has been reported that the hypothalamus and the pituitary gland function can be impaired by tumour cell invasion in leukaemia, lymphoma and in central nervous system tumours (Sabanegh and Ragheb, 2009) . Tumours can also release cytokines that affect sperm motility (Sabanegh and Ragheb, 2009).

In addition, cancer treatments have adverse effect on fertility. For example, In leukaemia, cancer treatments inlcuding cyclophosphamide or melphalan with total body irradiation can result in permanent sterility in at least 83% of patients (Dohle, 2010). For germinal testicular cancer, after cisplatin-based chemotherapy, sperm recovery occurred in 55–80% cases and carboplatin-based chemotherapy is known to be associated with less spermatogonial damage (Trottmann *et al.*, 2007). It has been reported that azoospermia was found in 85–90% of patients after more than 3 courses of MOPP therapy for Hodgkin's disease (Lee *et al.*, 2006); in contrast 90% returned to having normal sperm counts 12 months after ABVD therapy (Tal *et al.*, 2000).

With regard to female fertility, various chemotherapeutic agents have different toxicity levels on ovarian function. Alkylating agents, such as cyclophosfamide and busulfan, which are used in childhood cancer, are far more gonadotoxic than other chemotherapeutic agents (Nicholson and Byrne, 1993). In addition, total radiation exposure is associated with causing sterility in younger women and children (Lushbaugh and Casarett, 1976) and especially has a high risk of ovarian failure (Barrett *et al.*, 1987). Recently developed assisted reproductive techniques such as oocyte cryopreservation have led to pregnancies with more than 100 healthy newborns (Hourvitz *et al.*, 2008). However, the success rate is low and not regular (Hourvitz *et al.*, 2008). A recent study investigated the effect of cancer therapy on ART outcomes and reported that cancer survivors have significantly fewer oocytes retrieved and embryos available for transfer. In addition they have lower pregnancy and live birth rates (Barton *et al.*, 2011).

Numerous studies have implied that both testicular cancer and Hodgkin's lymphoma directly affect spermatogenesis, causing reduced fertility in patients even prior to treatments (Fossa *et al.*, 1989; Lass *et al.*, 1998; Meirow and Schenker, 1995; Petersen *et al.*, 1999; Rueffer *et al.*, 2001; Viviani *et al.*, 1991). A study from Petersen and colleagues has reported that in testicular cancer patients, sperm counts are approximately one-third lower than that seen in normal males (Petersen *et al.*, 1999). Studies looked at the sperm count of Hodgkin's lymphoma patients (Rueffer *et al.*, 2001; Viviani *et al.*, 1991) and reported that 66% of patients had a reduced sperm count. A recent study by Heracek and colleagues reported that cancer and its treatments cause reduced sperm count as well as impaired sperm motility and disorders in morphology and DNA integrity (Heracek *et al.*, 2010).

1.5. ART and preimplantation development

Human fertilisation is a relatively inefficient process since approximately 30% of pregnancies result in spontaneous losses (Dey, 2010). However ART has enabled millions of people in the world who had problems of conceiving naturally to have biological children (Ferraretti *et al.*, 2012).

ART is known to be an effective treatment in subfertility. ART include numerous new forms of technology such as *in vitro* fertilisation (IVF) intracytoplasmic sperm injection (ICSI), gamete intrafallopian transfer (GIFT), zygote intrafallopian transfer (ZIFT), use of donor egg or embryo and surrogacy (gestational carrier).

1.5.1. *In vitro* fertilisation (IVF)

IVF is the most commonly performed technique to assist reproduction in sub fertile patients. The world's first successful IVF pregnancy occurred in the late 1970s pioneered by Steptoe and Edwards at Oldham General hospital (Steptoe and Edwards, 1978). Since then, the number of IVF cycles carried out in the world has been significantly increased (Ferraretti *et al.*, 2012).

The procedure of IVF starts with ovarian hyper simulation with FSH in order to produce eggs. This procedure is known to take up to 12 days. Following the collection of eggs, sperm is added with an aim to forming embryos and incubated for16-20 hours. Then embryos that are fertilised transfed back to the uterus. Selection of embryos for transfer involves certain criteria published (Magli *et al.*, 2008). The number of embryos transferred generally depends on factors such as mother's age, embryo quality and patient history (Gianaroli *et al.*, 2000; Magli *et al.*, 2008). However, concerns have been raised regarding the outcome of IVF pregnancies related to multiple births, monozygotic twins, preterm deliveries and most importantly the increased risk for congenital malformations (Ericson and Kallen, 2001; Yang *et al.*, 2012).

1.5.2. Intracytoplasmic sperm injection (ICSI)

ICSI involves *in vitro* injection of a single sperm directly into an oocyte through the zona pellucid (Palermo *et al.*, 1992; Palermo *et al.*, 2000). Before the invention of

ICSI, during the IVF procedure, oocytes were incubated with sperm cells in a culture medium in order for the fertilisation to occur. However, this result in lower success of fertilisation; especially as this is not suitable for men with poor semen parameters (Palermo *et al.*, 1992; Palermo *et al.*, 2000; Van Steirteghem *et al.*, 2002a; Van Steirteghem *et al.*, 2002b). Because of ICSI, now it has become possible for men with severe oligozoospermia, asthenospermia or azoospermia to become fathers of their own biological children as ICSI can also be performed with spermatozoid extraction through testicular biopsy if necessary (Chan *et al.*, 2001; Janzen *et al.*, 2000; Schlegel *et al.*, 1997; Su *et al.*, 1999; Van Steirteghem *et al.*, 2002a; Van Steirteghem *et al.*, 2002b; Van Steirteghem, 2009).

Using ICSI, the first live birth was reported in the early 90's (Palermo *et al.*, 1992) and since then, 1000s of babies have been born around the world by use of ICSI. Several studies have reported successful live births from ICSI treatment using immotile testicular sperm (Cayan *et al.*, 2001; Kaushal and Baxi, 2007; McLachlan *et al.*, 2011). A study from Dam and colleagues reports successful live births using sperm from partial globozoospermia (presence of round-headed sperm cells lacking acrosomes) patients (Dam *et al.*, 2012). However it has been reported to be associated with a possible high risk for chromosomal aneuploidy of paternal origin as natural selection is over powered by ICSI (Durakbasi-Dursun *et al.*, 2008). An early study by Luetjens et al., (1999) postulated the fact that sperm used in ICSI have not gone through the acrosomal reaction. This could therefore result in impaired chromatin decondensation in the apical region of sex chromosomes. This negatively affects zygote progression into mitotic division, causing mitotic errors translated as sex chromosome abnormalities in ICSI offspring (Luetjens *et al.*, 1999). Also, a study

preformed in 2012 suggested that the origin of the sperm used in ICSI does not have a major influence on the early life of the offspring, but transgenerational and epigenetic effects may remain unknown (Halliday, 2012).

1.5.3. Preimplantation genetic diagnosis

Preimplantation genetic diagnosis (PGD) is a genetic diagnosis procedure which involves the diagnosis of single gene defects and chromosomal rearrangements such as translocations in the embryo generated by IVF prior to implantation (Frumkin *et al.*, 2008; Geraedts and De Wert, 2009; Griffin *et al.*, 1994; Handyside *et al.*, 1989; Handyside *et al.*, 1990; Handyside *et al.*, 1992; Handyside and Delhanty, 1997; Harper, 1996; Harper *et al.*, 2012; Munne *et al.*, 1993b; Munne *et al.*, 2000; Thornhill and Snow, 2002; Verlinsky *et al.*, 2004). This technique allows the identification of genetic abnormalities before implantation, thereby providing the opportunity to transfer the unaffected embryos without having to terminate the pregnancy. Generally, PGD is offered to patients who are at a high risk of transmitting a genetic disorder to their offspring (Geraedts *et al.*, 1999; Harper *et al.*, 2006; Harper and Sengupta, 2012; Harper *et al.*, 2012; Munne, 2003; Thornhill *et al.*, 2005) and is currently used coupled with IVF and/or ICSI (Altarescu *et al.*, 2006; Geraedts *et al.*, 2001; Handyside *et al.*, 2004; Harper *et al.*, 2004; Heng, 2006; Katz *et al.*, 2002; Munne, 2003; Robertson, 2003; Wells and Levy, 2003).

PGD requires patients to undergo IVF to generate embryos in vitro. By the 3rd day after fertilisation, the embryo is at the 8 cell stage. One, or in some cases two blastomeres are biopsied from the embryo to perform PGD. PGD can be achieved via

techniques such as PCR, FISH and more recently microarray. Only unaffected embryos are transferred with the hope of achieving a pregnancy (Braude, 2006; Geraedts and De Wert, 2009; Geraedts, 2010; Griffin *et al.*, 1994; Handyside *et al.*, 1990; Harper and Sengupta, 2012; Munne *et al.*, 1993b).

The fist clinical PGD case was performed using PCR to diagnose sex linked ornithine transcarbamylase deficiency (OTC) (Handyside *et al.*, 1990), followed by PGD for the diagnosis of cystic fibrosis (Handyside *et al.*, 1992). The first FISH based PGD was done in 1993 to prevent the risk of transmitting X linked disorders (Griffin *et al.*, 1993). More recently PGD performed using micro array techniques such as array CGH (Fiorentino *et al.*, 2011) and SNP arrays (Treff *et al.*, 2011a). As reviewed in Simpson 2010, to date, over 50,000 PGD cycles have been performed around the world and it is estimated that around 10,000 babies have been born. Currently, the use of PGD includes detection of monogenic disorders, mitochondrial disorders, translocations, numerical chromosome abnormalities, HLA matching (Geraedts *et al.*, 2010; Geraedts and De Wert, 2009; Harper *et al.*, 2012) and in some countries social sexing (Egozcue, 1993). Currently, PGD carried out using amplified DNA from biopsied blastomeres (Handyside *et al.*, 2004; Handyside *et al.*, 2009), polar bodies (Verlinsky *et al.*, 1990) as well as trophectoderm cells from blastocysts (Hahn *et al.*, 2000).

1.5.3.1. Polar bodies biopsy

Polar body biopsy was originally performed by biopsying only the first polar body (Verlinsky *et al.*, 1992). Later studies biopsied and analysed both polar bodies for more accurate analysis (Verlinsky *et al.*, 1998).

Polar bodies however provide maternal information only although most errors in human embryogenesis are of maternal origin (Delhanty, 2011). Also it is not possible to determine paternal errors (most importantly sex chromosome aneuploidies) which has a significantly high paternal origin and post fertilisation errors by looking at polar bodies.

A recent study by Kuliev and colleagues presented 938 PGD cycles for single gene disorders by polar body testing for 146 different monogenic conditions, resulting in 345 healthy child births (Kuliev and Rechitsky, 2011). This suggests that PB based PGD is a reliable and safe procedure, with an extremely high accuracy rate of over 99% (Kuliev and Rechitsky, 2011).

1.5.3.2. Blastocysts biopsy

Blastocyst biopsy is currently the most popular method of biopsy. Blastocyst biopsy has several advantages over cleavage stage blastomeres and polar bodies (Ly *et al.*, 2011). Blastocyst stage embryos can offer several cells for analysis allowing more detailed investigation of chromosomal abnormalities with both meiotic and mitotic errors that have both parental origins. It has also been reported that blastocyst stage is the optimal stage for diagnosis as embryos have less abnormalities and mosaicism due to a self correction mechanism that naturally limits proliferation of abnormal cells. This permits the results to be more reliable (Barbash-Hazan *et al.*, 2009). These mechanisms have only been recognised in the blastocyst stage but not in the early cleavage stage (Hernandez, 2009; Los *et al.*, 2004).

The first clinical applications of blastocyst biopsy were performed several years ago with success (Kokkali *et al.*, 2005; McArthur *et al.*, 2005). Followed by this many pregnancies following trophectoderm biopsy have been reported (Kokkali *et al.*, 2005; Kokkali *et al.*, 2007; Krieg *et al.*, 2009; McArthur *et al.*, 2005; McArthur *et al.*, 2008). The transfer of vitrified embryos has shown increased levels of pregnancy rates (Chang *et al.*, 2011; Zhu *et al.*, 2011). For many IVF centres, blastocyst culture and transfer has become routine (Harper and Sengupta, 2012).

It is well known that high levels of chromosome mosaicism is associated with cleavage stage blastomeres (Harper *et al.*, 1995; Munne *et al.*, 1995b) and it is expected that relatively low mosaicism in the blastocyst stage of embryos would be seen. However, mosaicism has also been seen in blastocyst stages (Harper and Sengupta, 2011). Recent data by Fragouli and colleagues have reported that mosaicism in blastocyst stage as well (Fragouli *et al.*, 2011). Another study looked at the association between blastocyst morphology and chromosomal status foun that there was a weak correlation between aneuploidy and embryo morphology in the blastocyst stage (Alfarawati *et al.*, 2011).

The other disadvantage in using the blastocyst is that *in vitro* embryo survival decreases after day 6 (Geraedts *et al.*, 2010; Geraedts and De Wert, 2009), therefore vitrification techniques would be required (Loutradi *et al.*, 2008; Rezazadeh Valojerdi *et al.*, 2009; Zhang *et al.*, 2009; Zhang *et al.*, 2011). Blastocyst biopsy is theerfore usually coupled with vitrification in order to cryopreserve blastocysts until the preparation of endometrium for implantation in the next cycle. This allows sufficient time (in fact unlimited time) to perform diagnosis, and this solves the problem

originally had with blastocyst transfer that was having only a 24 hour window to complete diagnosis.

1.5.4. Preimplantation genetic screening

PGS is a type of PGD designed to test for chromosome copy number abnormalities in embryos. It has been observed that IVF patients generate embryos with as high as 60%-70% chromosome aneuploidies (Delhanty *et al.*, 1997; Donoso and Devroey, 2007; Donoso *et al.*, 2007a; Munne *et al.*, 1993a). The aim of PGS is to increase the chance of a healthy pregnancy by selecting only chromosomally normal embryos to implant (Baart *et al.*, 2006; Baart *et al.*, 2007; Fritz, 2008; Harper *et al.*, 2008b; Harper *et al.*, 2010; Munne *et al.*, 1995b). The main indications for PGS are advanced maternal age, repeated implantation failure, repeated miscarriage or severe male factor infertility (Donoso and Devroey, 2007; Donoso *et al.*, 2007a; Gianaroli *et al.*, 1997b; Handyside *et al.*, 2004; Harper *et al.*, 2010; Munne and Cohen, 1998; Munne, 2003; Yakin *et al.*, 2008; Yakin and Urman, 2008). PGS aims to select only normal embryos for implantation, with the hope of increasing the chance of perpetuating pregnancy and reducing miscarriages and trisomy births (Anderson and Pickering, 2008; Baart *et al.*, 2006; Harper *et al.*, 2008a; Harper *et al.*, 2008b; Harper *et al.*, 2010; Jansen *et al.*, 2008; Staessen *et al.*, 2004).

Until the development of microarray techniques related to PGS, traditionally interphase FISH was used to determine aneuploidies in preimplantation embryos. When using FISH for PGS, one or two blastomeres are biopsied from day 3 IVF embryos and fixed onto a slide in order to perform chromosome analysis by FISH

(Harper *et al.*, 2004; Munne *et al.*, 1995b; Munne *et al.*, 2004b; Pellestor *et al.*, 2006; Zwirglmaier, 2005). FISH was used because numerous chromosome specific probes can be labelled simultaneously with different coloured fluorochromes (Griffin *et al.*, 1994; Handyside and Delhanty, 1997). In most clinics the chromosomes tested for PGS were usually 13, 15, 16, 18, 21, 22, X and Y (Donoso and Devroey, 2007; Donoso *et al.*, 2007a; Harton *et al.*, 2011), as these chromosomes are known to be involved in aneuploidies in spontaneous abortions and in trisomic live births.

1.5.5. PGS related controversy

The use of FISH based PGS for selecting embryos has been extensively debated; some studies reported benefits of PGS for groups of patients (Gianaroli *et al.*, 1999; Munne *et al.*, 1999; Munne, 2003; Munne *et al.*, 2003). For example, a significant reduction in spontaneous abortions after PGS has been reported (Colls *et al.*, 2006; Munne, 2006). Munne and colleagues (1999) originally found no increase in implantation rate, but an increase in ongoing pregnancy and live birth rate (Munne *et al.*, 1999). A more recent study reported that out of 230 cycles and 945 morphologically normal embryos, 314 embryos were diagnosed as chromosomally normal. Further embryo transfers resulted in 41 pregnancies with 37 healthy babies being delivered, with a take home baby rate of 24.2% (Ercelen *et al.*, 2011).

However, studies by Obasaju and colleagues (2001) found that in the case of advanced maternal age; PGS does not increase the implantation rate or clinical pregnancy rate (Obasaju *et al.*, 2001). A more recent randomised clinical trial by Mastenbroek and colleagues (2007) reported that preimplantation genetic screening

does not increase but significantly reduces the rates of ongoing pregnancies and live births after IVF (Mastenbroek *et al.*, 2007). The study was however heavily criticised by various groups due to their biopsy procedure, biopsy failures and the fact that undiagnosed embryos were transfered (Cohen and Grifo, 2007; Handyside and Thornhill, 2007; Munne *et al.*, 2007a; Munne *et al.*, 2007b; Sermondade and Mandelbaum, 2009; Simpson, 2008; Wilton, 2007). There are more randomised control trials by various groups that have been reported (Blockeel *et al.*, 2008; Chiamchanya *et al.*, 2008; Debrock *et al.*, 2009, , 2010; Garrisi *et al.*, 2009; Goossens *et al.*, 2008; Hardarson *et al.*, 2008; Jansen *et al.*, 2008; Mastenbroek *et al.*, 2008; Mersereau *et al.*, 2008a; Mersereau *et al.*, 2008b; Meyer *et al.*, 2009; Schoolcraft *et al.*, 2009; Staessen *et al.*, 2008), however the overall message from these reports is that PGS does not improve the pregnancy rate. Checa et al. (2009) postulated that, in women with poor prognosis, undergoing IVF and PGS is associated with lower pregnancy and live birth rates (Checa *et al.*, 2009).

1.5.5.1. PGS problems

Over the last few years various reasons for the failure of PGS have been published. Beyer et al. (2009) and unpublished data from the Griffin lab found that, culture medium can improve or negatively affect PGS success rates in patients aged less than 40 years old (Beyer *et al.*, 2009). The necessity of safer biopsy was underlined by Handyside and Thornhill in 2007 (Handyside and Thornhill, 2007). Also Cohen et al. 2007 suggested that extended times of biopsy should be avoided (Cohen and Grifo, 2007). In addition, until recently, PGS was performed using cleavage stage embryos which are now known to be associated with a higher level of chromosome instability

resulting in chromosome aneuploidies, mosaicism, uniparental disomies, segmental deletions and duplications (Vanneste *et al.*, 2009) which could also be a reason for the lack of success of PGS. It has also been suggested that in some centres PGS has been carried out with 2 or 3 embryos; this is much smaller than the minimum number of embryos (6-8) required for biopsy to increase live birth rates after PGS (Munne *et al.*, 2007b; Summers and Foland, 2009).

1.5.5.2. FISH related to PGS

The FISH technique used in PGS has a number of limitations. Following biopsy the blastomere has to be fixed on a glass slide in order to perform FISH analysis. Two methods exist to prepare the blastomere; Tween/HCl and methanol/acetic acid. It has been published that the methanol/acetic acid method facilitates better blastomere fixation, with less overlapping of signals and less cell loss (Velilla *et al.*, 2002). The available number of fluorochromes within the visible spectrum limits FISH diagnosis for a maximum of 5 chromosomes at a time. In addition, some probes can demonstrate cross hybridisation to sites on other chromosomes and some probes appear under more than one filter resulting in false positive results (Donoso and Devroey, 2007; Donoso *et al.*, 2007a; Wilton *et al.*, 2009).

Study by Ruangvutilert and colleagues have assessed the efficiency of FISH on metaphase and interphase nuclei. In this study, in order to assess FISH probe efficiency, skin fibroblast cultures from trisomic and triploid foetuses were analysed. Results suggested that expected FISH results were obtained in 100% of metaphases and only 80%-90% in interphase nuclei (Ruangvutilert *et al.*, 2000a). This is due to

hybridisation failure of probes to the target DNA. FISH probe hybridisation failure is possible if the target DNA is not fully denatured and this results in false negative results (Wilton *et al.*, 2009). On the other hand, split signals due to DNA conformation is also associated with misdiagnosis giving false positive results (Wilton *et al.*, 2009).

A study by Ruangvutilert and colleagues performed FISH analysis on day 5 arrested and blastocyst stage embryos and found out that in most of the blastocysts had a majority of diploid cells and arrested embryos had variable number of diploid and aneuploid cells (Ruangvutilert *et al.*, 2000b). In addition, Uher and colleagues have reported that poor quality embryos have a higher chance of having degenerate interphase chromatin, apoptotic cells or cytoplasm that can interfere with FISH signals (Uher *et al.*, 2009).

Despite the controversy related to FISH and PGS it has been suggested that screening for more chromosomes could provide a more comprehensive diagnosis in the detection of aneuploid embryos. Currently most clinics use 9 probes in a two layer experiment (Thornhill *et al.*, 2005). A study from Baart and colleagues involved screening for 15 chromosomes in cryopreserved day 4 and 5 embryos using three rounds of hybridisation and suggested that investigating 6 extra chromosomes allowed them to detect mainly chromosome aberrations of mitotic origin leading to a higher percentage of mosaic embryos (Baart *et al.*, 2007). Using three rounds of hybridisation, another study by Colls et al. (2009) screened for 12 chromosomes and found that embryos diagnosed as normal for the initial chromosome panel had extra

abnormalities that would not have been found without extended screening (Colls *et al.*, 2009).

1.5.5.3. Mosaicism and PGS

Chromosome mosaicism is a biological complexity responsible for PGS inaccuracies which occur due to post zygotic mitotic errors. This is one of the most common reasons for PGS misdiagnosis and embryo wastage, especially if the result is based on one cell that may not represent the chromosome status of the whole embryo (Donoso and Devroey, 2007; Donoso *et al.*, 2007a; Fauser, 2008; Fritz, 2008; Hernandez, 2009). For example, in a recent study, FISH was used to compare the chromosomal aneuploidies in day 3 single cells vs. day 5 whole embryos and found out that out of 198 abnormal embryos 164 were confirmed as aneuploid giving a positive predictive value of 83% however 17% of embryos were misdiagnosed as abnormal on day 3 when they are in fact normal (DeUgarte *et al.*, 2008). A large number of studies have observed a similar outcome when it comes to day 3 vs day 5 dignosis in human embryos (Mantzouratou and Delhanty, 2011; Santos *et al.*, 2010) . Mosaicism in blastomeres has also seen using comparative genomic hybridisation technique (CGH) by some studies (Voullaire *et al.*, 2000; Wells and Delhanty, 2000).

The level of mosaicism at cleavage stage embryos on day 3 has been reported to be as high as 57% (Donoso *et al.*, 2007a) and high levels of chromosomal instabilities have been reported (Vanneste *et al.*, 2009). A study by Santos and colleagues tested the chromosomal mosaicism in human embryos on days 4, 5 and 8 and reported 83% of day 4 embryos were mosaic reducng to 42% on Day 8 (Santos *et al.*, 2010).

Hernandez 2009 has also argued that the high 57% level of chromosome mosaicism seen in the 8 cell stage is reduced to 30% in miscarriages, 20% in still births and 0.3% in newborns, indicating the existence of a self correction mechanism (Hernandez, 2009).

1.5.5.4. PGS current status

The scientific and clinical community still remain divided regarding the application of PGS and its outcome with regard to improving pregnancy rate (Anderson and Pickering, 2008; ASRM, 2008; Geraedts *et al.*, 2010; Harper *et al.*, 2008a; Harper *et al.*, 2008b; Harper *et al.*, 2010; Harper and Harton, 2010; Jansen *et al.*, 2008; Mastenbroek *et al.*, 2008).

It has been agreed that PGS by FISH on cleavage stage embryos does not improve pregnancy. Therefore methods being developed to assess the full karyotype of embryos such as microarreays.

1.5.5.4.1. Microarray

In addition to the cell type used in PGS, the FISH technique has been overpowered by the modern microarray technique. Microarray involves hybridising DNA sequences from a test genome sequence to a glass slide fixed with thousands of DNA fragments (Le Caignec *et al.*, 2006), followed by computer based analyses of the colour emission of the hybridisation patterns (de Ravel *et al.*, 2007; Fiegler *et al.*, 2006).

Microarrays are preferable to FISH in several aspects. Microarrays output a huge amount of data at one time, equivalent to thousands of FISH experiments. They are considered to be more accurate as they use automation of the protocol and results analysed by *in silico* systems.

1.5.5.4.2. Comparative genomic hybridisation

Comparative genomic hybridisation is a genetic test which compares DNA from the sample of interest compared to a normal control. DNA from the test and control are labelled with different colour fluorochromes (either red or green). These are mixed and allowed to hybridise to metaphase spreads from a normal male control. Following that specialised computer program analyses the red green fluorescent ratio in each metaphase and output the ratios as ideograms. This technique first applied to blastomeres by Wells and colleagues and Voullaire and colleagues in 1999 (Voullaire *et al.*, 2000; Wells and Delhanty, 2000) with the successful live births (Wilton *et al.*, 2001). CGH has also use embryos in blastocyst stage (Schoolcraft *et al.*, 2010) as well as on polar bodies (Wells *et al.*, 2002).

1.5.5.4.3. Array CGH

Array comparative genomic hybridization (aCGH) is a high resolution technique that has the ability to analyse copy number variation using WGA amplified test DNA. aCGH use the same principle as in metaphase CGH with the use of array platform to bind DNA from the target and control. This technique scans the genome for gains or losses of chromosomal material through comparative hybridisation of a patient and

control DNA (de Ravel *et al.*, 2007). aCGH is now been used for the PGD cases (Fiorentino *et al.*, 2011; Traversa *et al.*, 2011) and detection of aneuploidy following PGS (Fishel *et al.*, 2010; Le Caignec *et al.*, 2006; Traversa *et al.*, 2011; Vanneste *et al.*, 2009), with successful results in some cases leading to a live birth (Fishel *et al.*, 2010). aCGH is also used on polar bodies (Geraedts *et al.*, 2011; Magli *et al.*, 2011), cleavage stage embryos (Hellani *et al.*, 2008) and in trophectoderm cells (Yang *et al.*, 2012) and currently, randomised trials performed on aCGH based PGD around the world in order to see the effectiveness on this method (Harper and Sengupta, 2012). However disadvantages of aCGH are including, unable to detect polyploidies, balanced translocations, inversions and alterations in DNA sequences such as point mutation and smaller aberrations less than 1MB for certain.

1.5.5.4.4. SNP arrays

SNPs are the most common form of genetic variation amongst the human population. The human genome contains millions of SNPs making them the ideal genetic marker for genome screening. Several studies have reported the application of SNP arrays clinically for PGD and PGS (Handyside *et al.*, 2009; Johnson *et al.*, 2010; Northrop *et al.*, 2010; Treff *et al.*, 2010a; Treff *et al.*, 2010b; Vanneste *et al.*, 2009). It has been reported that SNP arrays allow the most comprehensive screening of IVF embryos as they provide information regarding chromosomal abnormalities, single gene defects as well as distinguish the parent and phase that the abnormality originated from (Handyside, 2011).

1.5.5.4.5. Karyomapping

Karyomapping is a novel molecular cytogenetic method that uses SNP genotype analysis from parents and offspring to construct a 'Karyomap' by mapping crossovers between parental haplotypes (Handyside *et al.*, 2009).

Karyomapping can screen all 24 chromosomes and detect monosomies, trisomies, deletions, duplications, uniparental disomies, translocations and monogenic disorders simultaneously. It can also distinguish the parental origin and meiotic phase of the aneuploidy.

1.6. Thesis aims

As mentioned above, chromosome copy number and nuclear organisation play an important role in human development and any errors of these can cause diseases. The link between chromosome copy number and nuclear organisation has been studied in many cell types but human sperm and embryos still remain relatively under explored especially in relation to various medical conditions such as infertility and cancer.

Several studies of sperm chromo-centric arrangement of chromosomes have been published. A recent study in our lab compared the centromere localisation of normal and infertile groups with regard to all 24 chromosomes; however, telomere and subtelomere positions of normal and infertile patient groups have not been studied yet. Similarly, the effect of cancer on sperm cells thereby on fertility is studied in

many levels including sperm count, morphology, motility, chromosomal aneuploidy and DNA fragmentation. However, the effect of cancer and chemotherapy on sperm nuclear architecture still remains unknown. The first two chapters of this thesis aim to address issues related to sperm telomere localisation and the effect of cancer and chemotherapy on sperm nuclear architecture.

Among preimplantation embryos analysis of chromosome copy number and position is generally limited to a small sub set of chromosomes and a fewer number of embryos. Only recently, in our lab all 24 chromosomes in embryos were assessed, however this study has a limitation with regard to number and quality of the embryos as well as the method used to spread and fix embryos to the slide. Therefore, the second half of this thesis will focus on chromosomal copy number and nuclear organisation related issues in human preimplantation embryos using both 5/8 chromosome screening (chapter 5) and 24 chromosome screening (chapter 6 & 7).

The specific aims of this thesis are therefore as follows:

1. To test the hypothesis that telomere organisation is altered in men with severely compromised semen parameters.
2. To test the hypothesis that nuclear organisation in sperm heads is altered due to cancer and chemotherapy.
3. To assess the accuracy, reliability and success of PGS by comparing chromosome copy number abnormalities in day 3 vs. day 5 follow up embryos in a clinical data set.

4. To assess chromosome copy number abnormalities in human embryos with regard to maternal age, embryos day 3 morphology and embryo development stage using 24 chromosome screening.
5. To assess the nuclear organisation in human embryos using 24 chromosome screening and test the hypothesis that altered nuclear organisation is related to chromosome abnormalities, maternal age, embryo morphology and embryo development stage.

2. Materials and methods

2.1.Sperm studies

2.1.1. Sperm samples (for telomere study)

Control sperm samples were obtained from 10 chromosomally normal males from the donor insemination program at the London Bridge Fertility, Gynaecology and Genetics centre. Patient samples were obtained from 10 men undergoing male factor IVF treatment at the Embryogenesis Clinic in Athens, Hellas. All control donors and patients have given informed written consent for thier samples to be used in research. This research was approved by the Research Ethics Committees of the University of Kent (HFEA license 0700/L0070-18-c awarded to the Bridge clinic and by the University of Kent Local Research and Ethics Committee).

2.1.2. Sperm samples (for cancer and chemotherapy study)

10 patients (testicular cancer n = 5 and Hodgkin's lymphoma n = 5), visited McGill University Health Centre, Montreal and were recruited to this study. Testicular cancer patients were given 2–4 cycles of BEP chemotherapy (bleomycin, etoposide, cisplatin), and Hodgkin's lymphoma patients had 4–8 cycles of ABVD chemotherapy (doxorubicin, bleomycin, vinblastin, dacarbazine). None of the patients were treated with radiotherapy. All recruited patients have given informed consent, and this research project was approved by institutional ethics committees (HFEA license 0700/L0070-18-c awarded to the Bridge clinic and by the University of Kent Local Research and Ethics Committee).

Due to azoospermic conditions, samples could not be obtained from all patients' at every time point. Therefore this study focused on patients who donated samples at least at two time points; the first being after diagnosis of cancer (pre treatment) followed by at least one time point after treatment. According to this criterion, sperm from five testicular cancer patients (average 26 years, range 21–28 years), five Hodgkin's lymphoma patients (average 24.8 years, range 19–36 years) and 10 age-matched healthy control donors (average 24.9 years, range 18–32 years) were analysed.

Samples were collected from patient groups before and 6, 12 and/or 18–24 months after the initiation of treatment. FISH was performed in Department of Medical Genetics, University of Calgary by Prof.Renée Martin's research group in order to assess aneuploidy levels. Slides were then sent to University of Kent to perform nuclear organisation analysis. In order to make comparisons across the different time points between cancer types and with controls, samples from control males were also studied at the same time points.

2.1.3. Sperm sample preparation for telomere study (Chapter 3)

Standard protocol for sperm preparation for observation was used. This method can be applied to fresh ejaculate or cryopreserved sperm samples. The sample was first washed in sperm wash buffer (10mM NaCl/10mM Tris pH 7.0) (Sigma-Aldrich, UK. S7653/T1503) then centrifuged for 7 minutes at 1,900rpm (700g). The supernatant was removed without disturbing the pellet and re-suspended in sperm wash buffer. This was repeated 3-5 times depending on sample quality and amount. When the

pellet was clear, the sample was fixed in 3:1 methanol: acetic acid (Sigma-Aldrich, UK, 34860-2.5L-R, 320099-2.5L) solution, by adding fix solution drop wise until the final volume was 5ml. Tubes were then centrifuged at 1,900rpm for 7 minutes and the supernatant was removed without disturbing the pellet. This was repeated several times. Tubes were stored in the freezer at -20°C degrees to be used later for FISH. In order to check the density of the sample, approximately 5µl of sample was spread on a Poly-L-lysine coated slide (Sigma-Aldrich, UK. P0425) and air dried. The slide was then checked under a phase contrast microscope (Olympus, UK. BX60) for optimal density.

2.1.4. Sperm sample preparation for cancer and chemotherapy study (Chapter 4)

Sperm samples were prepared using the above standard protocol at the Department of Medical Genetics, University of Calgary. In order to perform the analysis of chromosome positions, slides were sent to University of Kent, Canterbury, UK. Slides were labelled with code numbers in order to ensure blind scoring.

2.1.5. Probes for sperm studies

Depending on the purposes of each chapter, different sets of probes were used. In chapter three telomere specific probes and q and p arm sub telomere probes for a sub set of chromosomes (table 2.1) were used.

Table 2.1: probes used in telomere study

Probe	Locus	Catologue Numbers
TelVysion 1p SpectrumGreen	CEB108/T7	Abbott Moleculer USA 05J03-091
TelVysion 2p SpectrumGreen	VIJyRM2052	Abbott Moleculer USA 05J03-092
TelVysion 3p SpectrumGreen	D3S4559	Abbott Moleculer USA 05J03-013
TelVysion 4p SpectrumGreen	GS10K2/T7; 4p02	Abbott Moleculer USA 05J03-014
TelVysion 5p SpectrumGreen	C84c11/T3	Abbott Moleculer USA 05J03-015
TelVysion 8p SpectrumGreen	D8S504	Abbott Moleculer USA 05J03-098
TelVysion 10p SpectrumGreen	10pTEL006	Abbott Moleculer USA 05J03-090
TelVysion 1q SpectrumOrange	VIJyRM2123, 1QTEL10	Abbott Moleculer USA 05J04-091
TelVysion 2q SpectrumOrange	D2S447	Abbott Moleculer USA 05J04-092
TelVysion 3q SpectrumOrange	D3S4560	Abbott Moleculer USA 05J04-093
TelVysion 4q SpectrumOrange	D4S2930	Abbott Moleculer USA 05J04-094
TelVysion 5q SpectrumOrange	D5S2907	Abbott Moleculer USA 05J04-095
TelVysion 8q SpectrumOrange	VIJyRM2053	Abbott Moleculer USA 05J04-098
TelVysion 10q SpectrumOrange	D10S2290	Abbott Moleculer USA 05J04-090
TelVysion 14q SpectrumOrange	D14S1420	Abbott Moleculer USA 05J04-024
TelVysion 15q SpectrumOrange	D15S936	Abbott Moleculer USA 05J04-025
TelVysion 21q SpectrumOrange	VIJyRM2029	Abbott Moleculer USA 05J04-031
TelVysion 22q SpectrumOrange	MS607;ACR	Abbott Moleculer USA 05J04-032

Table 2.3 shows the subtelomere probes used in the chapter 3 of this thesis (nuclear organisation of telomeres and subtelomeres), specific loci which probes bind to and catalogue numbers for each probe.

For the cancer and chemotherapy study (chapter 4), chromosomes 1, 13, 21, X and Y were analysed utilising two-colour FISH for chromosomes 13 (Vysis LSI 13 13q14 SpectrumGreen) (Abbott Moleculer, USA. 05J14-028) and 21 (Vysis LSI 21 21q22.13-q22.2 Spectrum Orange) (Abbott Moleculer, USA. 05J13-012) and three colour FISH for the sex chromosomes; Vysis CEP X Xp11.1-q11.1 Alpha Satellite DNA SpectrumGreen ^{TM} (Abbott Moleculer, USA. 05J10-033), Vysis CEP Y Yp11.1-q11.1 Alpha Satelite DNA SpectrumOrange) (Abbott Moleculer, USA. 05J08-034). Chromosome 1 (Abbott Moleculer, USA. 06J39-036) was used as an internal autosomal control to distinguish diploidy from sex chromosome disomy.

2.1.6. Fluorescent in situ hybridisation on sperm cells

2.1.6.1. Slides preparation

In sperm samples density was checked under a phase contrast microscope. Then sperm slides were aged for 1 hour at 70°C in a thermobrite. The slides were then de-condensed in 10mM DTT (Sigma-Aldrich, UK. D9779-10G) 0.1M Tris-HCl, pH: 8.0 (VWR, UK. 103156X) to, at RT for 20 minutes and rinsed in 2 X SSC (Fisher, UK. BPE1325-4). This was followed by slide dehydration by running through ethanol washes (70%, 80%, and 100%) for 3 minutes each. Slides were then treated with pepsin (Fisher Scientific, UK. 9001-75-6) to remove cytoplasm. Pepsin solution (49ml of ddH_2O and 0.5ml of 1N HCl) was pre-warmed at 37°C in a coplin jar (Fisher Scientific, UK. MNK-730-010F); pepsin was added just before slides were added in. The lides were incubated in pepsin solution for 20 min at 37°C followed by washes with ddH_2O and PBS (Invitrogen, UK. 20012-019) before being added to paraformaldehyde solution which was made by adding 500ml 37% formaldehyde (Fisher Scientific, UK. 50-00-0) to sodium hydrogen carbonate (Fisher Scientific, UK. BPE328-1) to saturate the solution. The slides were incubated in paraformaldehyde (1.34ml of 37% paraformaldehyde in 49ml of PBS) for 10 minutes at $4^{o}C$. Following paraformaldehyde treatment, the slides were rinsed with PBS (Invitrogen, UK. 20012-019) and ddH_2O and washed in an ethanol series for 2 minutes in each. The slides were then air dried and the probe mix was prepared.

2.1.6.2. Probe preparation

For telomeres, pantelomeric probe (Cambio, UK. 1696-B-02) was used; 1µl of this probe was added to 12.5µl of hybridisation mix (Cambio information sheet). The

probe/hybridisation mix was denatured for 10 minutes at 85°C and then quickly placed on ice until it was ready to be applied.

When vysis probes were used either for sub telomeres or centromere regions, the probes were taken out of the freezer and left at room temperature for 10 minutes. The probes were mixed in a vortex mixer (Labnet International, UK. VX100) and briefly spun in a mini centrifuge (Labnet International, UK. C1301).

2.1.6.3. Denaturation & Hybridisation

Different probes used in this study had different denaturing protocols. When telomere probes were used, the probes were separately denatured at 80°C for 10 minutes. The probes were then applied to the slide, covered with a cover slip (Fisher Scientific, UK.) and Parafilm® (Fisher Scientific, UK. SEL-400-050J) to prevent any probe leaking or drying out and co-denaturation was carried out at 75°C for 5 minutes. When sub telomere probes or centromeric probes (vysis) were used, probes were added into the sample and only co-denaturation was carried out at 75°C for 5 minutes. Following denaturation of any probe, hybridisation was performed by putting the slides in a thermobrite (Stretton Scientific Ltd, UK. 7J9120) at 37°C overnight.

2.1.6.4. Post hybridisation washes

When telomere probes were used, following hybridisation, the slides were removed from thermobrite (Stretton Scientific Ltd, UK. 7J9120) and the Parafilm® (Fisher Scientific, UK. SEL-400-050J) removed carefully. The slides were then placed in a coplin jar with 0.7 X SSC-0.3% Tween 20(Fisher, UK. BPE1325-4) and (Sigma-

Aldrich, UK. P2287) at RT until the cover slips floated off. This was followed by washing the slides in the same solution at 37°C for 10 minutes and then transferring them into 2x SSC (Fisher, UK. BPE1325-4) for 2 minutes at RT. Since telomeric probes with indirect labelling were used, detection with Cy3-streptavidin (GE Healthcare, UK. PA43001) was needed. Detection buffer was made by adding 4 X SSC, 0.05% Tween, 1.5% (Fisher, UK. BPE1325-4) and (Sigma-Aldrich, UK. P2287) BSA and Cy3-streptavidin (GE Healthcare, UK. PA43001) in a dilution of 1:200. 100µl of detection mix was added onto each slide, covered with a cover slip and incubated for 30 minutes at 37°C. The cover slips were then removed and the slides were washed in storage buffer for 10 minutes at RT followed by a rinse with ddH_2O. The slides were then air dried, mounted with vectershield with DAPI (Vector lab, UK. H1200) with cover slips applied and stored at 4°C until microscopic analysis.

When sub telomere probes were used, after hybridisation, slides were removed from thermobrite and the Parafilm® (Fisher Scientific, UK. SEL-400-050J) removed carefully. Slides were then placed in coplin jar with 0.7 X SSC-0.3% Tween (Fisher, UK. BPE1325-4) and (Sigma-Aldrich, UK. P2287) 20 at RT until the cover slips floated off. This was followed by washing slides in the same solution at 72°C for 2 minutes and then transferring into 2x SSC (Fisher, UK. BPE1325-4) for 2 minutes at RT. When sub telomeric probes were used, the slides were rinsed in ddH_2O, mounted with vectershield with DAPI (Vector lab, UK. H1200) with a cover slip applied and stored at 4°C until microscopic analysis.

However if the probe set contained a blue signal (for example XY1 probe set in cancer and chemotherapy study), the slides were stained with DAPI in a 0.1ng/ml

DAPI solution (Sigma-Aldrich, UK. D9542) for 10 minutes. The slides were then mounted with vectashield (Vector lab, UK. H1000), covered with a cover slip and stored at 4°C for microscopy analysis.

2.2. Embryo studies

2.2.1. Embryo samples

Human embryos used in clinical data analysis study (chapter 5) were from patients undergoing preimplantation genetic screening for aneuploidy at The London Bridge Fertility Centre (LBFC). In this part of the study, single cells and whole embryos were spread using HCL-tween method as discibed in (Coonen *et al.*, 1994; Harper *et al.*, 1994) . Single cell PGS and follow up studies were performed as a part of the clinical procedure by trained Molecular cytogenetisists worked in the clinic. I have performed FISH in approximately 5% of the PGS and follow up cases with in 2008-2010 during my training. Pre existing data was used for the purpose of research. This work was approved under the auspices of the treatment licence awarded by the HFEA to the London Bridge Fertility Centre. The Research and Ethics committee of the University of Kent have also approved this work (HFEA license 0700/L0070-18-c awarded to the Bridge clinic and by the University of Kent Local Research and Ethics Committee and the Greek Authority of Assisted Reproduction, 7/2009).

Human embryos used in results chapters 6 and 7 of this thesis were from patients undergoing PGS for aneuploidy at the Embryogenesis Clinic in Athens, Hellas. Patients have given written consent for these embryos to be used for research purposes. Embryos were fixed into slids by embryologists worked in Embryogenesis

Clinic in Athens and sent to University of Kent to perform 24 FISH. This work was approved by the local research and Ethics committee of the University of Kent.

2.2.1. Embryo preparation

Whole embryos were spread using HCL-tween, methanol: acetic acid combination method (Dozortsev and McGinnis, 2001) with an inside diameter of 175 microns pipet to transfer whole embryos from the biopsy dish to the spreading solution drop on a clean poly-L-lysine slide (Sigma-Aldrich , UK, P0425). Gentle agitation was used to dissolve the cell membrane. Slides were allowed to dry, denatured and the estimated number of blastomeres making up each embryo was recorded. Slides were stored at 4°C and sent to the University of Kent within a day of spreading.

2.2.2. Fluorescent in situ hybridisation on preimplantation genetic screening with single cells and follow-up embryos (Chapter 5)

2.2.2.1. Slide preparation

Slides with single cells were placed in PBS (Invitrogen, UK. 20012-019) at RT for 3 minutes and dehydrated for 3 minutes each in 70%, 80% and 100% alcohol. The slides were then examined under the phase contrast microscope to locate cells and positions were recorded. The slides were then immersed in pepsin solution for 20 min at 37°C followed by a brief rinse in distilled water and then in PBS (Invitrogen, UK. 20012-019). The slides were transferred into a Screw Coplin jar containing 1% paraformaldehyde/PBS (Invitrogen, UK. 20012-019) for 10 minutes at 4°C, rinsed

briefly in PBS (Invitrogen, UK. 20012-019) then washed twice in distilled water followed by dehydration for 3 minutes each in an ethanol series at room temperature.

2.2.2.2. Probe preparation

Multivision PB probe (Abbott, USA. 08L62-020) was taken out of the freezer and allowed to warm to room temperature for at least 15 minutes. The probe was mixed using a vortex and centrifuged using a minicentrifuge. The probe was applied in a range of 0.4 µl – 1 µl according to the size of coverslip used. Each slide was inverted with the marked area of the slide placed face down onto a coverslip. The area was the covered with a piece of Parafilm® (Fisher Scientific, UK. SEL-400-050J) to prevent any probe leaking or drying out.

2.2.2.3. Denaturation & Hybridisation

The prepared slides were placed in a thermobrite and co-denatured at 75°C for 5 minutes. Following probe denaturation, hybridisation was allowed in the thermobrite at 37°C for 2.5 hours.

2.2.2.4. Post hybridisation washes

Following hybridisation, the slides were removed from thermobrite and the Parafilm® was carefully removed. The slides were then placed in a coplin jar with 0.7 X SSC-0.3% Tween 20 (Fisher, UK. BPE1325-4) and (Sigma-Aldrich, UK. P2287) at RT until the cover slips floated off. This was followed by washing slides in the same solution at 72°C for 3 minutes and then transferring into 2x SSC (Fisher, UK. BPE1325-4) for 2 minutes at RT. The slides were then mounted with vectershield

without DAPI (Vector lab, UK. H100), covered with cover slips and immediately analysed microscopically.

2.2.2.5. Rescuer layer

After image capture of the hybridised slides, a rescuer layer of FISH was performed to verify any uncertainties that may be due to signals overlapping, split signals or other fluorescent blobs present on the cell. First, immersion oil was removed from the slides by wiping it off with lens tissue, immersing in 2x SSC (Fisher, UK. BPE1325-4) until the cover slips floated off and then transfering into fresh 2x SSC (Fisher, UK. BPE1325-4) for about 2 minutes. The slides were then immersed in distilled water at 72°C for 30 seconds to remove any remaining signals from the 1st layer. They were then dehydrated for 3minutes each in an ethanol series. XY15 probe mixture (Abbott Moleculer, USA. 06J36-025, 05J09-033, 05J10-034) or one of the rescuer probe mixtures (XY13, XY16, XY18, XY21 or XY22) was applied to normal blastomeres from the first layer depending on the first layer results; i.e depending on the chromosome gave inconclusive result. The slides were then placed in a thermobrite (Stretton Scientific Ltd, UK. 7J9120) and co-denatured at 75°C for 5 minutes as was done for the first layer followed by an overnight hybridisation at 37°C.

2.2.2.6. Follow up FISH

As part of the clinical procedure, some patients request follow-up analysis of their abnormal or poor quality embryos. Unlike PGS single cell FISH, follow up studies involve spreading the whole embryo onto a slide, allowing cell by cell analysis with FISH. At the start of follow up FISH, the slides were stained with DAPI and examined under the 10X lens of a phase contrast microscope (Olympus, UK. BX60)

to create a map with the relative locations of cells. DAPI staining was then removed and standard FISH protocol was followed as in section 2.4.2.1-2.4.1.5 above.

2.2.3. Sequential FISH (for 24 chromosomes) in preimplantation embryos (Chapter 6 and 7)

For results chapters 6 and 7, four layers of sequential FISH assay were performed in blastomeres using 24 chromosome probes from Kreatech diagnostic (Kreatech Diagnostics, Netherland. MultiStar 24 FISH KBI-40061, KBI-40062, KBI-40063 and KBI-40064). Sequential FISH allows information regarding all 24 chromosomes to be gained from a single blastomere. A lymphocyte slide was run in parallel for control purposes.

2.2.3.1. Pre hybridisation treatment

The slides with embryos were placed in PBS (Invitrogen, UK. 20012-019) for 3 minutes at RT followed by dehydration by running through an ethanol series (70-80-100%) for 3 minutes each. They were then placed in a pre warmed 1% pepsin solution (Fisher Scientific, UK. MNK-730-010F) at 37°C, for 20 minutes. Next, they were rinsed with ddH_2O and PBS (Invitrogen, UK. 20012-019) and placed in paraformaldehyde solution which was made by adding 500ml 37% formaldehyde (Fisher Scientific, UK. 50-00-0) to sodium hydrogen carbonate (Fisher Scientific, UK. BPE328-1) at 4°C for 10 minutes. While slides are in paraformaldehyde, appropriate amount of probes were aliquoted in to a tube and left in 4°C. After incubation, the

slides rinsed in PBS (Invitrogen, UK. 20012-019) and ddH_2O, followed by dehydration in an ethanol series and then they were allowed to air dry.

2.2.3.2. Probe denaturation and hybridisation of the first layer

During the dehydration of slides, probes were denatured at 73°C for 10 minutes. These were added to the slides, covered with a cover slip and sealed with Parafilm® (Fisher Scientific, UK. SEL-400-050J). The slides were then placed in thermobrite (Stretton Scientific Ltd, UK. 7J9120) for 3 minutes followed by co-denaturation and 15 to 45 minutes hybridisation at 37°C.

2.2.3.3. Post hybridisation washes using Kreatech probes

After hybridisation, the slides were removed from the thermobrite (Stretton Scientific Ltd, UK. 7J9120) and remove the Parafilm® (Fisher Scientific, UK. SEL-400-050J) carefully. Slides were then placed in coplin jar with 0.7 X SSC-0.3% Tween 20 (Fisher, UK. BPE1325-4) and (Sigma-Aldrich, UK. P2287) at RT until the coverslips floated off. This was followed by washing the slides in the same solution at 72°C for 1 minute and then transferring into 2x SSC (Fisher, UK. BPE1325-4) for 2 minutes at RT. The slides were then rinsed in ddH_2O and stained with DAPI in a 0.1ng/ml DAPI solution (Sigma-Aldrich, UK. D9542) for 10 minutes. They were then mounted with vectashield, covered with a coverslip and stored at 4°C for microscopy analysis.

2.2.3.4. Subsequent layer reprobing and post-hybridisation washes

After capturing images of chromosomes in the first layer, the slides were immediately cleaned and placed in a coplin jar with 2xSSC (Fisher, UK. BPE1325-4) to remove

the cover slips. The slides were washed at 70°C in ddH_2O to remove the probes from the first layer followed by rinses in an ethanol series. An appropriate amount of probes was dispensed into a tube and denatured in a 73°C water bath for 10 minutes. Denatured probes were then added the slides, covered with a coverslip and placed in a thermobrite for 3 minutes of co-denaturation followed by a 2nd hybridisation round for 15 to 45 minutes. The third and fourth layers also followed the same protocol for stripping and reprobing. Hybridisation for the third layer was for 15-45 minutes however the fourth layer required overnight hybridisation. After hybridisation the slides were washed in 0.7 X SSC 0.3% Tween (Fisher, UK. BPE1325-4) and (Sigma-Aldrich, UK. P2287) at room temperature to remove the cover slips, followed by washing in the same solution at 72°C for 60 seconds. The slides were then placed in a 2XSSC solution for 2 minutes followed by DAPI staining with 0.1ng/ml DAPI solution (Sigma-Aldrich, UK. D9542) for 10 minutes. They were then mounted with Vectashield, covered with a cover slip and stored at 4°C for microscopy analysis.

2.3. Control lymphocytes

2.3.1. Lymphocyte samples

Lymphocytes from a normal karyotype male were used as a control. This was approved by the Research Ethics Committees of the University of Kent.

2.3.2. Lymphocyte culture preparation from whole blood cultures

Blood was taken from a healthy karyotyped donor via standard phlebotomy using a heparin tube (6ml maximum) (Fisher, VCT, UK-090-070Q). Before processing blood,

the fume hood was radiated with UV for 30 minutes in order to sterilise the working area. PB Max karyotype media (Invitrogen, UK. 12557-039) was pre warmed to 37°C at water bath. Peripheral blood was added into PB Max karyotype media in a 1:19 ratio in tissue culture flasks (CELLSTAR, UK). This mixture was incubated at 37°C for 72 hours in a 5% CO_2 incubator. After 72 hours, 200µl of demecolcine solution (Sigma-Aldrich, UK. D1925 - 10g/ml in HBSS liquid, sterile filtered, AFC Qualified) was added in order to arrest cells in metaphase and they were incubated for 40 minutes at 37°C. During this incubation time 0.075M KCl (Sigma-Aldrich, UK. P9333) solution was allowed to warm to 37°C. At the end of incubation blood cultures were transferred into 15ml falcon tubes (10ml per falcon tube) and centrifuged at 1,900rpm for 5 minutes. The supernatant was removed and pre warmed KCl solution was added drop wise up to 6 ml while vortexing the mixture to allow constant agitation. Samples were incubate with KCl (Sigma-Aldrich, UK. P9333) for 12 minutes at 37 °C in order to allow red blood cell lysis. Tubes were then filled with freshly made 3:1 methanol: acetic acid (Sigma-Aldrich, UK. 34860-2.5L-R, 320099-2.5L) up to 14ml and centrifuged for 5 minutes at 1,900rpm. This was followed by the removal of the supernatant leaving up to 1ml of fix without disturbing the pellet. The pellet was then re- suspended in the remaining fix with more added to make up to 5ml and centrifuged for 5 minutes. This process was repeated 3- 5 times depending on sample quality and quantity; samples were then stored in the freezer at -20°C to be used in FISH experiments.

For clinical data analysis study (chapter 5), Vysis multivision probes were used in both day 3 and follow up analysis (Abbott Moleculer, USA. 08L62-020). The Vysis multivision probe set includes LSI 13 Spectrum Red (13q14) , LSI 21 Spectrum

Green (5 loci 21q2-22), CEP 18 Spectrum Blue (D18Z118p11.1- q11.1), CEP 16 Spectrum Aqua (16q11.2 and D16Z3) and LSI 22 (22q11.2). XY15 probes which include CEP15 (α sat) (Spectrum Orange) (D15Z4) (Abbott Moleculer, USA. 06J36-025), CEPX (Spectrum Aqua) (Xp11.1-q11.1/DXZ1) (Abbott Moleculer, USA. 05J09-033) and CEP Y (satellite III) (Spectrum Green) (Yq12/DYZ3) (Abbott Moleculer, USA. 05J10-034) were used for the second layer.

Multicolour probes designed by Kreatech Diagnostics for target all chromosomes were used in chapters 4 and 5, in order to investigate aneuploidy and nuclear architecture of the human blastomere. These probes consist of 4 different mixes (Kreatech Diagnostics, Netherland. MultiStar 24 FISH KBI-40061, KBI-40062, KBI-40063 and KBI-40064) each with sequences for 6 different chromosomes. The first 3 panels use centromeric sequences (panel 1: chromosomes 1,3,4,6,7,8; panel 2: chromosomes 9,10,11,12,17,20 and panel 3: chromosomes 2,15,16,18,X,Y) and panel 4 uses unique sequence probes for chromosomes 5,13,14,19,21,22 since centromeric sequences are not specific for these chromosomes.

2.3.3. FISH on control lymphocytes

2.3.3.1. Slide preparation and ageing

Superfrost slides were used after cleaning with 3:1methanol: acetic acid and allowing to air dry. Lymphocyte cultures were centrifuged for 5 minutes at 1,900rpm to concentrate the cells into pellet. The supernatant was removed leaving about 1ml of pellet which was resuspended. Approximately 0.5 ml of the lymphocyte sample was dropped onto a marked area of a clean glass slide, air dried and then checked for

optimum density under a phase contrast microscope. The slides were then allowed to age in the thermobrite (Stretton Scientific Ltd, UK. 7J9120) for 1 hour at 37°C.

2.3.3.2. Pre hybridisation washes

Together with embryos or sperm slides, control lymphocyte slides were rinsed with PBS (Invitrogen, UK. 20012-019) in a coplin jar and dehydrated by running through in an ethanol series (70%, 80% and 100%) for 3 minutes each. The slides were then air dried at room temperature. Next, they were placed in pepsin solution for 20 minutes at 37 °C to remove cytoplasm from the cells. After incubation with pepsin, the slides were briefly run through washes with ddH_2O and PBS (Invitrogen, UK. 20012-019) to wash off any left over pepsin solution. They were then transferred into a paraformaldehyde solution for 10 minutes in a fridge at 4°C. After incubation the slides were a rinsed in PBS (Invitrogen, UK. 20012-019) and ddH_2O, dehydrated in an ethanol series and allowed to air dry.

2.3.3.3. Denaturation, hybridisation and post hybridisation washes

Lymphocyte slides denature in only 3.5 minutes; this could be either co-denaturation with a probe or separate denaturation on a thermobrite (Stretton Scientific Ltd, UK. 7J9120) at 75°C or in a formamide solution (Fisher, UK. 18109-0010) depending on the protocol for each specific probe. Hybridisation and post hybridisation washes were usually the same as for test embryo or sperm slides depending on the probe used.

2.4. Image capturing

For all experiments other than clinical data analysis studies, slide images were captured using SmartCapture software (Digital Scientific, UK) on an Olympus BX-61 Epifluorescence microscope (Olympus, UK. BX-61) equipped with a cooled CCD digital camera (Olympus, UK). For telomere study, Cy3 and DAPI dedicated filters were used. For Sub telomeres, Cy3, FitC and DAPI filters were used. For cancer study, images were captured using FitC, Cy3, blue and DAPI filters. For 24 FISH studies, position 7 filters were used to accommodate all fluorochromes required (red, green, aqua, gold, blue, far red and DAPI) through the use of two communicating filter wheels (Digital Scientific, UK.). All images were captured using SmartCapture software (Digital Scientific, UK.). Appropriate levels of threshold were achieved by manually adjusting the histogram of the software and exporting as tiff files for further analysis. For the clinical data analysis study, FISH images of blastomeres were captured using an epifluorescence microscope with Cytovysion Software (Applied Imaging, UK).

2.5. Image Analysis

2.5.1. Analysis of radial chromosome positioning

Novel and automated systems based on methodology presented by (Croft *et al.*, 1999) were used in order to measure the chromosome position within the interphase nucleus to determine nuclear organisation. This method has been previously described in detail in (Ioannou and Griffin, 2010; Skinner *et al.*, 2009). Following capture, images were exported and analysed using a macro program written for ImageJ software (Digital Scientific, UK). During export, each of the images was converted into red,

green and blue binary masks, where signals were represented in red and green and DAPI counterstaining was represented in blue. For 6 colour FISH each 2 colours were converted into a binary mask with 3 planes consisting of red-far red, gold-blue and green-aqua, each with DAPI counter staining.

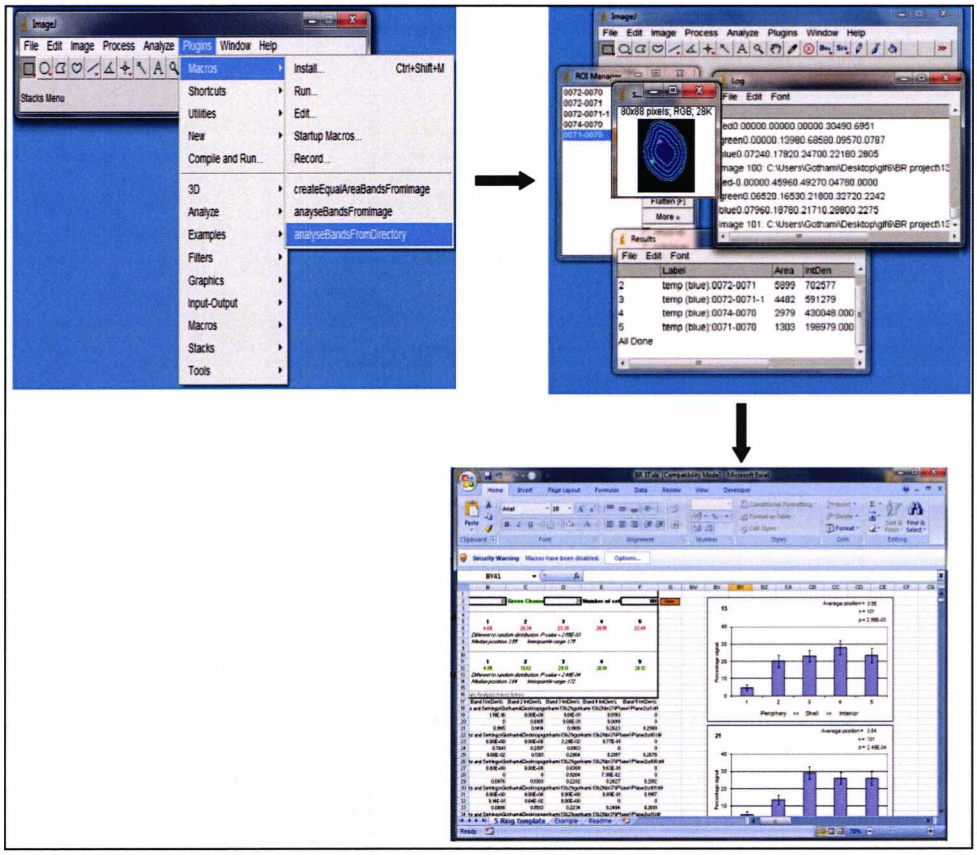


Figure 2.1: Radial chromosome position analysis by imageJ macro
In order to perform image analysis, captured images run through a macro via Image J program. Macro program recognise the nuclear periphery of the sperm head and divide nuclear into 5 rings with equal area which represent the equal volume in 3D. Software also output the percentage of red green and blue signal in each shell las a log file which can then be copied and pasted into excel to perform calculations.

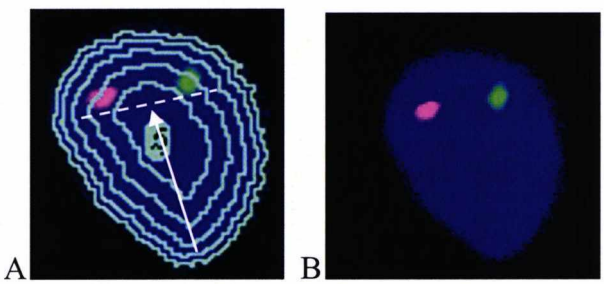


Figure 2.2: Radial and longitudinal analysis
Picture (A) is a sperm head after radial analysis, signal position is measured related to the concentric shells. Pcture (B) shows how longitudinal analysis perform; i.e. distance to the signal form the tail as a fraction of the length of the sperm cell.

When images were analysed with Image J Macro, the macro program recognised the nuclear periphery and divided the cell nuclei into five concentric shells of equal area which represent the equal volume in 3D. It also segmented the nuclei into red-green (signals) and a blue (counterstaining) binary mask. In each concentric shell, the amount of red-green and blue signal were measured and presented as a log of red-green and blue channels. The amount of signal in each channel within each shell was measured relative to the total signal for that specific channel within the area covered by the binary mask (Skinner *et al.*, 2009). This log value was transferred onto a spreadsheet for analysis.

In this thesis, embryos were analysed according to the DAPI density model. However sperm cells were analysed according to two different mathematical models. DAPI density and volumetric models were used in order to correct any errors may have occurred due to extrapolating 2D data from the 3D nucleus. In the DAPI density model the amount of signal within each shell was normalised against the DAPI intensity in order to balance the 2D extrapolation of the 3D nucleus as described in (Boyle *et al.*, 2001). The volumetric model involves calculating the volume of the cell and the pressure created in the cell when it was flattened in order to normalise the data.

Percentages of normalised chromosomal signals within each shell were calculated to enable direct comparisons to be made between graphs. In order to test our null hypothesis that "chromosomes are randomly distributed in the sperm head" a $\chi 2$ test was used at 4 degrees of freedom and p=0.05. Calculated p values were considered

statistically different when $p<0.05$. When the calculated p value was >0.05 the distribution was considered as insignificantlydifferent from a random distribution.

In addition to the chi squared test, the peak of the graphical distributions of signals and standard error of the mean were taken into account in order to assign the overall positions of signals. The criterion used to allocate signals into the shell of preference is as follows. Peripheral – Shell 1 or 1/2, Peripheral/Medial – Shell 2 or 1-3, Medial – Shell 3, 2/3, or 3/4, Central/Medial – Shell 4 or 3-5, Central – Shell 5 or 4/5.

The overall position for a chromosome of a sample is implicated by the median of the percentage signal within each shell in the whole population of nuclei analysed. Median positions from each test group and controls were compared using a Student T test in order to investigate if there is a significant difference between the chromosome positions in each group.

2.5.2. Longitudinal chromosome positioning

Captured images were also utilised in order to find the longitudinal positions of chromosome loci. The chromosome specific signal positions were measured as the fractional length from the sperm tail using ImageJ. For longitudinal measurements, at least 50 signals were measured per chromosome/patient/time point adhering to strict criteria. The mean longitudinal position of each chromosome was calculated for each of the controls and patients at different time points in order to compare the longitudinal position within the group across pre and post treatment time points. Theresults of TC, HD and controls were then compared with each other using a T test in order to identify the differences between the cancer and control groups.

3. To test the hypothesis that nuclear organisation is altered in men with severely compromised semen parameters by assaying the pan-telomeric sequence and sub-telomeric loci for 11 chromosomes

3.1. Background

Male infertility is a complex phenomenon caused by various factors, many of which are chromosomally related (Griffin and Finch, 2005; Shah *et al.*, 2003; Tempest *et al.*, 2004). Nuclear organisation in the sperm head has been extensively studied however a link between chromosome position and infertility has not been fully established. The sperm nucleus is thought to have a defined architecture with chromosomes adopting a chromo-centric model; i.e. centromeres locate at the centre and telomeres point towards the periphery (Zalensky and Zalenskaya, 2007). This highly ordered nuclear organisation is vital for successful fertilisation; therefore it is reasonable to hypothesise that any alterations in the nuclear organisation should be a cause of compromised fertility. Preliminary results from the Griffin laboratory (Finch *et al.*, 2008b) for three chromosomes (X, Y and 18) suggested that sex chromosomes adopt a more random position in infertile men compared to controls. However a more recent study (Ioannou and Griffin, 2010) has demonstrated that nuclear organisation in the sperm head is robust and did not alter significantly in infertile patients. The latter study mostly looked at centromeric chromosome loci, however some q arm loci for certain chromosomes that do not have specific centromere sequences were also observed. Telomeric and sub-telomeric nuclear positions on the other hand, are yet to

be investigated. The current study therefore tested the hypothesis that these loci in the sperm heads of men with normal semen parameters display a predominantly peripheral localisation and that this position is altered in sperm of men with impaired fertility [i.e. with OligoAsthenoTeratozoospermia (OAT)].

3.2. Aims and hypotheses

The purpose of this chapter was therefore to assess the relative nuclear organisation of all telomeres collectively and sub telomeric loci of 11 chromosomes in the sperm nuclei of normal healthy donors and OAT patients. All sperm nuclear organisation studies in this thesis (including this one) make use of 2 different mathematical models to extrapolate 3D information from flattened 2D specimens. The first (see materials and methods 2.5.1) is more established and corrects for the relative nuclear position of more peripheral signals that may appear more central by assigning relative scores to a signal through reference to DAPI density in the nucleus. There are adaptations to this model that have been developed in house using a "macro" written in ImageJ (Ioannou and Griffin, 2010; Skinner *et al.*, 2009). Henceforth, this is referred to as the **"DAPI density model."** Due to concerns that sufficient differentiation of DAPI signal would not be achieved by visualising a highly compacted sperm head, a second approach was developed "in house" by my colleague Dr Ali Hojjat. This second approach uses a mathematical algorithm that models the likely volumetric space that a spherical or elliptoid object would occupy if it were flattened. Henceforth, this is referred to as the **"Volumetric model."** Using these two models nuclear organisation in 10 normal fertile male donors and 9 OAT patients were assessed and compared. In these

analyses we assayed both individual men pooled data in order to ask the following scientific questions.

1. To test the hypothesis pan-telomeric and sub telomeric loci in the sperm head can be shown to adopt non-random peripheral nuclear position using both the 3D extrapolation models outlined above in normal fertile males.
2. To test the hypothesis that nuclear organisation (as assayed above) is significantly altered in OAT men (using either approach) and thus that aberrant nuclear organisation is a marker of compromised male fertility.

3.3. Results

The pan-telomere sequence (TTAGGG) specific probes were used to detect the telomere regions of all chromosomes in the 10 control and 9 OAT patients (Meterial and methods sections 2.1.1, 2.1.3, 2.1.5, 2.1.6). Seman parameters for patients and controls were perfomed by the clinical staff and results presented in the tables 3.1 and 3.2 below.

Table 3.1: Semen parameters of healthy donors

	Initial count			Post Thaw		
	Conc in millions/ml	Motility in millions/ml	Progression (1-3)	Conc in millions/ml	Motility in millions/ml	Progression (1-3)
1	60	35	2	22	7	2
2	52	33	2-3	32	6	2
3	52	33	2-3	No Post thaw conducted		
4	107	82	2-3	22	5	1-2
5	45	39	2-3	20	4	2
6	54	41	2-3	26	2	2
7	48.6	43.6	2	17.9	5.7	2
8	79	31	2	34	5	2
9	100	90	3	52	16	2
10	43	31	2-3	70	7	2

Table 3.1 presents the imitial and post thaw sperm count, motility and progression of sperm from 10 healthy dorners from London Bridge fertility Centre.

Table 3.2: Semen parameters of OAT patients

Patient number	Count (ml)	Motility (%)	Progressive motility (%)
1	18	30	20
2	1	5	2
3	10	20	10
4	1.5	2	1
5	5	5	1
6	8	10	5
7	5	5	2
8	6	5	1
9	2	1	0.1

Table 3.2 presents sperm count, motility and progression of sperm from 9 OAT patients from Genesis fertility clinic Athens.

Specific sub telomere loci were detected in 11 chromosomes including 1, 2, 3, 4, 5, 8, 10, 14, 15, 21 and 22. For chromosomes 1, 2, 3, 4, 5, 8 and 10 p and q arm specific sub telomere positions were analysed. For acrocentric chromosomes 14, 15, 21 and 22 only the q arm was analysed as no p arm probes were available.

For the pan-telomere analysis, 1900 cells were analysed; for sub telomere analysis 30400 signals from 20900 sperm cells were analysed. On average 100 images were analysed per probe per patient. Figures 3.1-3.3 shows a gallery of images (fig 3.1A and B is the pan-telomeric probe, fig 3.1C shows the sub-telomeric probes for chromosomes 1 where the p arm is red and the q arm is green; fig 3.1D is the sub-telomeric probe for chromosome 22 (q arm only).

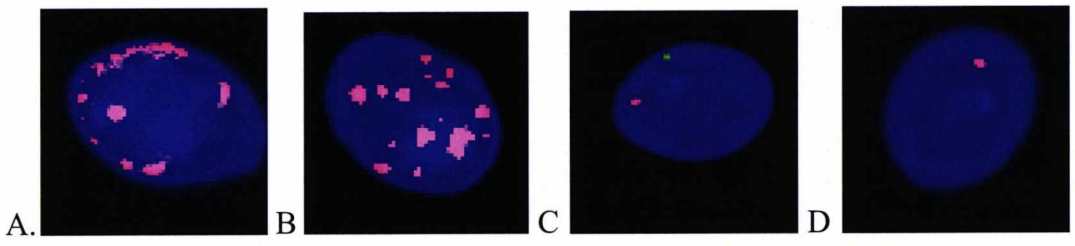


Figure 3.1: FISH images of the telomere sequence (TTAGGG) and subtelomere sequence on sperm cells. Image A shows a peripheral distribution of telomere signals in the sperm head and image B shows random localisation of telomere signals in the sperm head. Both images are from a contrl dorner. Image C shows FISH images of the sub-telomere sequence for chromosomes 1; p arm red and q arm green. Image D shows FISH images of the sub-telomere sequence for the q arm of chromosome 22 on sperm cells.

Using these two models outlined above, the following questions were addressed. Firstly "is there a non random pattern of nuclear organisation for each of the loci assayed?" This was asked both for individual loci/patients and for pooled data for each locus, considering the controls and patients as a group. Secondly we asked, if there was indeed a non-random pattern, which "shell" (shell 1 being the most peripheral, shell 5 being the most central) was predominantly represented. Third, to compare individuals and pools we asked what was the median position of all the signals assayed. To test for statistical significance for any differences, a two-tailed Student's T test was used. In a previous study in our lab (Ioannou and Griffin, 2010), all controls showed evidence of a "chromocentre" centromeric loci where all occupied a central position.

3.3.1. To test the hypothesis that pan-telomeric and sub-telomere regions in the sperm head adopt a non-random, peripheral position in the sperm heads of control males (using both models for 3D extrapolation).

Analysis of pan-telomeric and sub telomere positions in sperm cells of individual controls and patients (using both DAPI density and volumetric modes) produced a total of 722 graphs indicating the relative positions of all the loci in all the men studied. They are presented in the electronic appendix specific aim 1 section. In control males, visual inspection of the pan-telomeric probe on the sperm heads indicated a proportion of sperm heads for which a clear peripheral patterns could be seen (figure 3.1A). In other sperm heads however, the pattern was not so clear cut (figure 3.1B) and, indeed, in the final analysis only 1 control male showed a graph for which we were confident of predicting a peripheral pattern (figure 3.2). In

the majority however, regardless of which model was used, an apparently random pattern was the most common outcome (figure 3.2).

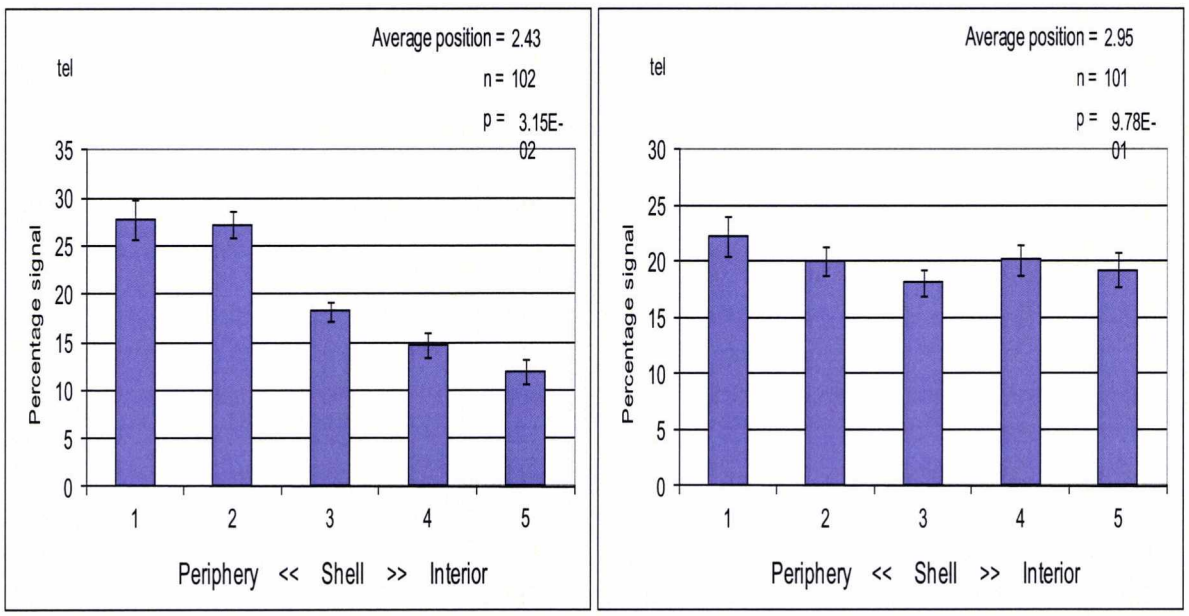


Figure 3.2: Results for nuclear organisation analysis, control male, peripheral (left) and random (right) pattern, pan telomere probe.
100 sperm images run through a macro program which analyse the nuclear positions of FISH signals. Software divides the each sperm head into 5 concentric shells and output percentage of signals in each shell which is presented as a graph in figure 3.2. Positions of signals were analysed with chi squared test and when p value is less than 0.05 results consideres as significant. Depending on the shell number (1to 5) majority of signals located, distribution is catogorised as peripheral, medial or central. When p value is higher than 0.05 results were considered as random.

Table 3.3 shows the numbers of controls for whom each type of pattern (e.g. peripheral, central, random) was seen. In each case, there is no clear evidence for a peripheral location of the pan-telomeric sequence (except in one case), despite the obvious peripheral distribution in certain sperm heads.

Table 3.3: Number of controls with each type of distribution – pan-telomeric sequence – both types of analysis models compared.

Chromosome number	Peripheral	Peripheral medial	Medial	Central medial	Central	Random	Total
DAPI	1	0	0	0	1	8	10
Volumetric	0	2	3	0	0	5	10

Controls with different sperm nuclear organisation patterns; Peripheral – Shell 1 or 1/2, Peripheral/Medial – Shell 2 or 1-3, Medial – Shell 3, 2/3, or 3/4, Central/Medial – Shell 4 or 3-5, Central – Shell 5 or 4/5.

Analysis then proceeded to individual chromosomes, albeit using sub-telomeric sequence specific probes. In this case, there was not such an obvious preference for any of the probes to occupy a peripheral position and, indeed, this was borne out by the analysis in which a peripheral location was rarely seen. Using the DAPI normalisation model, a total of 1/180 for any chromosome arm had peripheral localisation compared to 75/180 central and 38/180 random (tables 3.4 and 3.6). Using the volumetric model a total of 7/180 for any chromosome arm had peripheral localisation compared to 11/180 central and 27/180 random (tables 3.5 and 3.6). Table 3.4 illustrates all of the data interpreted using the DAPI density model, table 3.5 is the same but using the volumetric model and table 3.5 compares the total numbers of distributions for both models of analysis.

Table 3.4: Number of controls with different types of distribution per sub-telomeric loci analysed using DAPI density model.

Chromosome number	Peripheral	Peripheral medial	Medial	Central medial	Central	Random	Total
1p	1	0	1	4	1	3	10
1q	0	0	0	2	5	3	10
2p	0	0	4	0	0	6	10
2q	0	0	0	2	4	4	10
3p	0	0	0	1	7	2	10
3q	0	0	0	3	6	0	10
4p	0	0	0	2	8	0	10
4q	0	0	1	2	3	3	10
5p	0	0	0	3	5	2	10
5q	0	0	0	3	7	0	10
8p	0	0	2	3	1	4	10
8q	0	0	2	1	7	0	10
10p	0	0	0	5	3	2	10
10q	0	0	0	6	3	0	10
14q	0	0	1	4	5	0	10
15q	0	0	2	0	1	7	10
21q	0	0	1	2	4	2	10
22q	0	0	0	5	5	0	10

Using DAPI density model, controls with different sperm nuclear organisation patterns for various subtelomere loci; Peripheral – Shell 1 or 1/2, Peripheral/Medial – Shell 2 or 1-3, Medial – Shell 3, 2/3, or 3/4, Central/Medial – Shell 4 or 3-5, Central – Shell 5 or 4/5.

Table 3.5: Number of controls with different types of distribution per sub-telomeric loci analysed using volumetric model.

Chromosome number	Peripheral	Peripheral medial	Medial	Central medial	Central	Random	Total
1p	3	2	3	2	0	0	10
1q	0	1	4	3	0	2	10
2p	2	3	2	0	0	2	10
2q	0	0	4	3	0	3	10
3p	1	0	2	2	1	3	10
3q	0	1	4	3	1	1	10
4p	0	0	1	6	2	1	10
4q	0	2	4	1	1	2	10
5p	0	1	1	6	0	0	10
5q	0	1	2	5	2	0	10
8p	1	2	3	3	0	1	10
8q	0	0	7	0	1	2	10
10p	0	0	3	3	0	4	10
10q	0	1	2	4	1	2	10
14q	0	0	8	1	0	1	10
15q	0	2	6	0	0	2	10
21q	0	1	7	1	0	1	10
22q	0	0	4	3	2	0	10

Using volumetric model, controls with different sperm nuclear organisation patterns for various subtelomere loci; Peripheral – Shell 1 or 1/2, Peripheral/Medial – Shell 2 or 1-3, Medial – Shell 3, 2/3, or 3/4, Central/Medial – Shell 4 or 3-5, Central – Shell 5 or 4/5.

Comparison of the two approaches for measurement (table 3.6) showed that the DAPI density model was more likely to suggest that the distributions were random, whereas the volumetric model was more likely to display a peripheral or peripheral medial pattern. In both cases however, considering all probes analysed, there was no evidence to support a peripheral nuclear address of (sub) telomeric sequences, with the caveat that visual inspection suggested a peripheral location in a subset of sperm cells for the pan-telomeric sequence alone.

Table 3.6: Total numbers of distributions for both models of analysis.

	Peripheral	Peripheral medial	Medial	Central medial	Central	Random	Total
Total DAPI density	1	0	14	48	75	38	180
Total volumetric	7	17	67	46	11	27	180

Comparison of DAPI density and volumetric models; controls with different sperm nuclear organisation patterns for various subtelomere loci; Peripheral – Shell 1 or 1/2, Peripheral/Medial – Shell 2 or 1-3, Medial – Shell 3, 2/3, or 3/4, Central/Medial – Shell 4 or 3-5, Central – Shell 5 or 4/5.

3.3.2. To test the hypothesis that nuclear organisation (as assayed above) is significantly altered in OAT men (using either approach) and thus that aberrant nuclear organisation is a marker of compromised male fertility.

As mentioned above, a total of 9 patients with OAT were assayed by the above approach. The first analysis was by visual inspection. Figure 3.3 shows an image that was typical of an OAT patient. In general terms, there was not the clear-cut preference for a peripheral location in any of the OAT patients

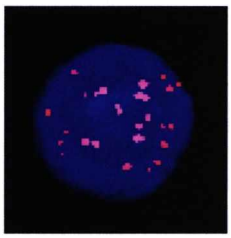


Figure 3.3: Telomeric location in a typical sperm heads from an OAT patient.
FISH images of the telomere sequence (TTAGGG) on a single sperm cell. Signals are randomly distributed.

Despite the fact that the analysis of the control data, using the approaches outlined, did not show a clear preference for a peripheral location (see discussion section 8.1.2 for reasons why that might be the case), there were clear and demonstrable differences between patient and control groups. That is, by both models of analysis, there was a different distribution, for instance the tendency to a central location for the telomeres in most of the OAT patients when the DAPI density model was used and a central-medial location when the volumetric model was used (table 3.7). All individual graphs for patients and controls are in the electronic appendix specific aim 1 section. Graphs from pooled data are included in the paper appendix 10.1.1 and 10.1.2 sections.

Table 3.7: Comparison of telomeric sequence nuclear position in controls and patients using both models of analysis

	Peripheral	Peripheral medial	Medial	Central medial	Central	Random	Total
Total DAPI density control	1	0	0	0	1	8	10
Total DAPI density patients	0	0	0	0	7	2	9
Total volumetric control	0	2	3	0	0	5	10
Total volumetric patients	0	0	1	1	4	3	9

Comparison of DAPI density and volumetric models; for both controls and patients with different sperm nuclear organisation patterns for telomere loci; Peripheral – Shell 1 or 1/2, Peripheral/Medial – Shell 2 or 1-3, Medial – Shell 3, 2/3, or 3/4, Central/Medial – Shell 4 or 3-5, Central – Shell 5 or 4/5.

When analysis then turned to individual sub-telomeric regions, the following distributions were seen (tables 3.8, 3.9 and 3.10).

Table 3.8: Number of patients with different types of distribution per sub-telomeric loci analysed using DAPI density model.

Chromosome number	Peripheral	Peripheral medial	Medial	Central medial	Central	Random	Total
1p	0	0	0	0	9	0	9
1q	0	0	0	3	5	1	9
2p	0	0	0	1	8	0	9
2q	0	0	1	1	7	0	9
3p	0	0	0	0	1	8	9
3q	0	0	0	1	3	5	9
4p	0	0	0	2	6	1	9
4q	0	0	4	1	3	0	9
5p	0	0	1	2	1	5	9
5q	0	0	1	0	8	0	9
8p	0	0	1	5	2	1	9
8q	0	0	0	3	6	0	9
10p	0	0	1	0	6	1	8
10q	0	0	0	1	7	0	8
14q	0	0	1	5	2	0	8
15q	0	0	1	2	3	3	9
21q	0	0	0	5	1	3	9
22q	0	0	0	1	8	0	9

Using DAPI density model, patients with different sperm nuclear organisation patterns for various subtelomere loci; Peripheral – Shell 1 or 1/2, Peripheral/Medial – Shell 2 or 1-3, Medial – Shell 3, 2/3, or 3/4, Central/Medial – Shell 4 or 3-5, Central – Shell 5 or 4/5.

Table 3.9: Number of patients with different types of distribution per sub-telomeric loci analysed using volumetric model.

Chromosome number	Peripheral	Peripheral medial	Medial	Central medial	Central	Random	Total
1p	0	0	0	7	2	0	9
1q	0	1	5	2	1	0	9
2p	0	0	4	3	0	2	9
2q	0	0	4	3	0	2	9
3p	3	0	1	0	1	4	9
3q	0	1	3	0	0	5	9
4p	0	1	5	2	0	1	9
4q	0	3	5	1	0	0	9
5p	0	1	1	2	0	5	9
5q	0	0	5	3	1	0	9
8p	0	1	4	2	0	2	9
8q	0	0	0	6	1	2	9
10p	0	1	2	3	1	1	8
10q	0	0	1	6	0	0	8
14q	0	0	4	3	0	1	8
15q	0	1	3	2	0	2	9
21q	0	1	5	1	0	2	9
22q	0	0	4	2	3	0	9

Using volumetric model, patients with different sperm nuclear organisation patterns for various subtelomere loci; Peripheral – Shell 1 or 1/2, Peripheral/Medial – Shell 2 or 1-3, Medial – Shell 3, 2/3, or 3/4, Central/Medial – Shell 4 or 3-5, Central – Shell 5 or 4/5.

Again, when comparing the two modes of analysis, there were differences. For patients using the DAPI normalisation model, 0/159 for any chromosome arm had peripheral localisation compared to 86/159 central and 28/159 random (tables 3.6 and 3.8). Using the volumetric model 3/159 for any chromosome arm had peripheral localisation compared to 10/159 central and 29/159 random (tables 3.7 and 3.8). Overall patients showed more internal localisation for sub-telomeric loci compared to controls.

Table 3.10: Comparison of total sub-telomeric sequence for nuclear position in controls and OAT patients using both DAPI density and volumetric models of analysis.

	Peripheral	Peripheral medial	Medial	Central medial	Central	Random	Total
DAPI density control	1 (0.6)	0 (0.0)	14 (7.8)	48 (26.7)	75 (41.7)	38 (21.1)	180
DAPI density patients	0 (0.0)	0 (0.0)	11 (6.9)	33 (20.8)	86 (54.1)	28 (17.6)	159
volumetric control	7 (3.9)	17 (9.4)	67 (37.2)	46 (25.6)	11 (6.1)	27 (15)	180
volumetric patients	3 (0.0)	11 (6.9)	56 (35.2)	48 (30.2)	10 (6.3)	29 (18.2)	159

Comparison of DAPI density and volumetric models; for both controls and patients with different sperm nuclear organisation patterns for sub telomeric loci; Peripheral – Shell 1 or 1/2, Peripheral/Medial – Shell 2 or 1-3, Medial – Shell 3, 2/3, or 3/4, Central/Medial – Shell 4 or 3-5, Central – Shell 5 or 4/5.

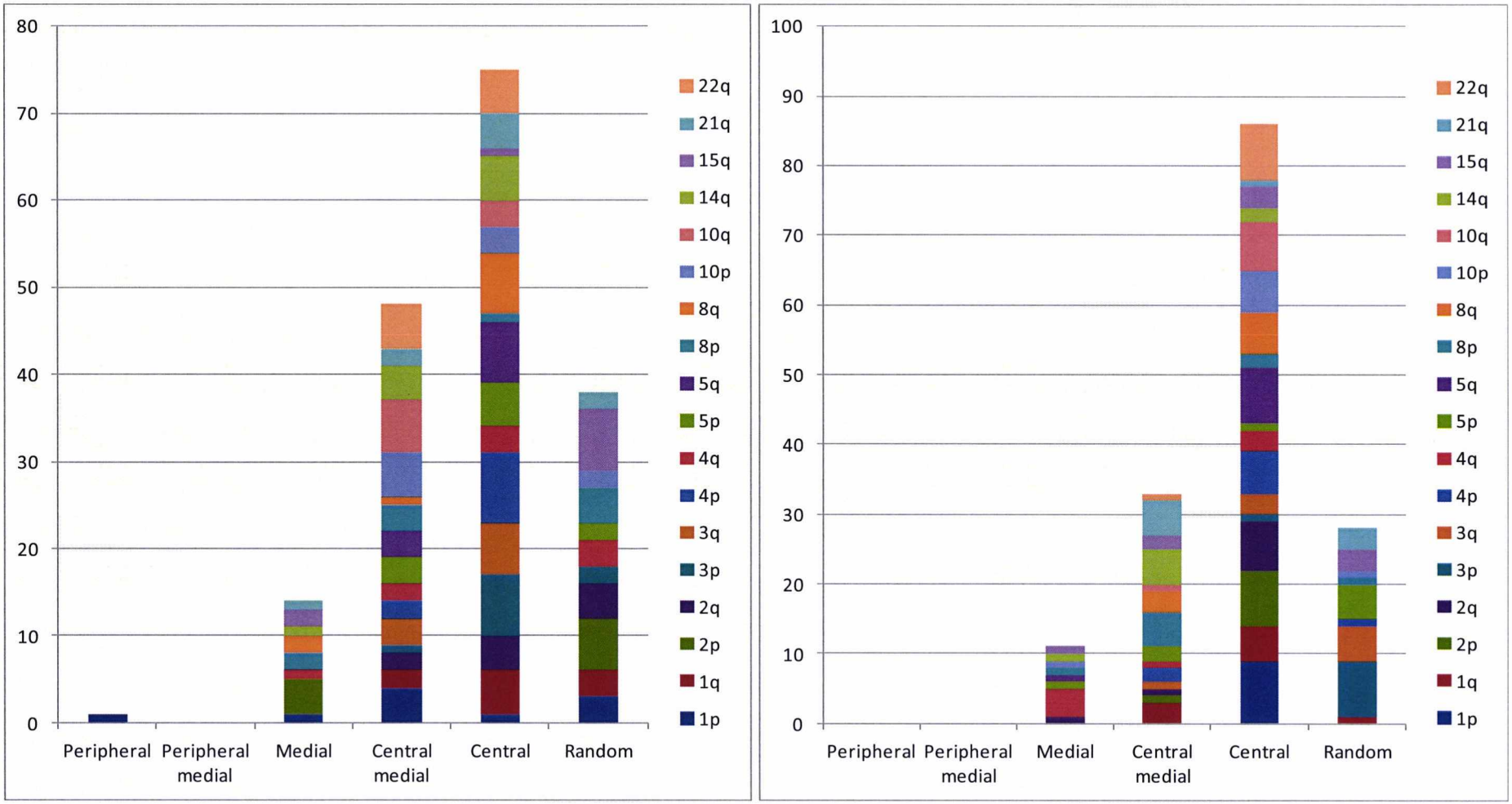


Figure 3.4: Overall, number of fertile controls (left) and OAT patients (right) with different types of distribution per sub-telomeric loci analysed using DAPI density model.
Controls and patients with different sperm nuclear organisation patterns for all of the subtelomere loci analysed; Peripheral – Shell 1 or 1/2, Peripheral/Medial – Shell 2 or 1-3, Medial – Shell 3, 2/3, or 3/4, Central/Medial – Shell 4 or 3-5, Central – Shell 5 or 4/5.

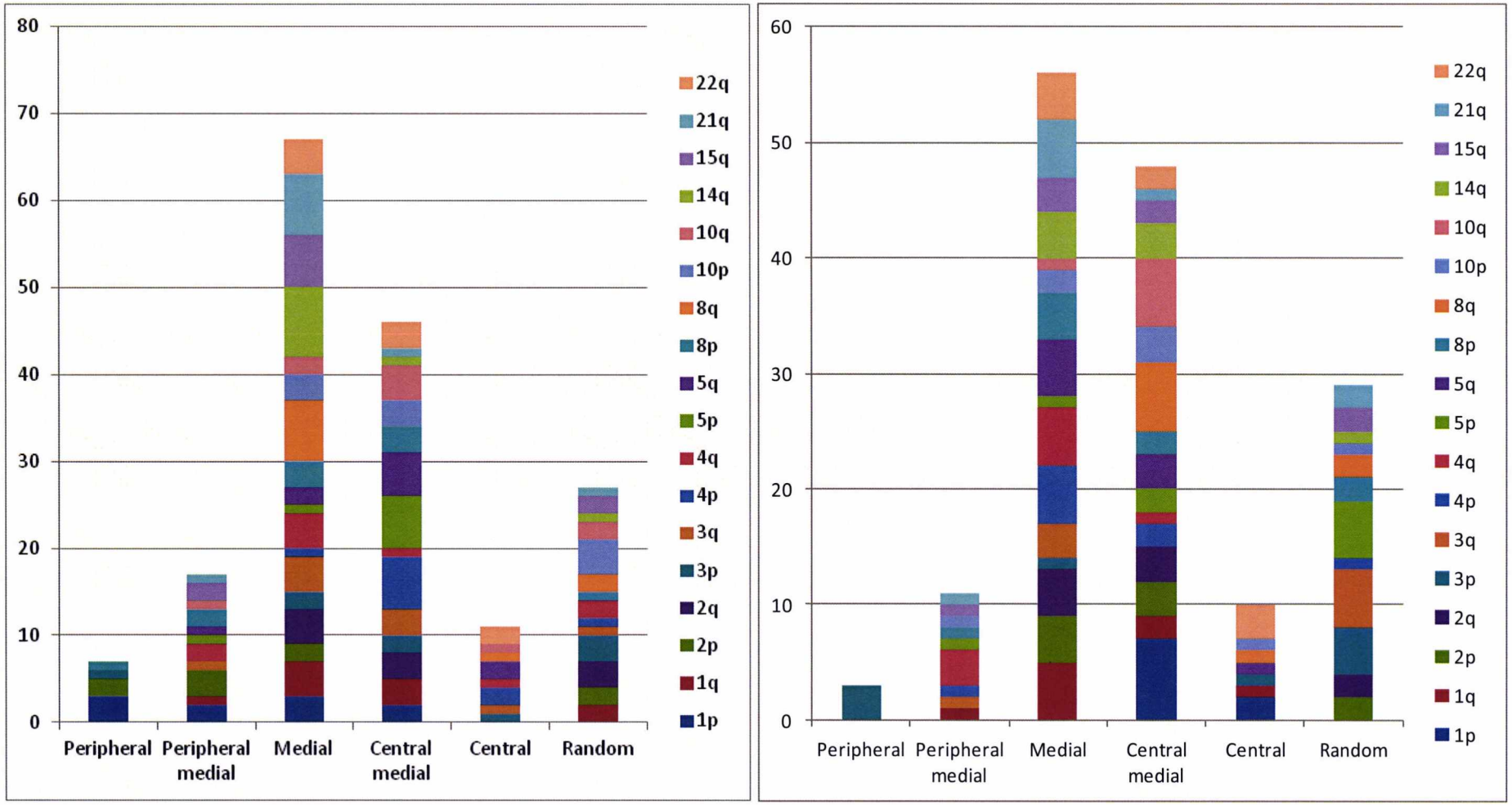


Figure 3.5: Overall, number of normal controls (left) and OAT patients (right) with different types of distribution per sub-telomeric loci analysed using volumetric model.
Controls and patients with different sperm nuclear organisation patterns for all of the subtelomere loci analysed; Peripheral – Shell 1 or 1/2, Peripheral/Medial – Shell 2 or 1-3, Medial – Shell 3, 2/3, or 3/4, Central/Medial – Shell 4 or 3-5, Central – Shell 5 or 4/5.

In general terms, the differences between patients and controls were subtle: That is, the overall graphs (Figures 3.4 and 3.5 above) did not display noticeably different patterns. As mentioned above however, I also analysed the median position of each of the sequences probed, on each of the sperm heads and asked whether they were significantly different (Student's T-test – two tailed). When $P \leq 0.05$, results were significantly different between control and patients groups (at 95% confidence) and those were highlited in yellow. When $P \leq 0.1$, results were significantly different between control and patients groups (at 90% confidence) and those were highlited in pink. The results are presented in tables 3.11 and 3.12. Results suggested (with the DAPI density model) that chromosomes 1p, 2pq, 3pq, 4p sub-telomeric regions (and of course telomeres) show a significant difference in OAT patients compared to controls. Using the volumetric model sub-telomeric regions 1p, 2pq, 3pq, 4p, 8p, 10q (and the telomeres) show significant difference in OAT patients vs. controls. The inference would be therefore that the larger chromosomes tend to show altered nuclear organisation in patients vs. controls.

Table 3.11: Comparison of median position in normal controls vs. OAT patients using DAPI density model

	1		2		3		4		5		8		10		14	15	21	22	TEL
	p	q	p	q	p	q	p	q	p	q	p	q	p	q	q	q	q	q	
C1	2.90	3.65	2.90	3.65	3.06	3.90	4.01	3.71	3.99	4.00	3.51	3.72	3.61	4.18	3.82	3.25	3.43	3.70	2.43
C2	3.16	3.71	3.16	3.69	4.00	3.96	3.94	3.77	3.60	4.00	2.88	3.30	3.47	3.56	3.90	3.07	3.54	3.96	3.00
C3	3.00	3.97	3.02	3.60	4.00	3.07	4.13	3.69	2.34	3.97	3.09	3.54	3.80	3.24	3.83	3.42	3.72	4.02	3.08
C4	3.35	3.78	2.95	3.99	4.00	4.39	4.65	3.90	4.57	4.49	3.58	4.09	3.24	3.65	3.84	3.21	3.98	4.32	3.27
C5	3.64	3.94	3.42	4.00	3.67	3.71	4.00	3.50	3.07	4.00	3.83	3.90	3.66	4.00	3.92	3.34	3.80	3.89	3.58
C6	3.79	3.19	3.00	3.09	2.96	4.00	3.83	3.20	3.94	3.97	2.94	3.80	3.86	3.90	3.55	3.14	3.71	3.60	2.95
C7	3.00	3.49	3.10	3.50	3.80	3.95	4.00	3.86	3.34	3.66	3.35	4.00	3.66	4.00	3.53	3.13	3.00	3.85	3.29
C8	2.42	3.35	2.97	3.68	4.00	4.00	4.00	3.47	4.00	3.94	3.51	3.67	3.01	4.00	4.00	3.00	3.22	4.00	3.05
C9	4.00	4.00	3.60	3.44	3.64	3.99	4.00	4.00	4.41	4.00	3.67	3.89	4.00	4.00	3.73	3.46	3.54	4.05	2.94
C10	3.91	4.00	3.52	3.25	4.00	4.03	4.00	3.26	3.91	3.91	3.00	3.99	3.75	3.36	4.00	3.98	3.30	3.95	3.21
OAT1	4.13	3.70	3.89	3.85	4.00	3.81	3.63	3.11	3.23	4.00	3.00	3.98	3.71	3.95	3.45	3.99	3.70	4.00	3.92
OAT2	4.54	3.20	3.97	4.00	3.28	3.73	3.99	3.79	3.58	4.12	3.09	3.97	4.00	4.00	3.81	3.86	3.79	4.00	3.59
OAT3	4.52	4.00	3.89	4.00	2.86	3.24	4.01	3.32	3.34	4.00	3.81	3.84	4.00	4.07	4.00	3.25	3.93	4.17	3.95
OAT4	4.00	3.82	3.88	4.00	3.30	3.51	3.79	3.45	3.65	3.99	4.00	4.00	3.99	3.97	3.43	3.65	3.19	4.00	3.76
OAT5	4.00	3.83	3.85	4.00	2.97	3.33	3.91	3.07	3.00	4.00	3.80	3.91	4.00	4.00	3.86	3.34	3.81	3.92	3.84
OAT6	4.00	3.86	3.98	4.00	2.34	3.50	3.43	3.87	3.03	4.00	3.97	3.97	4.00	4.01	4.00	3.22	3.00	4.00	3.42
OAT7	4.06	4.00	4.00	3.81	3.00	3.30	4.00	4.00	3.75	4.15	3.89	4.02	3.31	4.00	3.75	3.73	3.51	4.00	3.75
OAT8	4.48	3.54	3.95	3.86	2.98	3.84	3.82	3.75	3.21	4.01	3.41	4.00	3.00	4.00	4.00	3.07	3.84	4.00	2.78
OAT9	4.10	3.84	3.97	4.00	3.28	3.38	3.73	3.00	3.03	3.66	3.89	4.00				3.37	3.56	4.00	3.28
T test	2E-04	0.714	1E-07	0.002	0.006	0.01	0.021	0.326	0.108	0.983	0.073	0.047	0.382	0.08	0.798	0.169	0.632	0.287	0.005

Using DAPI density model, medin positions obtained for each chromosome loci by each control and patient. Medians were compared using studnts T test and p values are presented in the table for each chromosome loci. When P≤ 0.05, results were significantly different between control and patients groups (at 95% confidence) and those were highlited in yellow. When P≤ 0.1, results were significantly different between control and patients groups (at 90% confidence) and those were highlited in pink.

Table 3.12: Comparison of median position in normal controls vs. OAT patients using volumetric model

	1		2		3		4		5		8		10		14	15	21	22	TEL
	p	q	p	q	p	q	p	q	p	q	p	q	p	q	q	q	q	q	
C1	1.91	2.70	1.91	2.70	2.24	3.00	3.31	2.73	3.03	3.14	2.41	2.76	2.64	3.48	3.04	2.49	2.51	2.81	1.74
C2	2.11	2.77	2.11	2.76	2.78	2.91	3.18	2.86	2.67	3.05	1.90	2.49	2.66	2.66	2.86	2.38	2.45	3.08	2.34
C3	2.07	2.82	2.32	2.67	2.87	2.25	3.44	2.67	1.86	3.16	2.21	2.65	2.71	2.50	2.97	2.58	2.73	3.31	2.56
C4	2.59	3.00	2.20	3.18	3.17	3.60	3.80	2.78	3.75	3.70	2.66	3.48	2.44	2.60	2.81	2.49	3.04	3.61	2.84
C5	2.72	2.95	2.65	3.00	2.65	2.70	3.43	2.55	1.98	3.22	2.82	2.95	2.72	2.88	3.01	2.38	2.54	2.93	2.95
C6	2.99	2.31	1.83	2.43	1.73	2.95	2.66	2.42	2.78	2.83	1.94	2.79	2.88	2.85	2.68	2.25	2.89	2.74	2.30
C7	2.26	2.76	2.01	2.12	2.84	2.93	2.94	2.43	2.48	2.74	2.61	3.17	2.67	2.88	2.68	2.25	2.16	2.87	2.69
C8	1.53	2.56	2.04	2.60	3.53	3.55	3.33	2.54	3.26	3.03	2.56	2.72	2.23	3.13	3.12	2.18	2.43	3.17	2.50
C9	3.18	3.10	2.73	2.52	2.60	2.97	3.22	3.18	3.74	3.46	2.41	2.98	3.07	3.14	2.91	2.44	2.62	3.33	2.24
C10	2.93	3.20	2.54	2.61	3.27	2.86	3.20	2.40	2.94	2.99	2.30	2.96	2.62	2.55	3.11	3.02	2.54	3.02	2.66
OAT1	3.58	2.98	2.96	2.63	3.25	2.82	2.83	2.36	2.35	3.33	2.04	3.18	2.65	2.88	2.58	3.14	2.73	3.21	3.50
OAT2	3.73	2.46	2.90	2.90	1.91	2.63	2.96	2.92	2.68	3.36	2.41	2.83	3.12	3.10	2.88	2.79	2.82	3.13	3.06
OAT3	3.77	3.06	2.81	3.14	1.78	2.46	3.29	2.38	2.39	3.22	2.79	2.83	3.23	3.50	3.24	2.41	3.08	3.32	3.45
OAT4	3.27	2.89	2.85	3.10	1.98	2.46	2.79	2.51	2.62	2.85	3.13	3.31	2.99	3.11	2.66	2.69	2.39	3.38	3.32
OAT5	3.31	2.67	2.91	2.90	1.81	2.50	2.96	2.15	2.03	3.00	2.77	2.88	3.13	3.13	2.98	2.45	2.86	3.17	3.41
OAT6	3.36	2.97	2.92	3.01	1.46	2.44	2.74	2.68	2.14	3.12	2.89	3.14	2.86	3.25	3.07	2.47	2.20	3.03	2.94
OAT7	3.58	2.98	2.90	2.77	2.13	2.62	3.04	3.58	2.87	3.54	2.91	3.40	2.42	3.16	2.68	2.73	2.50	3.19	3.24
OAT8	3.75	2.67	2.96	3.02	1.94	2.71	2.79	2.72	2.49	3.41	2.52	3.19	2.45	3.23	3.14	2.25	2.92	3.39	2.52
OAT9	3.61	2.98	2.97	3.14	1.97	2.34	2.74	2.33	1.98	2.80	3.00	2.26				2.25	2.50	3.29	2.93
T test	2E-05	0.757	8E-06	0.017	0.005	0.007	0.008	0.849	0.07	0.698	0.035	0.468	0.151	0.026	0.875	0.299	0.544	0.152	4E-04

Using volumetric model, medin positions obtained for each chromosome loci by each control and patient. Medians were compared using studnts T test and p values are presented in the table for each chromosome loci. When P≤ 0.05, results were significantly different between control and patients groups (at 95% confidence) and those were highlited in yellow. When P≤ 0.1, results were significantly different between control and patients groups (at 90% confidence) and those were highlited in pink.

3.4. Concluding remarks

Taken together, the results suggest preferential locations of some individual sequences, but apparently random patterns in others. A complete discussion of results are presented in section 8.1. The apparently peripheral location of the telomeric array (observed by visual inspection) was not borne out in the analysis. Similarly we found no evidence (visual or otherwise) of the sub-telomeric regions probed showing a peripheral location. This may suggest a considerable relative distance between telomere and sub-telomere, more so than would have been expected by visualisation on a metaphase chromosome. Despite this, a clear and demonstrable difference between OAT patients and normal controls was observed for the telomeric sequences (and, to some extent the sub-telomeric loci) suggesting that nuclear organisation is altered in OAT males.

4. To test the hypothesis that nuclear organisation is altered in patients who have had cancer and treatment for it

4.1. Background

Cancer has long been associated with infertility. Firstly, certain forms of cancer, such as Hodgkin's lymphoma (HD) and testicular cancers (TC) can cause infertility. Secondly, improved survival rate can raise the concern about the effects of anti cancer treatments on germ cell lines and therefore potential risk to the future offspring. Thirdly, an unrelated or independent factor may cause both cancer and infertility together in certain individuals (Meirow and Schenker, 1995).

To date, several studies have reported the high incidence of sex chromosome aneuploidy and autosomal aneuploidy associated with sperm in TC and HD patients compared with healthy donors, both during (Frias *et al.*, 2003; Martinez-Pasarell *et al.*, 1999; Robbins *et al.*, 1997a) and after (De Mas *et al.*, 2001b; Frias *et al.*, 2003; Martin *et al.*, 1995a; Martin *et al.*, 1997; Martinez-Pasarell *et al.*, 1999; McInnes *et al.*, 1998; Robbins *et al.*, 1997a; Thomas *et al.*, 2004) treatment. However, an association between the above and nuclear organisation remains to be established.

Given that certain cancers and chemotherapy lead to high levels of chromosome aneuploidy, it is a reasonable hypothesis that nuclear organisation is also altered in cancer (TC & HD) and chemotherapy patients. In other words, if altered nuclear organisations can be considered a marker for "nuclear health" of the sperm head, it could be a useful marker to assess whether

fertility had returned to normal in cancer and chemotherapy patients. Therefore the aim of this study was to test the hypothesis that altered chromosome positioning is associated with TC and HD patients before and after undergoing chemotherapy.

4.2. Aims and hypotheses

Specifically, the primary purpose of this chapter was to assess the relative nuclear organisation (radial and longitudinal) for five chromosome loci in the sperm nuclei of 10 men with normal semen parameters, in five men with TC and in five men with HD at five different time points in their treatment. Patient information is given in section 2.1.2 and 2.1.4 in material and methods chapter. This study utilised two different mathematical models (DAPI density and Volumetric – as outlined in the chapter 3) to assess radial nuclear organisation and, in this case, the integrity of the sperm heads also allowed measurements of the longitudinal position of the loci. The following hypotheses were tested:

1. That there is a non-random nuclear organisation in the sperm of normal healthy controls for the loci assayed in this study.
2. That nuclear organisation is significantly altered in the sperm heads of TC and HD patients compared to controls for the loci examined.
3. That chemotherapy treatment significantly alters the nuclear organisation patterns in the sperm of the above patients and that it returns to normal over time.

In this chapter, the analysis was restricted to pre-prepared sperm FISH slides previously analysed for sperm aneuploidy. For this reason, I was only able to capture sperm images (15900 images of single sperm nuclei) from the prepared slides and had no input into the design of probes and samples were not available for re-analysis. The probes for

chromosomes 1, X and Y recognised the centromeric regions whereas the probes for chromosomes 13 and 21 were locus-specific (approximately half way down the chromosome). Timepoints for analysis were "0 months" i.e. before treatment. Treatment occurred within six months from this date and thus the next timepoint "6 months" could be considered to be relatively soon after the treatment cycle. For further timepoints "12, 18 and 24 months" no further treatment was administered and thus this allowed measurement of whether the sperm head returned to normal.

4.3. Results

FISH was performed as described in material and method section 2.1.6 and a total of 29,960 signals for chromosome 1, 13, 21 X and Y in 14,980 nuclei from 10 healthy men with normal semen parameters, five TC patients and five HD patients at various treatment timepoints were analysed (see figure 4.1 for examples). Both radial and longitudinal analysis (as described in material and methods 2.5.1 and 2.5.2 sections) for all five chromosomes was performed.

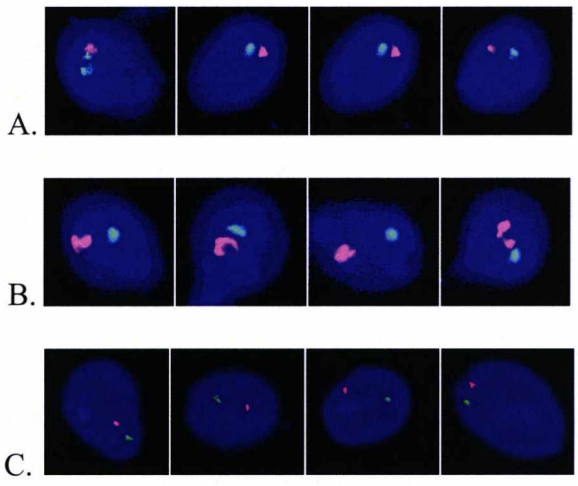


Figure 4.1: FISH images of sperm heads form a control doner probed for chromosome X, Y, 1, 13 and 21.
A. Chromosome X (red) and 1 (green)
B. Chromosome Y (red) and 1 (green)
C. Chromosome 13 (red), and 21 (green)

As a result of the analysis, 520 graphs (260 for DAPI density and 260 for volumetric model) were generated (for each patient per chromosome per time point); these are in the electronic appendix 'specific aim 2' section. The results are summarised in the tables below for the DAPI density analysis and in the appendix 10.2.1 and 10.2.2 sections for the volumetric analysis:

Table 4.1: DAPI density analysis for chromosome 1 (centromere), 13 and 21 (locus specific probes)

	Patient code	0 Month	6 Month	12 Month	18 Month	24 Month
Chromosome 1	1	Central	Central	Central	Central	
	2			Central	Central	
	3	Central		Central	Central	
	4	Central	Central	Central	Central	
	5	Central	Central	Central		
	6	Central	Central	Central		
	7	Central	Central			
	8	Central		Central		
	9	Central	Central	Central		
	10	Central				
	TC1	Central	Central	Central		
	TC2	Central	Central		Central	Central
	TC3	Central			Central	
	TC4	Central	Central		Central	
	TC5			Central		
	HD1	Central	Central			Central
	HD2	Central	Central	Central		
	HD3	Central	Central	Central	Central	
	HD4	Central				
	HD5	Central	Central			
Chromosome 13	1	Central/Medial	Central/Medial	Central/Medial	Central	
	2			Central/Medial	Central/Medial	
	3	Central/Medial	Central	Medial	Central/Medial	
	4	Central		Central	Central/Medial	
	5	Central/Medial	Central	Central		
	6	Medial	Central/Medial	Central		
	7		Central/Medial	Central/Medial		
	8	Central/Medial		Central/Medial		
	9	Central/Medial	Central/Medial	Central/Medial		
	10	Central				
	TC1	Central/Medial	Medial	Central		
	TC2	Central/Medial	Central/Medial		Central	Central/Medial
	TC3	Central/Medial			Central/Medial	
	TC4	Central	Medial	Central/Medial		
	TC5			Medial		
	HD1	Central/Medial	Central/Medial			Central/Medial
	HD2	Central	Central	Central		
	HD3	Medial	Central	Medial	Random	
	HD4	Central				
	HD5	Central	Central			
Chromosome 21	1	Central/Medial	Medial	Central/Medial	Medial	
	2			Medial	Central/Medial	
	3	Central	Random	Central/Medial	Random	
	4	Medial		Central/Medial	Central	
	5	Central/Medial	Medial	Central/Medial		
	6	Medial	Central	Central		
	7		Central	Central/Medial		
	8	Central		Central		
	9	Central/Medial	Central/Medial	Medial		
	10	Medial				
	TC1	Central/Medial	Random	Medial		
	TC2	Random	Central/Medial		Central	Medial
	TC3	Central			Medial	
	TC4	Central/Medial	Medial	Random		
	TC5			Medial		
	HD1	Central	Medial			Central
	HD2	Central/Medial	Medial	Central		
	HD3	Medial	Medial	Central/Medial	Central/Medial	
	HD4	Central/Medial				
	HD5	Central/Medial	Central/Medial			

Nuclear organisation of controls, Testicular cancer and Hodgkin's lymphoma patients at different time points; 0 months refers to after diagnosis of cancer before any treatments, 6, 12 and 18-24 months refers to number of months after completion of treatments. Controls and patients with different organisation patterns for all of the autosomal loci analysed; Peripheral – Shell 1 or 1/2, Peripheral/Medial – Shell 2 or 1-3, Medial – Shell 3, 2/3, or 3/4, Central/Medial – Shell 4 or 3-5, Central – Shell 5 or 4/5.

In all analyses, chromosome 1 (centromere) displayed a central nuclear organisation, consistent with the presence of a chromocentre. The locus specific probes however were generally more medially located.

Table 4.2: DAPI density analysis for chromosome X & Y (centromeres)

	Patient code	0 Month	6 Month	12 Month	18 Month	24 Month
Chromosome X	1	Central	Central	Central	Central	
	2			Central	Central	
	3	Central		Central	Central	
	4	Central	Central	Central	Central	
	5	Central	Central	Central		
	6	Central	Central	Central		
	7	Central	Central			
	8	Central		Central		
	9	Central	Central	Central		
	10	Central				
	TC1	Central	Central	Central		
	TC2	Central	Central		Central	Central
	TC3	Central			Central	
	TC4	Central	Central		Central	
	TC5			Central		
	HD1	Central	Central			Central
	HD2	Central	Central	Central		
	HD3	Central	Central	Central	Central	
	HD4	Central				
	HD5	Central	Central/Medial			
Chromosome Y	1	Central	Central	Central	Central	
	2			Central	Central	
	3	Central		Central	Central	
	4	Central	Central	Central	Central	
	5	Central	Central	Central		
	6	Central	Central	Central		
	7	Central	Central			
	8	Central		Central		
	9	Central	Central	Central		
	10	Central				
	TC1	Central	Central/Medial	Central		
	TC2	Central	Central		Central	Central
	TC3	Central			Central	
	TC4	Central	Central		Central	
	TC5			Central		
	HD1	Central	Central			Central
	HD2	Central	Central	Central		
	HD3	Central	Central	Central	Central	
	HD4	Central				
	HD5	Central	Central/Medial			

Nuclear organisation of controls, Testicular cancer and Hodgkin's lymphoma patients at different time points; 0 months refers to after diagnosis of cancer before any treatments, 6, 12 and 18-24 months refers to number of months after completion of treatments. Controls and patients with different organisation patterns for all of the sex chromosome loci analysed; Peripheral – Shell 1 or 1/2, Peripheral/Medial – Shell 2 or 1-3, Medial – Shell 3, 2/3, or 3/4, Central/Medial – Shell 4 or 3-5, Central – Shell 5 or 4/5.

In all analyses, chromosome X and Y (centromeres) displayed a central nuclear organisation, consistent with the presence of a chromocentre and/or central sex chromosome "body". As in the previous chapter, DAPI density and volumetric analyses were compared. By and large the results were similar, with the volumetric analysis showing distributions toward the

nuclear periphery. That is chromosomes X, Y and 1 showed central patterns, whereas chromosomes 13 and 21 (locus specific probes) were more medial.

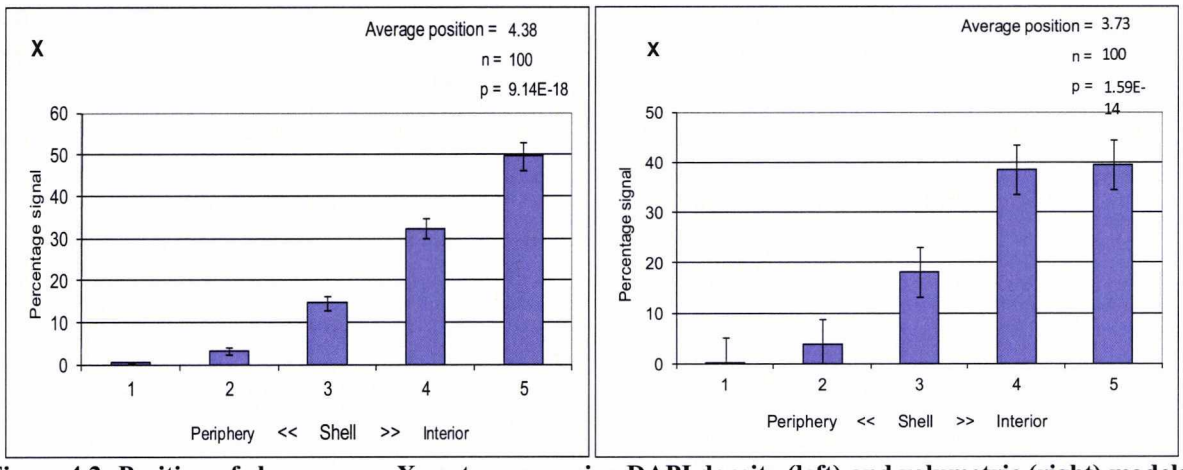


Figure 4.2: Position of chromosome X centromere using DAPI density (left) and volumetric (right) models 100 sperm images run through a macro program which analyse the nuclear positions of FISH signals. Software divides the each sperm head into 5 concentric shells and output percentage of signals in each shell which is presented as graphs in figure 4.2. Positions of signals were analysed with chi squared test and when p value is less than 0.05 results consideres as significant. Depending on the shell number (1to 5) majority of signals located, distribution is catogorised as peripheral, medial or central. When p value is higher than 0.05 results were considered as random.

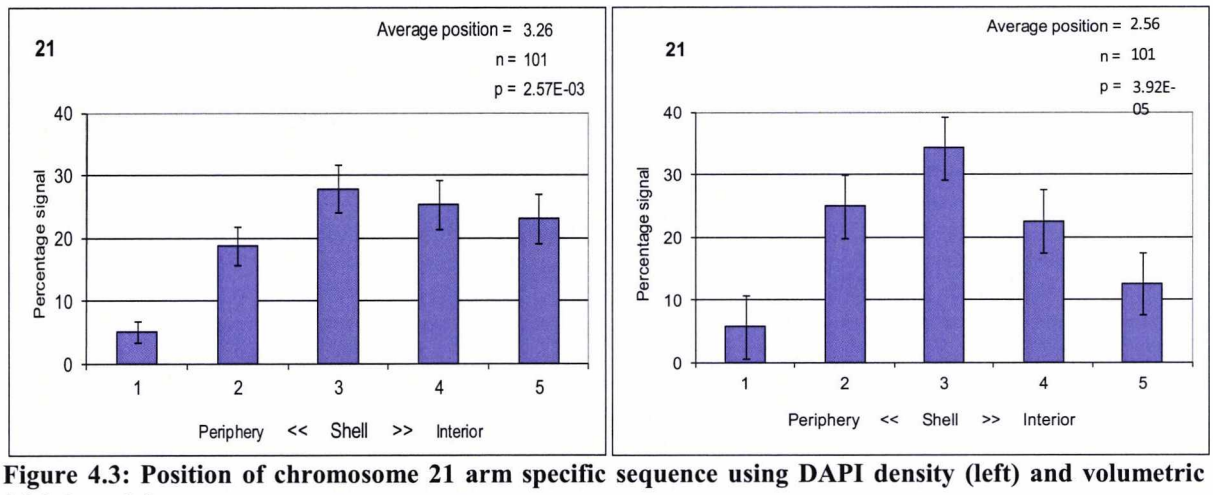


Figure 4.3: Position of chromosome 21 arm specific sequence using DAPI density (left) and volumetric (right) models
100 sperm images run through a macro program which analyse the nuclear positions of FISH signals. Software divides the each sperm head into 5 concentric shells and output percentage of signals in each shell which is presented as graphs in figure 4.2. Positions of signals were analysed with chi squared test and when p value is less than 0.05 results consideres as significant. Depending on the shell number (1to 5) majority of signals located, distribution is catogorised as peripheral, medial or central. When p value is higher than 0.05 results were considered as random.

Longitudinal analysis for each chromosome locus generated numbers between 0 (tail end) and 1 (head end) – standard deviations are given in brackets. In all cases, results were between 0.4 and 0.8, presented in tables 4.3 and 4.4.

Table 4.3: Longitudinal analysis of chromosome 1 (centromere), 13 & 21(locus specific probes).

	Patient code	0 Month	6 Month	12 Month	18 Month	24 Month
Chromosome 1	1	0.6 (0.24)	0.7 (0.16)	0.62 (0.17)	0.62 (0.16)	
	2			0.54 (0.19)	0.55 (0.22)	
	3	0.59 (0.14)		0.55 (0.16)	0.56 (0.17)	
	4	0.57 (0.16)	0.60 (0.15)	0.60 (0.15)	0.57 (0.20)	
	5	0.51 (0.20)	0.54 (0.17)	0.49 (0.23)		
	6	0.60 (0.16)	0.60 (0.13)	0.59 (0.16)		
	7	0.61 (0.18)	0.60 (0.19)			
	8	0.55 (0.18)		0.60 (0.18)		
	9	0.50 (0.19)	0.59 (0.19)	0.61 (0.17)		
	10	0.60 (0.15)				
	TC1	0.63 (0.15)	0.63 (0.15)	0.59 (0.18)		
	TC2	0.57 (0.17)	0.63 (0.15)		0.55 (0.23)	0.50 (0.220
	TC3	0.58 (0.17)			0.50 (0.22)	
	TC4	0.58 (0.17)	0.55 (0.0.17)		0.49 (o.20)	
	TC5			0.57 (0.18)		
	HD1	0.59 (0.19)	0.58 (0.20)			0.61 (0.16)
	HD2	0.51 (0.19)	0.54 (0.21)	0.48 (0.18)		
	HD3	0.51 (0.20)	0.61 (0.14)	0.50 (0.18)	0.50 (0.20)	
	HD4	0.56 (0.13)				
	HD5	0.60 (0.17)	0.54 (0.18)			
Chromosome 13	1	0.61 (0.22)	o.63 (0.22)	0.63 (0.20)	0.63 (0.20)	
	2			0.62 (0.21)	0.59 (0.22)	
	3	0.61 (0.21)	0.54 (0.24)	0.62 (0.21)	0.62 (0.23)	
	4	0.63 (0.21)		0.61 (0.21)	0.60 (0.22)	
	5	0.65 (0.19)	0.64 (0.20)	0.62 (0.21)		
	6 (51)	0.66 (0.19)	0.63 (0.22)	0.65 (0.23)		
	7		0.64 (0.19)	0.61 (0.23)		
	8 (53)	0.62 (0.24)		0.61 (0.23)		
	9	0.68 (0.20)	0.61 (0.23)	0.64 (0.20)		
	10	0.54 (0.21)				
	TC1	0.56 (0.23)		0.64 (0.19)		
	TC2	059 (0.26)	0.71 (0.20)		0.65 (0.19)	0.63 (0.20)
	TC3	0.66 (0.18)			0.66 (0.20)	
	TC4	0.63 (0.18)	0.69 (0.19)	0.64 (0.21)		
	TC5			0.71 (0.19)		
	HD1	0.61 (0.21)	0.63(0.24)			0.59 (0.21)
	HD2	0.61 (0.19)	0.57 (0.19)	0.56 (0.23)		
	HD3	0.67 (o.20)	0.64 (0.20)	0.68 (O.22)	0.66 (0.22)	
	HD4	0.64 (0.21)				
	HD5	0.65 (0.18)	0.59 (0.19)			
Chromosome 21	1	0.51 (0.18)	0.61 (0.21)	0.62 (0.20)	0.47 (0.27)	
	2			0.61 (0.23)	0.51 (0.24)	
	3	0.61 (0.21)	0.59 (0.22)	0.55 (0.23)	0.61 (0.21)	
	4	0.52 (0.25)		0.56 (0.22)	0.56 (0.21)	
	5	0.60 (0.21)	0.62 (0.22)	0.55 (0.21)		
	6	0.60 (0.22)	0.64 (0.16)	0.56 (0.21)		
	7		0.52 (0.22)	0.51 (0.27)		
	8	0.53 (0.21)		0.55 (0.23)		
	9	0.60 (0.20)	0.51 (0.23)	0.53 (0.24)		
	10	0.52 (0.20)				
	TC1	0.59 (0.21)		0.58 (0.21)		
	TC2	0.56 (0.22)	0.56 (0.25)		0.50 (0.23)	0.54 (0.23)
	TC3	0.58 (0.23)			0.52 (0.23)	
	TC4	0.57 (0.22)	0.62 (0.23)	0.58 (0.22)		
	TC5			0.60 (0.23)		
	HD1	0.56 (0.20)	0.55 (0.23)			0.59 (0.22)
	HD2	0.54 (0.21)	0.59 (0.21)	0.55 (0.20)		
	HD3	0.55 (0.23)	0.52 (0.24)	0.49 (0.20)	0.56 (0.21)	
	HD4	0.55 (0.25)				
	HD5	0.52 (0.22)	0.62 (0.18)			

Longitudinal position for controls testicular cancer patients, and Hodgkin's lymphoma patients over time points, as average fractional length of the sperm cell from the tail for 100 cells and standard deviation; values closer to 0 indicate signals located near to the tail and closer to 1 indicate signals locate near the acrosome region.

Table 4.4: Longitudinal analysis of chromosome X & Y (centromere).

	Patient code	0 Month	6 Month	12 Month	18 Month	24 Month
Chromosome X	1	0.60 (0.16)	6.60 (.014)	0.63 (0.14)	0.64 (0.19)	
	2			0.57 (0.15)	0.61 (0.17)	
	3	0.60 (0.11)		0.63 (0.14)	0.60 (0.13)	
	4	0.58 (0.18)	0.59 (0.20)	0.55 (0.19)	0.58 (0.22)	
	5	0.57 (0.13)	0.58 (0.14)	0.56 (0.18)		
	6	0.68 (0.12)	0.60 (0.15)	0.62 (0.14)		
	7	0.68 (0.17)	0.67 (0.12)			
	8	0.59 (0.13)		0.64 (0.14)		
	9	0.59 (0.16)	0.59 (0.15)	0.64 (0.13)		
	10	0.58 (0.16)				
	TC1	0.56 (0.20)	0.66 (0.16)	0.54 (0.15)		
	TC2	0.58 (0.15)	0.59 (0.17)		0.64 (0.15)	0.66 (0.14)
	TC3	0.60 (0.17)			0.65 (0.12)	
	TC4	0.50 (0.22)	0.53 (0.22)		0.62 (0.18)	
	TC5			0.52 (0.22)		
	HD1	0.59 (0.17)	0.59 (0.17)			0.65 (0.13)
	HD2	0.59 (0.11)	0.62 (0.16)	0.61 (0.15)		
	HD3	0.64 (0.15)	0.63 (0.13)	0.67 (0.11)	0.66 (0.11)	
	HD4	0.59 (0.16)				
	HD5	0.67 (0.16)	0.61 (0.17)			
Chromosome Y	1	0.60 (0.18)	0.60 (0.16)	0.61 (0.15)	0.64 (0.13)	
	2			0.59 (0.20)	0.62 (0.14)	
	3	0.59 (0.13)		0.60 (0.15)	0.59 (0.17)	
	4	0.56 (0.14)	0.58 (0.17)	0.58 (0.14)	0.55 (0.20)	
	5	0.59 (0.15)	0.50 (0.16)	0.54 (0.17)		
	6	0.62 (0.14)	0.58 (0.14)	0.64 (0.12)		
	7	0.63 (0.14)	0.63(0.14)			
	8	0.60 (0.12)		0.60 (0.18)		
	9	0.59 (0.17)	0.58 (0.15)	0.60 (0.17)		
	10	0.62 (0.11)				
	TC1	0.65 (0.14)	0.66 (0.12)	0.61 (0.15)		
	TC2	0.58 (0.16)	0.62 (0.14)		0.57 (0.17)	0.60 (0.15)
	TC3	0.60 (0.14)			0.64 (0.15)	
	TC4	0.64 (0.13)	0.55 (0.21)		0.64 (0.16)	
	TC5			0.58 (0.16)		
	HD1	0.61 (0.17)	0.61 (0.18)			0.59 (0.14)
	HD2	0.59 (0.15)	0.59 (0.15)	0.60 (0.15)		
	HD3	0.62 (0.10)	0.61 (0.13)	0.67 (0.11)	0.66 (0.11)	
	HD4	0.59 (0.14)				
	HD5	0.62 (0.14)	0.54 (0.21)			

Longitudinal position for controls testicular cancer patients, and Hodgkin's lymphoma patients over time points,as average fractional length of the sperm cell from the tail for 100 cells and standard deviation; values closer to 0 indicate signals located near to the tail and closer to 1 indicate signals locate near the acrosome region

4.3.1. Is there a non-random nuclear organisation in the sperm of normal healthy controls for the loci assayed in this study?

In the control males, clear examples of a non-random pattern of nuclear organisation were seen, regardless of which method of analysis was used. Apparently random patterns were rare (only two examples in fact). All centromeric loci showed significant (chi square test $p<0.05$) non-random, central positions whereas the q arm probes for chromosomes 13 and 21 were more medially located (in both models of analysis) indicating the presence of a chromocentre and/or sex chromosome central body.

In the longitudinal axis, all of the chromosome loci localised towards the middle of the nuclei; the mean position and standard deviation for chromosome X was 0.61 (0.03), chromosome Y was 0.59 (0.03), chromosome 1 was 0.58 (0.04), chromosome 13 was 0.62 (0.03) and chromosome 21 was 0.56 (0.04).

4.3.2. Does nuclear organisation alter significantly in the sperm heads of TC and HD patients compared to controls for the loci examined?

Using the DAPI density model for analysis, for centromeric probes for chromosome 1, X and Y, the nuclear position appears to be central in both TC and HD patients before treatment. Similar results were obtained for chromosome 13 however, for chromosome 21, one TC patient show an apparently random organisation. Using the volumetric model for analysis, TC patients show central organisation for all centromeric probes, however two HD patients showed medial position for chromosome X and Y. For chromosome 21 patterns seen were similar to controls; however one TC patient showed random position for chromosome 13. Taken together therefore, there was initially little or no evidence to support the hypothesis that the presence of HD or TC significantly altered nuclear organisation, at least for the loci examined.

In a second analysis (Comparion of medians using T Test) however, the median radial position of each probe was examined and pairwise comparisons made between each group (control, HD and TC) using the data from all individuals pooled. T test analysis revealed four examples of a significant difference (see table 4.5 and 4.6) Similar analysis of the

longitudinal position revealed a single significant difference. Isolated examples of differences between groups can therefore be identified, but not easily.

Table 4.5: Comparison of the radial positions of chromosomes between testicular cancer, Hodgkin's lymphoma and controls using T test P<0.05 are significant at 95%, P<0.1 are significant at 90%

		0 months	6 months	12 months	18-24 months
	Chromosome 21				
Volumetric	TC & C	0.209	0.252	0.002	0.666
	HD & C	0.929	0.536	0.593	0.042
	TC & HD	0.162	0.327	0.161	0.075
DAPI normalisation	TC & C	0.595	0.423	0.012	0.622
	HD & C	0.623	0.761	0.739	0.039
	TC & HD	0.262	0.513	0.174	0.063
	Chromosome 13				
Volumetric	TC & C	0.723	0.688	0.171	0.411
	HD & C	0.385	0.525	0.584	0.702
	TC & HD	0.635	0.918	0.867	0.415
DAPI normalisation	TC & C	0.707	0.289	0.127	0.184
	HD & C	0.888	0.722	0.685	0.741
	TC & HD	0.743	0.203	0.651	0.539
	Chromosome 1				
Volumetric	TC & C	0.002	0.004	0.482	0.566
	HD & C	0.872	0.510	0.594	0.327
	TC & HD	0.042	0.043	0.925	0.499
DAPI normalisation	TC & C	0.024	0.009	0.902	0.885
	HD & C	0.923	0.614	0.855	0.451
	TC & HD	0.199	0.079	0.802	0.432
	Chromosome X				
Volumetric	TC & C	0.247	0.239	0.779	0.992
	HD & C	0.324	0.235	0.832	0.700
	TC & HD	0.923	0.630	0.674	0.695
DAPI normalisation	TC & C	0.145	0.418	0.792	0.945
	HD & C	0.733	0.679	0.918	0.710
	TC & HD	0.381	0.677	0.806	0.748
	Chromosome Y				
Volumetric	TC & C	0.010	0.194	0.247	0.992
	HD & C	0.082	0.492	0.643	0.700
	TC & HD	0.777	0.556	0.925	0.695
DAPI normalisation	TC & C	0.185	0.201	0.402	0.263
	HD & C	0.624	0.425	0.583	0.591
	TC & HD	0.528	0.586	0.600	0.336

Testicular cancer and Hodgkins's lymphoma patients and controls were compared to each other using student T test (2 tailed). This was performed for all the time points, for each chromosome (21,13,1,X and Y), using both DAPI density and volumetric models. Obtained P values are presented in the table. When p<0.05 results considerd as significant at 95% and highlited in yellow. P<0.1 are significant at 90% and highlited in gray.

Table 4.6: Comparison of the longitudinal positions of chromosomes between testicular cancer, Hodgkin's lymphoma and controls using T test P<0.05 are significant at 95%, P<0.1 are significant at 90%

		0 months	6 months	12 months	18-24 months
Chromosome 21					
	TC & C	0.440	0.842	0.076	0.620
	HD & C	0.340	0.719	0.390	0.331
	TC & HD	0.013	0.641	0.257	0.092
Chromosome 13					
	TC & C	0.594	0.005	0.225	0.035
	HD & C	0.574	0.751	0.965	0.743
	TC & HD	0.347	0.009	0.600	0.646
Chromosome 1					
	TC & C	0.327	0.963	0.505	0.020
	HD & C	0.516	0.206	0.005	0.779
	TC & HD	0.170	0.328	0.024	0.560
Chromosome X					
	TC & C	0.116	0.792	0.005	0.066
	HD & C	0.714	0.649	0.434	0.027
	TC & HD	0.086	0.664	0.142	0.275
Chromosome Y					
	TC & C	0.383	0.446	0.934	0.647
	HD & C	0.553	0.714	0.464	0.607
	TC & HD	0.555	0.577	0.445	0.787

Testicular cancer and Hodgkins's lymphoma patients and controls were compared to each other using student T test (2 tailed). This was performed for all the time points, for each chromosome (21,13,1,X and Y), using both DAPI density and volumetric models. Obtained P values are presented in the table. When p<0.05 results considerd as significant at 95% and highlited in yellow. P<0.1 are significant at 90% and highlited in gray.

4.3.3. Does chemotherapy treatment significantly alter the nuclear organisation patterns in the sperm of the above patients and does it return to normal over time?

The above tables (4.5 and 4.6) also show the results for each timepoint, six months is essentially immediately after treatment and the remainder represent a potential "recovery" phase. When using the DAPI density model, one out of five TC patients displayed an apparently random distribution for chromosome 21 at the six months and 12 months timepoints indicating that they alone may have displayed an effect of chemotherapy on their

sperm nuclear organisation. Other chromosomes for TC and HD show similar chromosome positioning patterns to the controls. Using the volumetric model, two TC patients showed peripheral and peripheral medial localisation for chromosome 21 at the six month and 12 month timepoints compared to central/medial in controls – again an effect of the treatment may be indicated. Another TC patient showed medial localisation for chromosome Y at the six month timepoint and three HD patients showed medial position for chromosome 1, X and Y at six months after chemotherapy. In all cases therefore a shift from the nuclear centre towards the periphery may by an effect of the therapy.

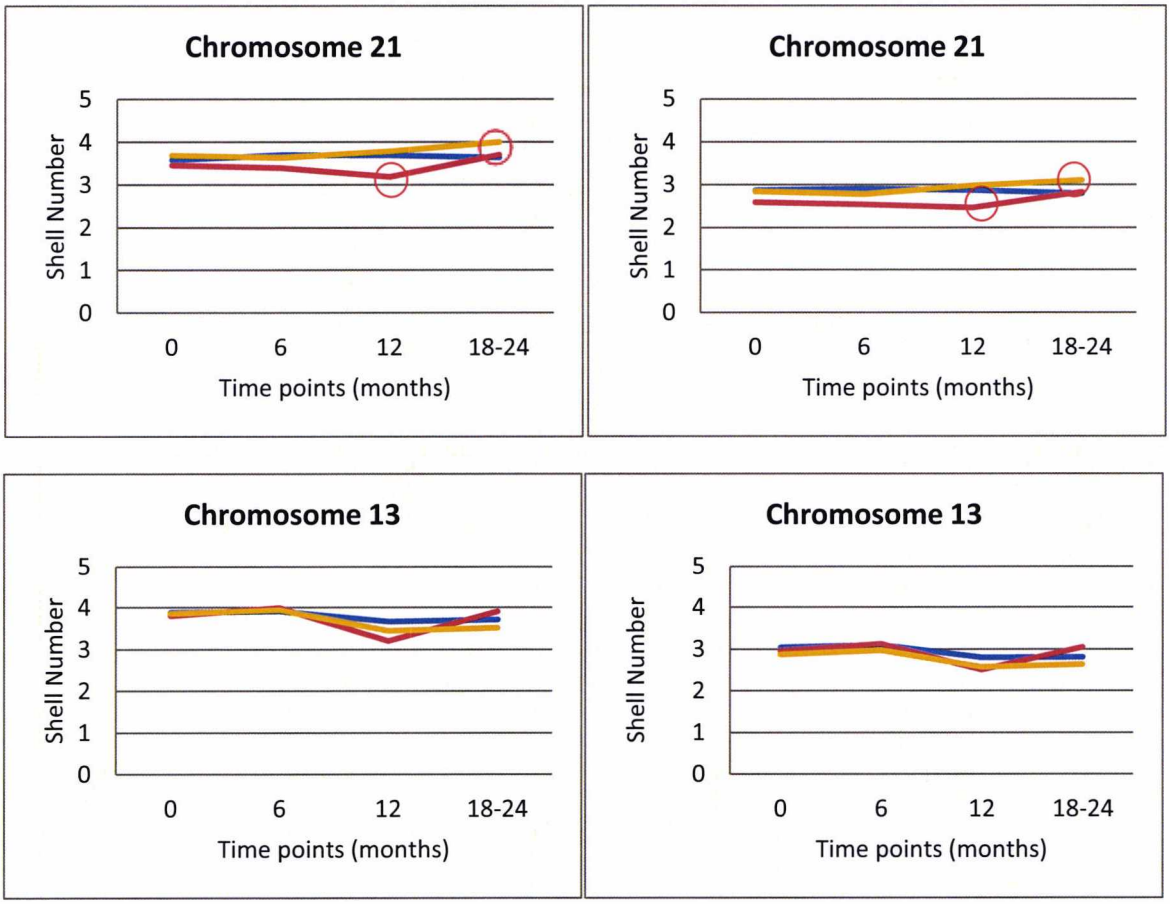
Similar T test to the above was then used in pairwise comparison of the pooled data to investigate if there is a significant difference between cancer and control groups at different time points (see tables 4.5 and 4.6 above). According to this analysis, the chromosome 1 position was significantly different six months after chemotherapy between TC and control groups as well as TC and HD groups. Also position of chromosome Y is significantly different between TC and control groups and HD and control groups at six months after therapy. By 12 months after therapy position of chromosome 21 appears to be significantly different to controls. At 18 to 24 months after chemotherapy the position of chromosome 21 in HD patients appears to be significantly different to controls and TC patients. The individual graphs from the pooled analyses are presented in the appendix 10.2.3 section.

Longitudinal chromosome positions after chemotherapy showed some significant differences compared to controls. The longitudinal position of chromosome 13 of TC patients after chemotherapy was significantly different compared to the controls and HD patients. At 12 months, the position of chromosome 21 and X in TC patients are significantly different compared to controls and chromosome 1 in TC patients significantly difference to HD

patients. After 18 to 24 months time point, longitudinal position of chromosome 13 and 1 from TC patients are significantly different to controls. The position of chromosome 21 is significantly different in TC patients compared to HD patients and longitudinal position of chromosome X is significantly different compared to the controls. Significnat data highlited in yellow in table 4.5 and 4.6 and circled in red in figures 4.4 and 4.5.

The graphs below (figure 4.4) show how pooled median positions of controls, TC and HD patients change over time. Other than chromosome 21 position in HD patients, all other chromosomes analysed do not show a significant difference to that of the controls 18 months after completion of chemotherapy treatments. For longitudinal positions however only chromosome Y did not have a significant difference to control for any cancer patients at 18 months after chemotherapy.

DAPI density model **Volumetric model**



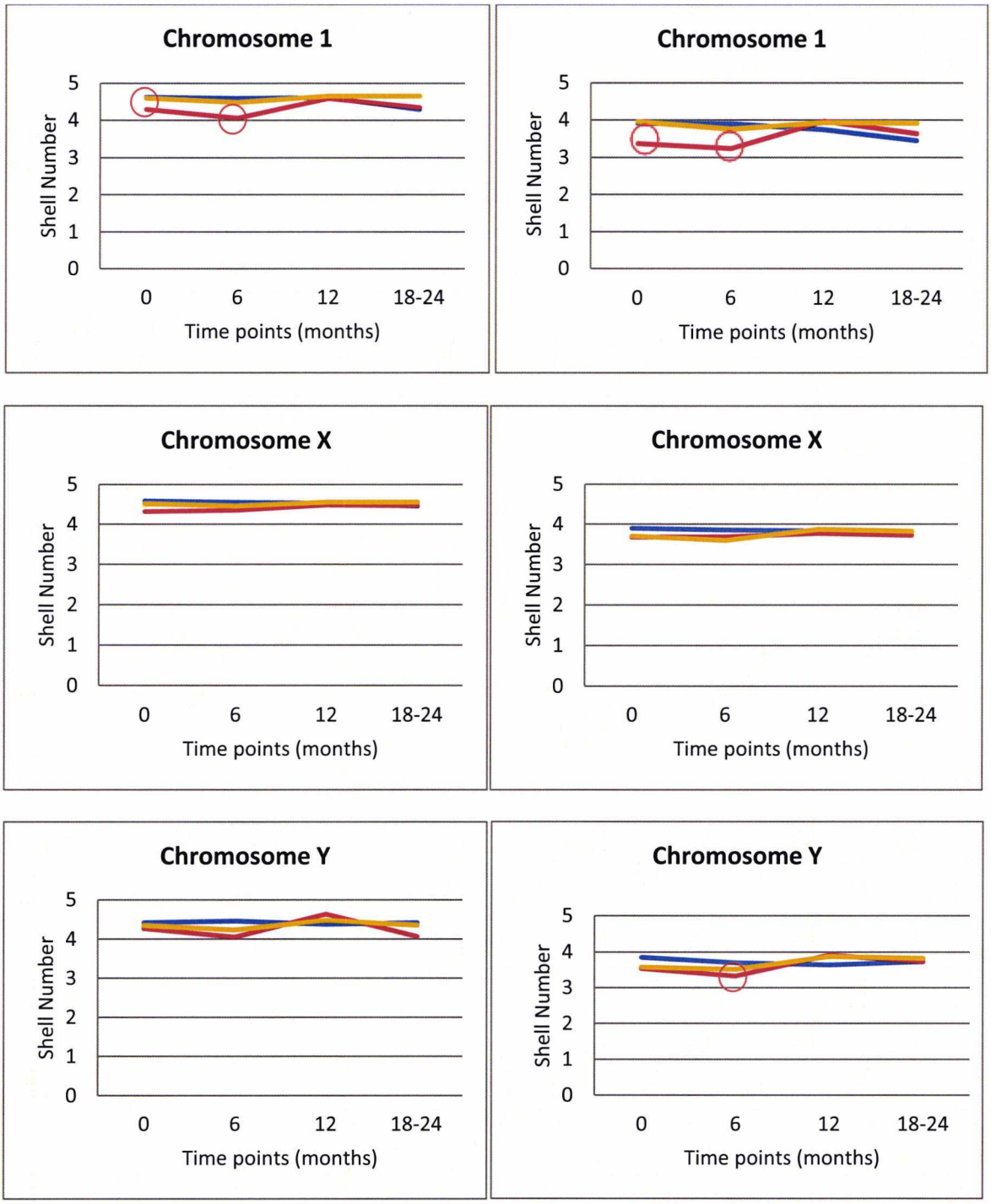


Figure 4.4: Average median position for chromosome 21,13,1 X and Y from controls, TC and HD patients before (0) and 6, 12, 18-24 months after chemotherapy using DAPI density (left) and volumetric models (right). Medians were compared to each other using 2 tailed T test and timepoints gave significant differences with 95% confidence are circled in red.

Longitudinal positions

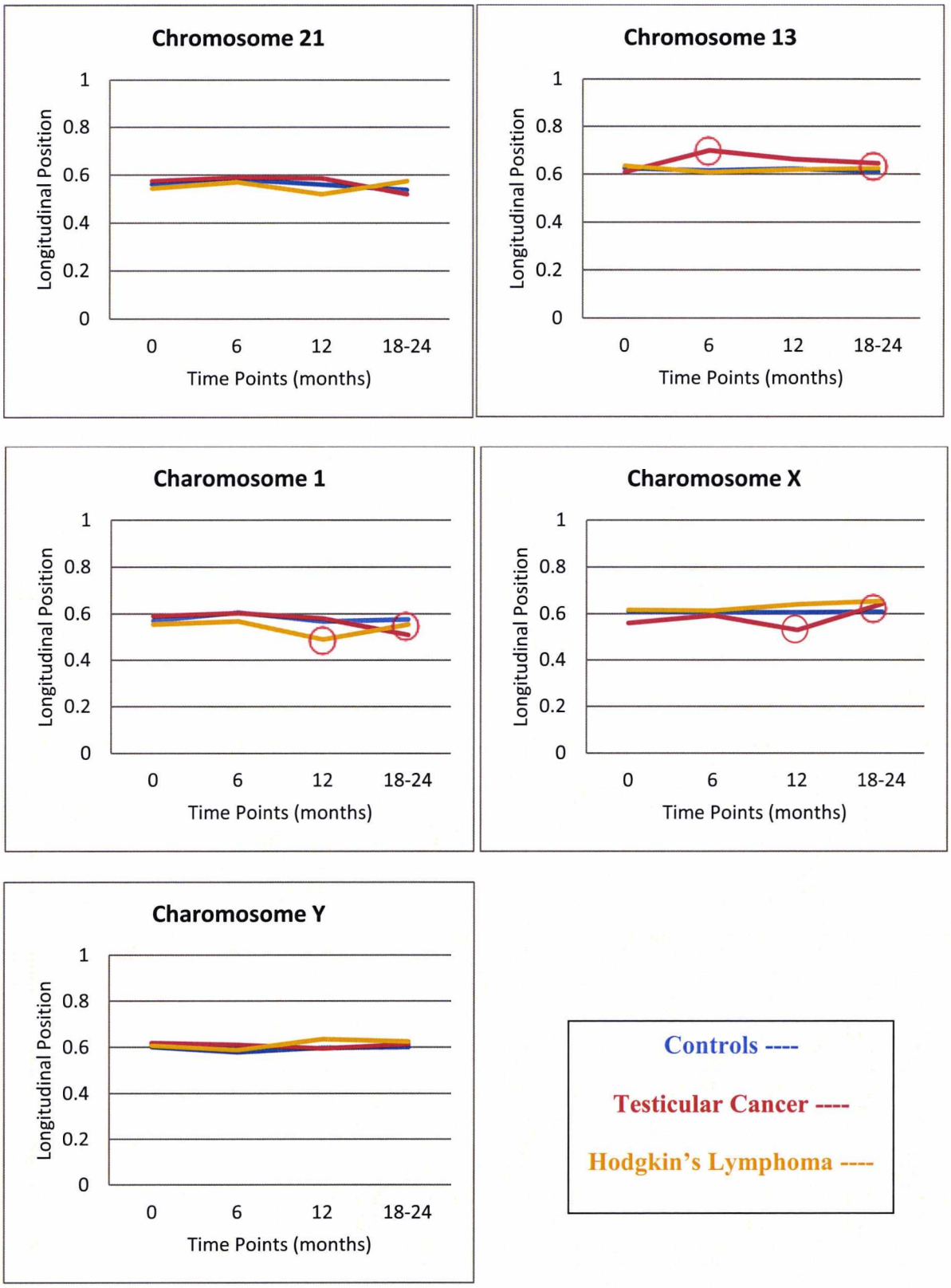


Figure 4.5: Average longitudinal position for chromosome 21,13,1 X and Y from controls, TC and HD patients before (0) and 6, 12, 18-24 months after chemotherapy. Medians were compared to each other using 2 tailed T test and timepoints gave significant differences with 95% confidence are circled in red.

4.4. Conclusions

With regards to the initial hypotheses, clear patterns of non-random nuclear organisation could easily be observed using both methods of analysis for radial position and when assaying for longitudinal position. This was the case for all five loci examined. Some changes associated with cancer and cancer therapies were observed however these were relatively subtle. Results in this chapter are discussed in depth in section 8.2. To sum up, it seems reasonable to conclude therefore that chemotherapy can alter nuclear organisation however the results were nowhere near as dramatic as the increases in sperm disomy observed in the exact same samples.

5. Assessment of FISH based PGS outcomes of clinical cases

5.1. Background

Use of PGS for selecting embryos has been extensively debated; some studies reported benefits of PGS for groups of patients (Gianaroli *et al.*, 1999; Gianaroli *et al.*, 2005; Munne, 2003; Munne *et al.*, 2007b) while others report that PGS do not increase the implantation rate or clinical pregnancy rate (Blockeel *et al.*, 2008; Debrock *et al.*, 2009; Hardarson *et al.*, 2008; Mastenbroek *et al.*, 2007; Mersereau *et al.*, 2008a; Meyer *et al.*, 2009; Schoolcraft *et al.*, 2009; Staessen *et al.*, 2004; Staessen *et al.*, 2008). Many reasons for the failure of PGS have been published over last few years including culture related issues, biopsy techniques (Cohen and Grifo, 2007; Handyside and Thornhill, 2007), FISH limitations and embryo mosaicism (Baart *et al.*, 2006; Coonen *et al.*, 1994; Coonen *et al.*, 2004; Delhanty *et al.*, 1997; Harper *et al.*, 1995; Mastenbroek *et al.*, 2007; Vanneste *et al.*, 2009). This study aims to analyse incidence of aneuploidy of PGS single cell biopsies and follow up embryos in a large clinical data set for a small subset of chromosomes asking number of scientific questions related to PGS accuracy, sensitivity and specificity.

5.2. Specific aims

With the above in mind, in this chapter, results are presented of a retrospective analysis of 241 PGS cycles that were performed at the London Bridge Fertility Centre in the period 2004 to 2010. In these cycles, PGS performed by biopsing a single cell from an embryo and testing for abnormalities in certain chromosomes (chromosomes 13, 16, 18, 21, 22 and, in 82% of cases, XY). This is called single cell FISH analysis and results available for 670 embyo

biopsies from PGS cases at day 3 after fertilisation. Of these, 452 embryos which were dignosed as abnormal or had poor embryo morphology therefore not suitable for cryopreservation at day 5 were used for follow up analysis. Follow up FISH was performed with the same probe set on day 5-7 producing results on an average of 18 nuclei per embryo. Referral categories included advanced maternal age (AMA), recurrent implantation failure (RIF) and small numbers of recurrent miscarriage. As described in section 2.2.1 this study analyse readily available clinical data however approximately 5% of PGS and follow up cases I carried out personally according to the standered protocols described in material and methods section 2.2.2. This data allowed me to test the following scientific questions and hypotheses relevant to the above issues associated with the problems of PGS:

1. Of those patients that did not make it to embryo biopsy and/or had an inconclusive FISH analysis, was a particular referral category especially over-represented?
2. What is the incidence of chromosome abnormality for each chromosome assayed and are some chromosomes more prone to errors than others
    a. In the PGS single cells?
    b. In the follow up embryos?
3. That the original PGS result was an accurate predictor of the subsequent embryo karyotype
4. That trisomy and monosomy is related to maternal age
    a. In the PGS single cells
    b. In the follow up embryos
5. That the recurrent implantation failure (RIF) referral category has a significantly different pattern of chromosome abnormality compared to the rest of the cohort.
    a. In the PGS single cells
    b. In the follow up embryos

5.3. Results

5.3.1. Of those patients that did not make it to embryo biopsy (case cancellations) and/or did not have a conclusive FISH analysis (either no FISH signal or FISH signals impractical to interpret), was a particular referral category especially over-represented?

Chi suqared test was used to analyse data in this section. The P values <0.05 are considered as statistically significant. The results in table 5.1 suggest that case cancellation was more likely in older age groups i.e. only 3% of cases were cancelled in the younger age group whereas this figure was 8% and 9% in the older age groups, however this data is not statistically significant (P=0.21). The inability to distinguish a signal did not change however, surprisingly, lost cells or no FISH signal was more prevalent in the younger age group (P=0.005). Results also show that there was no difference in case cancellations between RIF and non-RIF groups (note, the full data set was not used for "non-RIF" in order to age-match the groups – i.e. the older ones were removed to make the analysis meaningful) nor was there a significant difference in the ability to distinguish a signal. Nonetheless, in the RIF group, the likelihood of losing a cell or not having a FISH signal was greater (P=0.17).

Table 5.1: Case cancelations, inconclusive and no result related to indication and age groups.

Study groups	Total number of cases	Case Cancellations		Total number of embryos	FISH signal could not be interpreted		Lost cell or no FISH signal	
		Number	%		Number	%	Number	%
Total data set	241	22	**9**	670	25	**4**	89	**13**
≤35	31	1	**3**	107	4	**4**	27	**25**
36-39	71	6	**8**	227	9	**4**	19	**8**
≥40	136	12	**9**	292	11	**4**	35	**12**
RIF	22	0	**0**	182	5	**3**	39	**21**
Non RIF (age matched)	49	1	**2**	180	8	**4**	23	**13**

Case cancelations, inconclusive FISH results and no FISH results as percentage for the total data set, when data analysed according to maternal age groups and when data analysed according to RIF indication group vs the rest of the cases.

5.3.2. What is the incidence of chromosome abnormality for each chromosome assayed and are some chromosomes more prone to errors than others in the PGS single cells and in the follow up embryos?

At day 3, total of 670 embryos were analysed for abnormalities in chromosome 13, 16, 18, 21, 22, X and Y with average 3 embryos per cycle. Of those FISH diagnosed embryos, 182 (27%) were normal and 339 (51%) were aneuploid, 35 (5%) had another abnormalities such as haploid and tetraploidy. A summary of the different chromosomal constitutions in day 3 PGS embryos is presented in table 5.2 below.

Table 5.2: chromosome constitutions found in day 3 PGS embryos

Normal	Aneuploid	Other abnormalities	Inconclusive	No result
182	339	35	25	89
27%	51%	5%	4%	13%

Normal, aneuploid, other abnormal, inconclusive and noresults embryo from day 3 PGS cses; whole numbers and percentages.

At day 5 a total of 452 embryos were analysed in the follow up study for abnormalities in chromosome 13, 16, 18, 21, 22, X and Y. A total of 6906 nuclei were analysed with the average of 18 cells per embryo. Of these, 39 (9%) were normal and 163 (36%) were uniform aneuploid, 59 (13%) major mosaic, 52 (12%) minor mosaic, 38 (8%) were chaotic. A summary of the different chromosomal constitutions in follow up embryos is presented in table 5.3 below.

Table 5.3: chromosome constitutions found in PGS follow up embryos

Uniform diploid	Uniform aneuploid	Major mosaic	Minor mosaic	Chaotic	Other	No result
39	163	59	52	38	26	75
9%	36%	13%	12%	8%	6%	16%

Uniforma diploid, uniform aneuploid, major and minor mosaic, chaotic, other and no results embryos from follow up analysis; whole numbers and percentages are presented.

Note, abnormalities would be expected to be higher in day 5 embryos because those diagnosed as normal at day 3 were normally transferred.

Of the 339 single cells diagnosed as aneuploid at day 3, 606 incidences of chromosomal abnormalities were found (1.79 per cell on average). Of these, 380 (63%) were monosomies, 186 (31%) were trisomies and 40 (7%) were nullisomies. Monosomy 16 was the most common (16% of all abnormalities). Trisomy 22 and 13 were the most common trisomies.

In the 452 embryos diagnosed as aneuploid on day 5 however there were 163 that were considered as uniform aneuploid – these were compared with the single cell analyses above. A total 223 incidences of uniform aneuploidies were observed. Similar to day 3, incidences of monosomies were twice those of trisomies (66% vs 33%) in follow up embryos. Among those, monosomy 22 was the most common (20.6% of all abnormalities) with chromosome 22 also having the most common trisomy (14.3%).

Table 5.4: number of aneuploidies and percentage in day 3 PGS single blastomeres

Chromosome	Monosomy	Trisomy	Nullisomy	Total
13	52 (8.6%)	44 (7.3%)	5 (0.8%)	101
16	97 (16%)	35 (5.8%)	9 (1.5%)	141
18	63 (10.4%)	28 (4.6%)	5 (0.8%)	96
21	71 (11.7%)	28 (4.6%)	6 (1.0%)	105
22	74 (12.2%)	44 (7.3%)	7 (1.2%)	125
X	21 (3.5%)	3 (0.5%)	5 (0.8%)	29
Y	2 (0.3%)	4 (0.7%)	2 (0.3%)	8
Total	380 (63%)	186 (31%)	40 (7%)	606

Incidence of monosomies, trisomies and nullisomies for each cheomosome at day 3 PGS studies, whole numbers and percentage

Table 5.5: number of aneuploidies and percentage in day 5 uniform aneuploid embryos

Chromosome	Monosomy	Trisomy	Nullisomy	Total
13	15 (6.7%)	9 (4.0%)	0 (0.0%)	24
16	31 (13.9%)	12 (5.4%)	1 (0.4%)	44
18	24 (10.8%)	9 (4.0%)	0 (0.0%)	33
21	25 (11.2%)	11 (4.9%)	1 (0.4%)	37
22	46 (20.6%)	32 (14.3%)	1 (0.4%)	79
X	5 (2.2%)	0 (0.0%)	0 (0.0%)	5
Y	1 (0.4%)	0 (0.0%)	0 (0.0%)	1
Total	147 (66%)	73 (33%)	3 (1%)	223

Incidence of monosomies, trisomies and nullisomies for each cheomosome at day 5 follow up studies, whole numbers and percentage

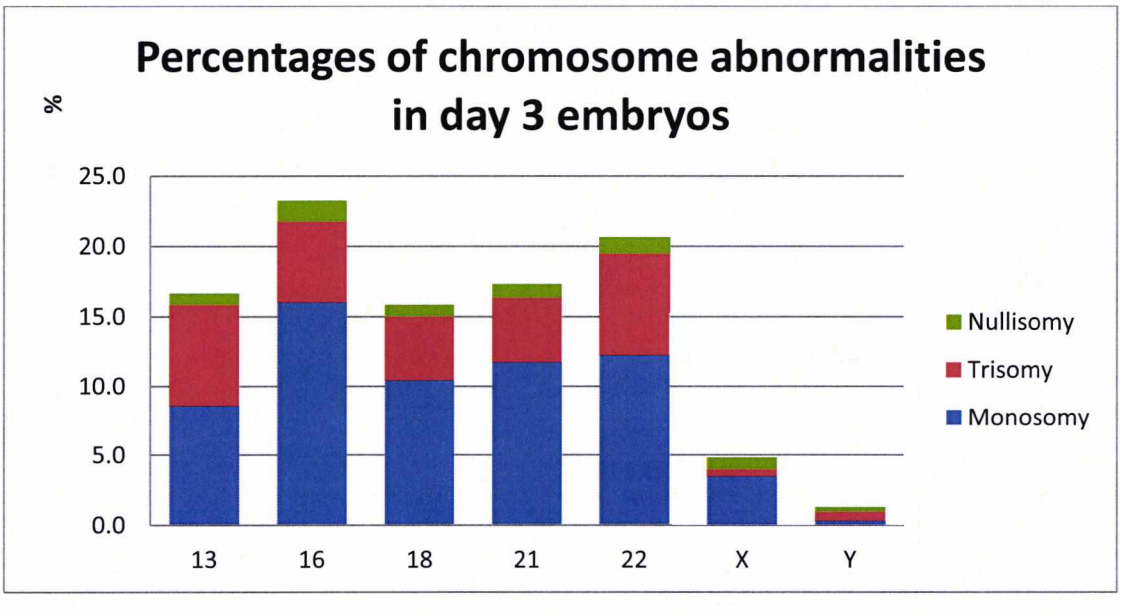


Figure 5.1: Percentages of aneuploidies in day 3 embryos related to each chromosome. For each chromosome (13, 16, 18, 21, 22, X and Y) percentage of monosomies, trisomies and nullisomies are presented

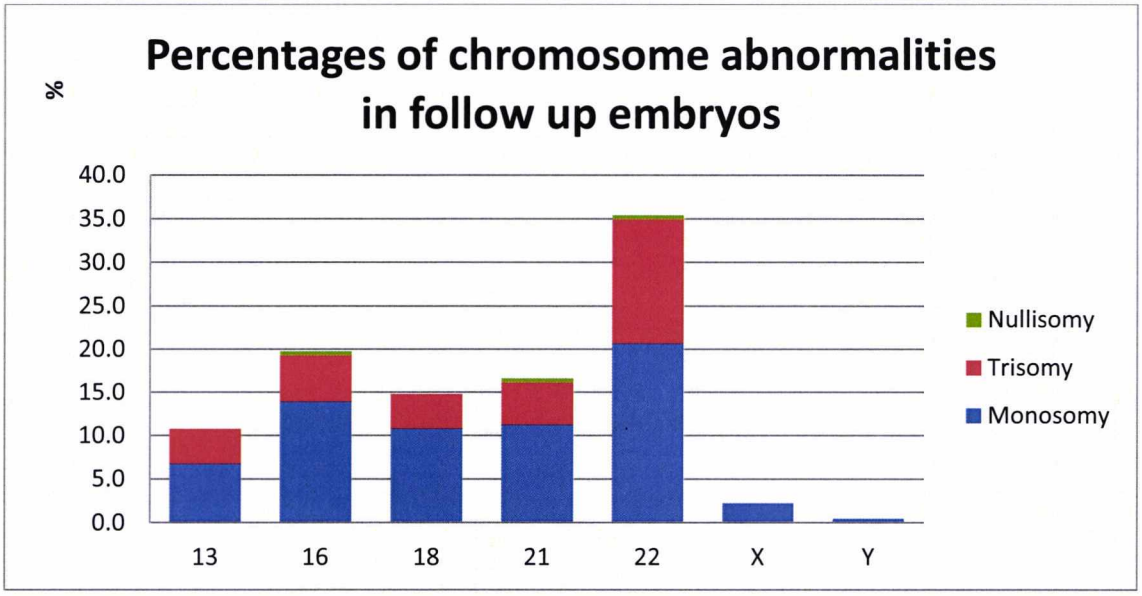


Figure 5.2: Percentages of aneuploidies in follow up embryos related to each chromosome
For each chromosome (13, 16, 18, 21, 22, X and Y) percentage of monosomies, trisomies and nullisomies are presented

The most notable results was the relatively greater number of abnormalities of the autosomes compared to the sex chromosomes and the fact that chromosome 22 had by far the greatest number of abnormalities at day 5 but a similar proportion to the others at day 3.

5.3.3. That the original PGS result was an accurate predictor of the subsequent embryo karyotype

Here, comparisons were made between single cell PGS (day 3) and follow up embryo (day 5) results in order to investigate at what extend day 3 data represent the actual ploidy of the whole embryo. Comparing the two datasets, embryos were categorised into 10 different groups as shown in the table 5.6.

Table 5.6: 10 Outcome from day 3 and day 5 analyses.

Day 3	Day 5	Category	Number	Percentage
Normal (disomy for all chromosomes)	Uniform disomy	1	39	11
	Minor mosaic	2	18	5
	Uniform aneuploidy/major mosaic	3	3	1
Abnormal (for at least one chromosome)	Uniform disomy/minor mosaic	4	6	2
	Minor mosaic (abnormality match)	5	52	15
	Major mosaic (abnormality match)	6	59	17
	Uniform aneuploidy exact match	7	79	22
	Uniform aneuploidy (match) plus others	8	46	13
	Other abnormality (excluding chaotic)	9	12	3
	Chaotic	10	38	11
			352	100

Overall, PGS and follow up concordance shown in the table 5.6 above; day 3 outcome as normal or abnormal and various day 5 outcomes when whole embryos were analysed. Green shading = precise concordance; Yellow shading = partial concordance; Red shading = false positive or negative

In the total data set from day 3 vs. day 5 diagnosis, number of embryos had exactly the same diagnosis was 118 (33%). 173 embryos (49%) had either some abnormality seen in the day 3 analysis or found more abnormalities than in day 3. 61 (18%) embryos did not agree with day 3 results at all (false positive or negative). Table 5.6 above and figure 5.3 below shows the percentages of different outcomes obtained when comparing day 3 vs. follow up embryos in the total data set. Categories 1-10 are according to the table 5.6 above.

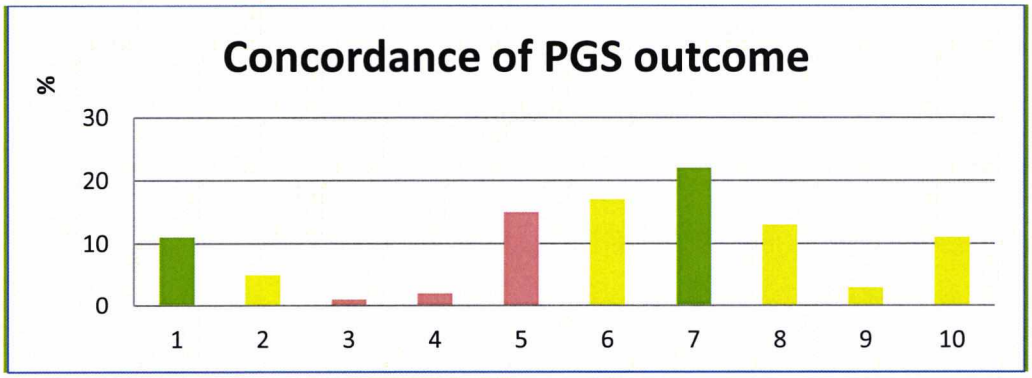


Figure 5.3: PGS accuracy in terms of different outcomes obtained comparing day 3 vs. follow up embryos. Categories 1-10 are as in the table above; categories 1,2,3 normal at day 3 and category 1:uniform disomy, 2: minor mosaic, and 3: Uniform aneuploidy/major mosaic at day5. Catogories 4,5,6,7,8,9,10 abnormal at day 3 and category 4: Uniform disomy/minor mosaic, 5: Minor mosaic (abnormality match), 6: Major mosaic (abnormality match), 7: Uniform aneuploidy exact match, 8: Uniform aneuploidy (match) plus others, 9: Other abnormality (excluding chaotic), 10: Chaotic at day 5. Coloured bars correspond to shaded cells in table; Green shading = precise concordance; Yellow shading = partial concordance; Red shading = false positive or negative.

The red cells and bars could also be broken down into "false negative" i.e. a normal diagnosis was given but the embryo was in fact abnormal or "false positive" i.e. the diagnosis was abnormal but the embryo was in fact predominantly normal. Only a 1% false negative rate was seen (table 5.7).

Table 5.7: Concordance between single cell and follow up embryos and false positive and negative values

	Concordance			Error rate	
	Yes	**Partial**	**No**	False +ve	False -ve
Total data set	118 (33%)	173 (49%)	61(18%)	58 (17%)	3 (1%)

Concordance between day3 single cell results and day5 follow up results. Agreements, disagreements and partial agreements as whole numbers and percentages. Disagreements of day 3 and day 5 results then catogorised as false positive and false negative; whole numbers and percentages for each category are presented.

5.3.4. That trisomy and monosomy is related to maternal age in the PGS single blastomeres and in the follow up embryos

As discussed in introduction section 1.4.2.1, the maternal age effect for trisomy is well described in live births and spontaneous abortions. It is less well established however whether the effect extends to monosomy (since monosomy can also arise by anaphase lag as well as non-disjunction) and whether abnormalities of different chromosomes are differentially represented (since abortus and live birth figures are compounded by differential survival rates). Questions of maternal age effect on trisomy and monosomy as well as differential effects on different chromosomes were addressed in both day 3 single blastomeres and follow up embryos (day 5) as there has been little opportunity for effects of differential survival to come into play. Patients were divided into 3 groups i.e. ≥35, 36-39 and ≤40. Average maternal age, standard deviation, minimum and maximum ages in the groups are presented in table 5.8 below.

Table 5.8: Age groups with maximum and minimum ages, average and standard deviations

Age groups	≤35	36-39	≥40
Mean age	32.71	38.19	42.18
Standard deviation	2.41	0.94	1.73
Maximum age within the group	35.00	39.00	48.00
Minimum age within the group	26.00	36.00	40.00
Number of cycles within the group	30	65	124

Different age groups included in this study are presented with mean age, standered diviations, maximum and minimum age within each group and number of cycles per each group.

In day 3 biopsied blastomeres a clear effect of maternal age was observed as indicated in table 5.9. That is, in the younger age group 32% were aneuploid, rising to 48% and 59% in the subsequent age groups which is highly significant (statistical analysis using chi test, $p<0.01$).

Table 5.9: Chromosome constitutions found in day 3 PGS embryos related to different age groups

Age group	No of blastomeres	Normal	Aneuploid	Other abnormalities	Inconclusive	No result
≤35	107	36 (34%)	34 (32%)	6 (6%)	4 (4%)	27 (25%)
36-39	227	80 (35%)	109 (48%)	10 (4%)	9 (4%)	19 (8%)
≥40	292	56 (19%)	172 (59%)	18 (6%)	11 (4%)	35 (12%)

Number of total blastomeres, normal, aneuploid, other abnormalities inconclusive and no results found in each age group.

Similarly, when considering patterns of abnormality, age specific differences were noted. That is, the proportion of abnormal cells that were trisomic increased incrementally in the age groups (18%, 28% and 34% respectively) and this increase is highly significant (statistical analysis using chi test, $p<0.01$). Whereas the proportion that were monosomic did not – thereby indicating a maternal age effect for trisomy but not monosomy (table 5.10).

Table 5.10: Incidences of aneuploidies in terms of monosomies, trisomies and nullisomies, related to chromosomes and age groups.

		Monosomy	Trisomy	Nullisomy	Total
≤35	13	5	5	3	13
	16	12	5	1	18
	18	10	2	1	13
	21	8	0	1	9
	22	6	1	1	8
	X	4	0	3	7
	Y	0	0	2	2
	Total	45 (63%)	13 (18%)	13 (18%)	71
36-39	13	18	14	0	32
	16	40	6	4	50
	18	15	5	4	24
	21	19	6	2	27
	22	22	17	3	42
	X	6	2	0	8
	Y	0	3	0	3
	Total	120 (65%)	53 (28%)	13 (7%)	186
≥40	13	27	22	2	51
	16	41	22	3	66
	18	33	19	0	52
	21	37	18	3	58
	22	42	23	3	68
	X	11	1	2	14
	Y	2	1	0	3
	Total	193 (62%)	106 (34%)	13 (4%)	312

Incidence of monosomies, trisomies and nullisomies for each chromosome according to each age group; regardless of the chromosome, total monosomies, trisomies or nullisomies seen in each age group also presented as percentages.

Similarly, the individual chromosomes that were more or less affected by maternal age seemed to be different. That is, in the younger age group chromosome 16 was the most affected however chromosome 22 became more subject to maternal age. In figure 5.4 below, the y-axes are normalised to allow comparison.

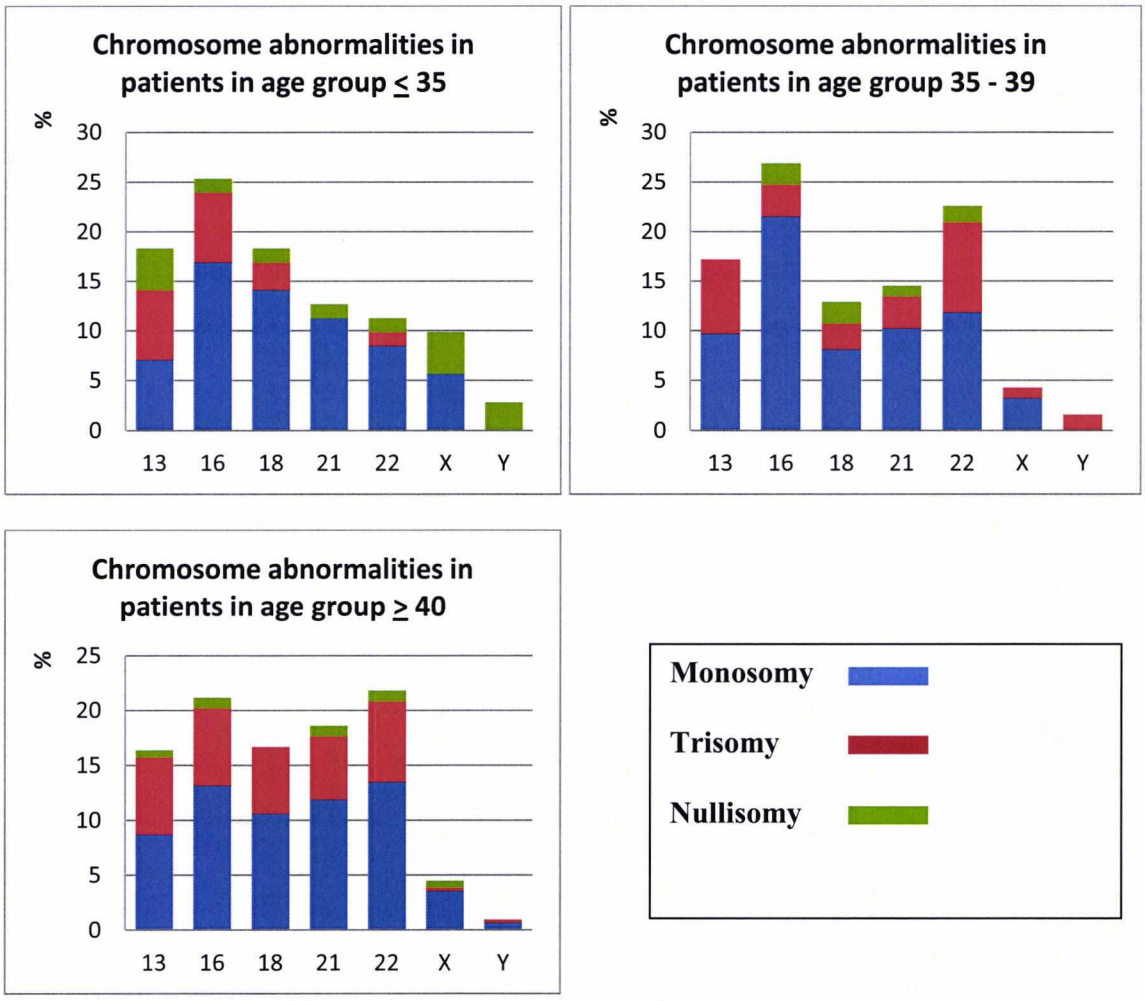


Figure 5.4: Incidences of aneuploidies in terms of monosomies, trisomies and nullisomies, related to chromosomes and maternal age groups. For each chromosome (13, 16, 18, 21, 22, X and Y) percentage of monosomies, trisomies and nullisomies are presented.

Looking at day 5 follow up cases, a maternal age effect was also observed. That is, the embryos that were uniformly aneuploidy (or the uniform aneuploids and the major mosaics combined) showed an incremental and significant increase in each of the age groups (statistical analysis using chi test, $p<0.01$).

The percentages of normal embryos were comparatively highest in 36-39 age group (16%). Levels of uniform aneuploidies were highest (45%) in the 40 and older age group and this is statistically significant at $p<0.01$. Table 5.11 below present different chromosomal constitutions related to various maternal age groups

Table 5.11: Chromosome constitutions found in follow up embryos related to different indication groups

Age group	Number of Embryos	Uniform diploid	Minor mosaic	Uniform aneuploid	Major mosaic	Chaotic	Other	No result
≤35	71	5 (7%)	15 (21%)	13 (18%)	16 (23%)	8 (11%)	9 (13%)	5 (7%)
36-39	135	22 (16%)	22 (16%)	43 (32%)	13 (10%)	10 (7%)	5 (4%)	20 (15%)
≥40	296	8 (4%)	14 (7%)	89 (45%)	24 (12%)	18 (9%)	12 (6%)	31 (16%)

Number of total embryos, uniform diploids, uniform aneuploids, minor and major mosaic, chaotic, other abnormalities and no results found in each age group as whole numbers and percentages.

As with the day 3 single blastomeres the relative proportion of trisomies increased with maternal age however, in this case, it was vast the expense of the monosomies, rather than the nullisomies (table 5.12)

Table 5.12: Incidences of aneuploidies in terms of monosomies, trisomies and nullisomies, related to chromosomes and age groups at day 5 follow up embryos.

		Monosomy	Trisomy	Nullisomy	Total
35 and younger	13	0	1	0	1
	16	2	1	0	3
	18	3	1	0	4
	21	2	0	0	2
	22	5	0	0	5
	X	0	0	0	0
	Y	0	0	0	0
	Total	12 (80%)	3 (20%)	0 (0%)	15
36-39	13	2	4	0	6
	16	9	2	0	11
	18	5	0	0	5
	21	9	2	0	11
	22	11	13	0	24
	X	0	0	0	0
	Y	0	0	0	0
	Total	36 (63%)	21 (37%)	0 (0%)	57
40 and older	13	12	4	0	16
	16	17	9	1	27
	18	14	6	0	20
	21	11	7	0	18
	22	26	16	0	42
	X	5	0	0	5
	Y	1	0	0	1
	Total	86 (67%)	42 (33%)	1 (1%)	129

Incidence of monosomies, trisomies and nullisomies for each chromosome according to each age group; regardless of the chromosome, total monosomies, trisomies or nullisomies seen in each age group also presented as percentages.

Chromosome specific differences were also apparent in each of the age groups (see figure 5.5, note y axes have been normalised for comparative purposes) with a relative increase in trisomy 21 the most notable increase.

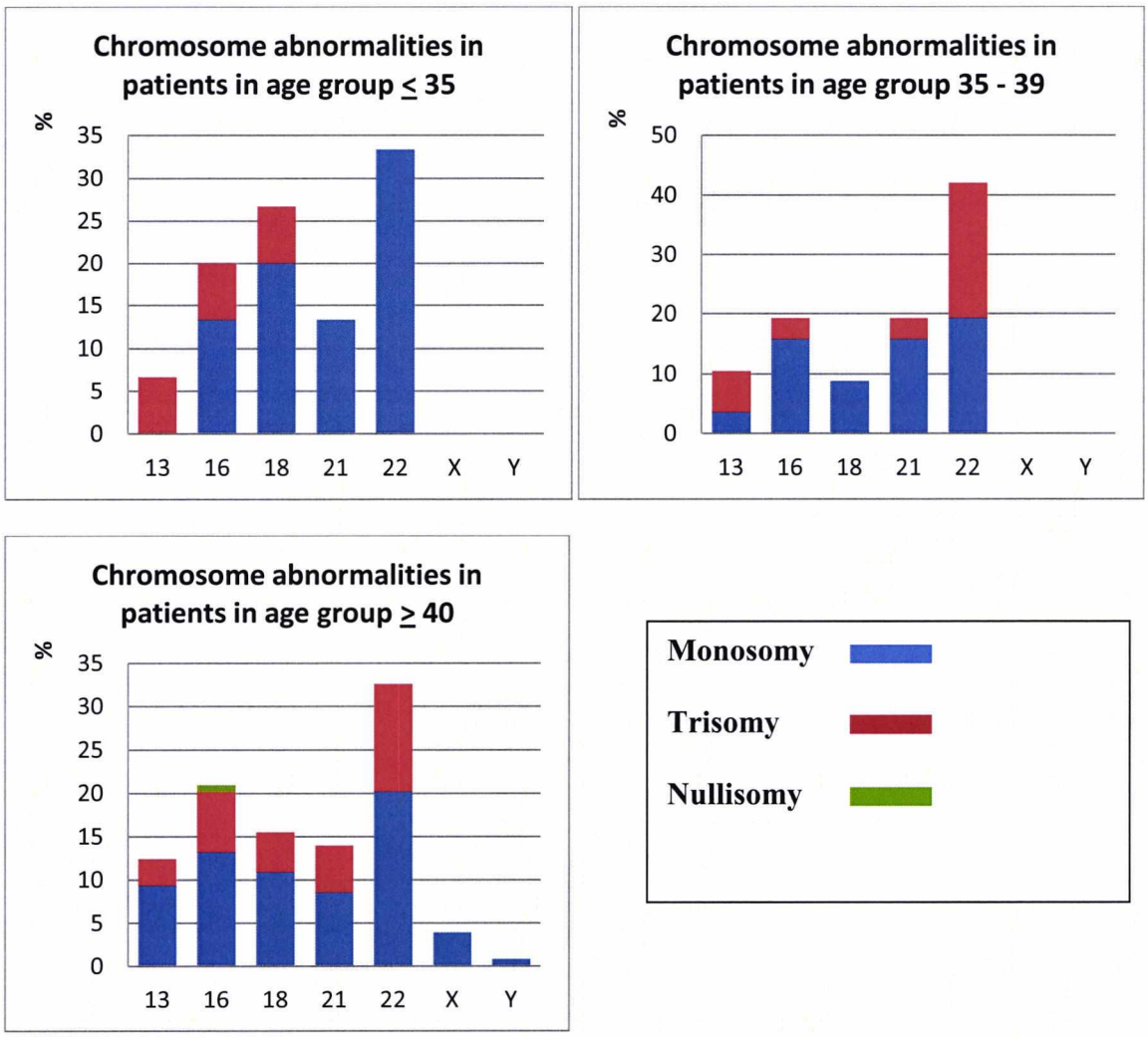


Figure 5.5: Incidences of aneuploidies in terms of monosomies, trisomies and nullisomies, related to chromosomes and maternal age groups at day 5 follow up studies. For each chromosome (13, 16, 18, 21, 22, X and Y) percentage of monosomies, trisomies and nullisomies are presented.

In conclusion, there is a clear and noticeable effect maternal age however the effect seems to be restricted to trisomy rather than monosomy and there are also chromosome specific patterns between the age groups.

5.3.5. That the recurrent implantation failure (RIF) referral category has a significantly different pattern of chromosome abnormality compared to the rest of the cohort.

In the PGS single cells

In the follow up embryos

It has been suggested that individual referral categories need to be looked are more closely (Harper *et al.*, 2008a) to establish patterns of abnormality. In our data set, numbers of cases were not sufficient to analyse chromosomal abnormalities related to all indication groups. However this study analysed chromosome abnormalities related to RIF patients and compare with non RIF patients. The RIF group was compared with the remainder of the cohort however the "non-RIF" cohort was "age-matched" for comparative purposes to control for the confounding effects of maternal age (i.e. some of the older patients were removed from the "non-RIF" group to allow a fair comparison). The following table 5.13 shows the age statistics.

Table 5.13: RIF and non-RIF group with maximum and minimum ages, average and standard deviations

Indication groups	RIF	Non-RIF
Average age	35.41	37.30
Standard deviation	4.15	2.60
Maximum age within the group	45.00	39.00
Minimum age within the group	28.00	26.00
Number of cycles within the group	34.00	48.00

Different indication groups (recurrent implantation failure vs. the other) included in this study are presented with mean age, standered diviations, maximum and minimum age within each group and number of cycles per each group.

Table 5.14 shows, for the day 3 biopsied cells indicate that the proportion of normal and aneuploid cells was very similar in both groups (data analysis with chi squared test, $p<0.05$).

The only differences were small but not significant proportions of aneuploid vs "no result" outcomes. This is also indicated in figure 5.6.

Table 5.14: Chromosome constitutions found in day 3 PGS embryos in RIF and non RIF groups

Indication	Average maternal age	No of embryos	Normal	Aneuploid	Other abnormalities	Inconclusive	No result
RIF	35	182	56 (31%)	75 (41%)	7 (4%)	5 (3%)	39 (21%)
Non- RIF	37	180	55 (31%)	85 (47%)	9 (5%)	8 (4%)	23 (13%)

Number of total blastomeres, normal, aneuploid, other abnormalities inconclusive and no results found in each age group.

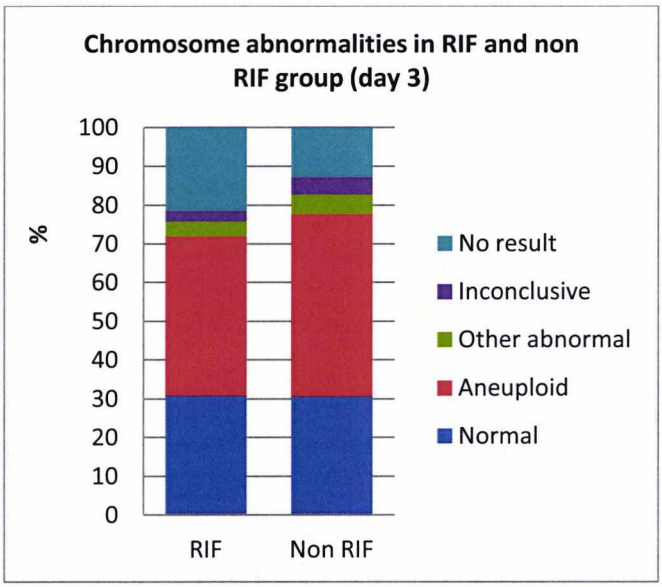


Figure 5.6: Day 3 results according to indication groups (RIF and non RIF)
Percentages of normal, aneuploid, other abnormalities inconclusive and no results found in each age group.

In table 5.15 the abnormalities are broken down by type and chromosome and analysis with chi squared test, $p<0.05$), again the patterns are similar for RIF and non-RIF groups – see also figure 5.7.

Table 5.15: Incidences of aneuploidies in terms of monosomies, trisomies and nullisomies, related to chromosomes and indication groups.

		Monosomy	Trisomy	Nullisomy	Total
RIF	13	9	11	2	22
	16	24	7	2	33
	18	11	5	1	17
	21	15	6	1	22
	22	12	10	1	23
	X	7	0	2	9
	Y	0	0	2	2
	Total	78 (61%)	39 (30%)	11 (9%)	128
Non RIF	13	14	6	1	21
	16	24	8	3	35
	18	12	3	2	17
	21	20	6	0	26
	22	14	13	1	28
	X	9	2	1	12
	Y	0	3	0	3
	Total	93 (65%)	41(29%)	9(6%)	143

Incidence of monosomies, trisomies and nullisomies for each chromosome according to RIF and non RIF groups; regardless of the chromosome, total monosomies, trisomies or nullisomies seen in each age group also presented as percentages.

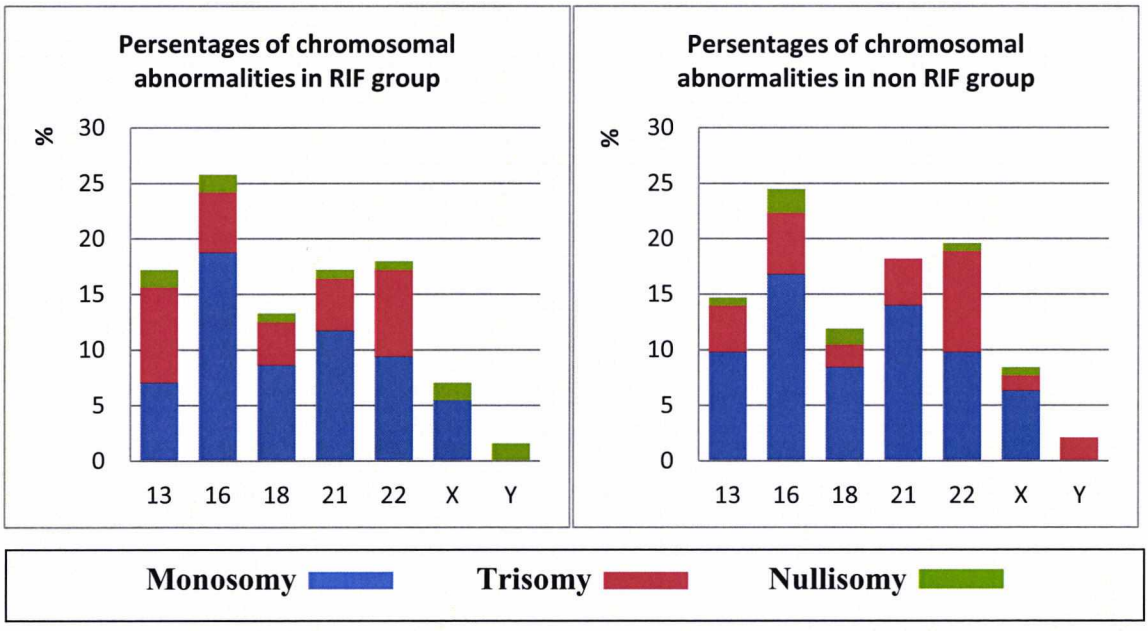


Figure 5.7: Incidences of aneuploidies in terms of monosomies, trisomies and nullisomies, related to chromosomes in RIF and non RIF group (day 3). For each chromosome (13, 16, 18, 21, 22, X and Y) percentage of monosomies, trisomies and nullisomies are presented.

When considering similar questions for the day 5 follow up embryos there were also 2 groups, RIF and non RIF (age matched). Number and percentages of chromosomal

constitutions in both groups are presented in the table 5.16 and figure 5.8 below. Again, data was analysis with chi squared test, at $p<0.05$ and patterns are not statistically similar from one another.

Table 5.16: Chromosome constitutions found in day 5 embryos - RIF compared to non RIF groups

Indication	No of embryos	No of nuclei	Uniform diploid	Uniform aneuploid	Major mosaic	Minor mosaic	Chaotic	Other abnormalities	No result
RIF	118	3284	11 (9%)	39 (33%)	11 (9%)	18 (15%)	13 (11%)	8 (7%)	18 (15%)
Non RIF	117	1908	14 (12%)	36 (31%)	15 (13%)	23 (20%)	9 (8%)	8 (7%)	12 (10%)

Number of total embryos, uniform diploids, uniform aneuploids, minor and major mosaic, chaotic, other abnormalities and no results found in RIF and non RIF group as whole numbers and percentages.

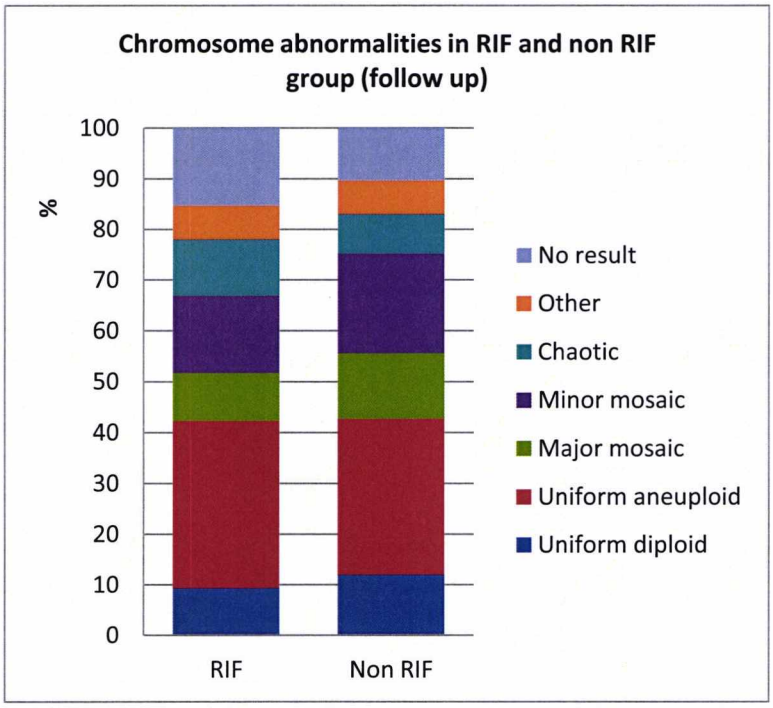


Figure 5.8: Follow up results according to indication groups (RIF and non RIF)
Percentage of uniform diploids, uniform aneuploids, minor and major mosaic, chaotic, other abnormalities and no results found in RIF and non RIF groups.

Finally, when broken down by chromosome, patterns were very similar in both groups with abnormalities of chromosome 22 the most common, followed by chromosomes 21, 16, 13, 18, 13 and the sex chromosomes respectively (figure 5.9).

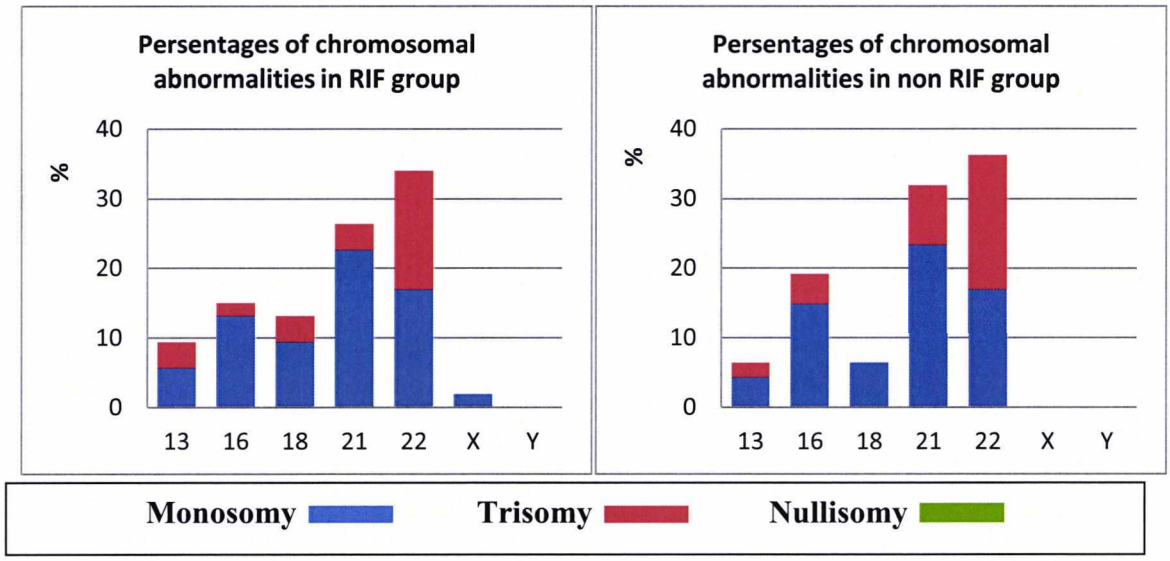


Figure 5.9: Incidences of aneuploidies in terms of monosomies, trisomies and nullisomies, related to chromosomes in RIF and non RIF groups (day 5). For each chromosome (13, 16, 18, 21, 22, X and Y) percentage of monosomies, trisomies and nullisomies are presented.

In conclusion therefore little or no evidence could be found, either in day 3 biopsied blastomeres or day 5 "follow-up" embryos that the recurrent implantation failure (RIF) referral category has a different pattern of chromosome abnormalities compared to the rest.

5.4. Conclusion remarks

Results in this chapter suggested that the overall levels of monosomy were significantly higher than trisomies in single cells from day 3, and in follow up embryos from day 5. Also, evidence is provided that PGS is a reasonable, though not entirely accurate, predictor of the karyotype of the rest of the embryo, that a maternal age effect could be detected and that there were not any significant differences in the RIF referral category compared to the others. A complete discussion of the results in presented in the section 8.3. In conclusion, PGS inaccuracies due to biological causes such as mosaicism cannot be not totally prevented. However technical aspects of causing inaccuracies such as quality of the FISH and the number of chromosomes tested can be improved; our approaches to solve some of these issues were presented in the next chapter.

6. To apply a 24 chromosome FISH strategy to investigate the incidence of aneuploidy in human embryos and possible correlates of chromosome abnormality

6.1. Background

Chromosomal aneuploidy studies both for PGS and "follow up" embryos (i.e. those surplus to requirements) have traditionally been performed using probes for a limited subset of chromosomes (typically 13, 16, 18, 21, 22 plus others) as in the previous chapter. However a recent study in our lab (Ioannou and Griffin, 2010; Ioannou *et al.*, 2011) has shown that 24 chromosome FISH can be achieved in a "4 layer, 6 fluorochrome" strategy. This method has produced 46/46 signals in approximately 60% of the nuclei in known diploid cells (lymphocytes). The aforementioned study also performed 24 chromosome FISH in 25 human embryos and found extensive chromosome loss in human follow up embryos. This initial study provided proof of principle for the approach however concerns were raised internally about the spreading method used to fix the nuclei to a glass slide and the quality of the embryos themselves. In other words it was unclear whether the extensive chromosomal loss seen was due to technical or biological reasons. The controversy surrounding the accuracy of the PGS, i.e. whether single cell diagnosis is an accurate reflection of the rest of the embryo was founded, in part, through worries about mosaicism. A cell by cell appraisal of mosaicism in human preimplantation embryos is thus a priority.

6.2. Aims and hypotheses

Given the above, the purpose of this chapter was to assess chromosomal copy number for loci from all 24 chromosomes in human embryos fixed with two different spreading methods with the following specific aims in mind:

1. To assess chromosome copy number in human cleavage stage embryos for all 24 chromosomes on a cell by cell basis in day 5 human embryos
2. To test the hypothesis that certain chromosomes are more prone to aneuploidy than others.
3. To test the hypothesis that chromosome loss is more common than chromosome gain as suggested by previous studies
4. To assess the level of chromosome mosaicism in human preimplantation development (at least for the embryo cohort studied here) for all chromosomes
5. To test the hypothesis that chromosome abnormality in human embryos is related to maternal age
6. To test the hypothesis that the day 3 morphological quality of the embryo is a reasonably accurate indicator of chromosome abnormality
7. To test the hypothesis that chromosomal abnormalities are less in number, in more advanced developmental stages (i.e. that there is some mechanism of "self-correction" or differential survival of euploid lines)
8. To test the hypothesis that different fixation methods give markedly different results in the above
9. Test the hypothesis that PGS diagnosis (for 8 chromosomes) is an accurate predictor of the ploidy status of the rest of the embryo.

6.3. Results

Control cells were assayed for each experiment, and the following table 6.1 idicates the hybridization efficiency for each probe (mean of all experiments). All probes hybridised at >95% efficiency apart from chromosome 1 (94.1%) and chromosome 14 (90.1%). All but 10 hybridised with >97% efficiency.

Table 6.1: Hybridisation efficiency for each probe on control lymphocyte material.

Chromosome probe	Percentage of nuclei with correct number of signals
1	94.1
2	96.1
3	100.0
4	98.0
5	96.1
6	100.0
7	99.0
8	96.1
9	95.1
10	99.0
11	95.1
12	100.0
13	96.1
14	90.3
15	97.0
16	93.2
17	95.1
18	97.0
19	97.8
20	98.0
21	99.0
22	98.1
X	100.0
Y	100.0

Useing each layer of probes separately, FISH was performed in control lymphocytes and hybridisation efficiency for each probe is presented in the table 6.1

The table 6.1 above for control lymphocytes is only indivigual layers of FISH resutls. Sequensial FISH have been performed on lymphocytes (as described in material and methods section 2.2.3) everytime when perform FISH with an embryo slide to make sure each layer of probes work in lymphocyte as well. However unlike blastomeres, lymphocyte cells do not keep their strcture with 24 FISH. After 2nd layer cell start getting damaged and likely to lose parts. Sequntial FISH on lymphocytes was performed in our lab (Ioannou *et al.*, 2011), with

the 60% sucessfull hybridisation to all 4 layers, therefore this study did not replicate the same experiments. However in comparison to lymphocytes embryo cells are much more robust and cells can handle atleast 6 layers of hybridisation rounds.

6.3.1. To assess chromosome copy number in human cleavage stage embryos for all 24 chromosomes on a cell by cell basis in day 5 human embryos

As described in the material and method section 2.2.1, embryos were already fixed in slide by embryologist from Genesis fertility clinic Athens and sent to University of Kent for the purpose of this study. In order to assess the chromosomal copy number for all 24 chromosomes, human embryos were fixed using a method that involved a methanol: acetic acid and tween HCl combination. This compared with a prior study by Dimitris Ioannou, PhD thesis, 2010 in which only an acid-tween fixation approach was used – for aim 8 (section 6.3.7) the results generated in this thesis were compared with those prior ones by Ioannou. In the current study 42 human embryos were examined at day 5 post-fertilization. The number of cells in the embryo that we felt confident of scoring varied from 4 – 120, with the average of 50, in part depending on whether arrest had occurred. Emrbyo details are given in the table 6.2 bellow.

Table 6.2: Information of embryos used in this study

Patient ID	Embryo number	Maternal age	Day 3 morphological quality	Day 5 Morphology	Number of cells
A	A1	32	2	Arrested	50
	A2		2	Blastocyst	38
	A3		2	Arrested	60
	A4		2.5	Blastocyst	62
	A5		2.5	Blastocyst	60
	A6		3	Arrested	25
B	B1	36		Morula	0
	B2		2	Arrested	19
	B3		2	Blastocyst	63
	B4		2	Arrested	17
	B5		2	Morula	29
	B6		3	Arrested	10
C	C1	38	3	Blastocyst	23
	C2		3	Morula	12
	C3		3	Morula	23
	C4		2	Blastocyst	25
	C5		2.5	Morula	28
D	D1	40	2	Hatching Blastocyst	60
	D2		2	Blastocyst	61
	D3		2	Blastocyst	56
	D4		2.5	Blastocyst	53
	D5		2.5	Morula	31
	D6		3	Morula	8
E	E1	39	2	Morula	22
	E2		1.5	Hatching Blastocyst	68
	E3		2	Hatching Blastocyst	49
	E4		2.5	Morula	43
F	F1	33	1	Morula	44
	F2		1	Blastocyst	101
	F3		2	Arrested	20
	F4		2.5	Morula	40
	F5		2.5	Morula	16
G	G1	37	3	Degenerate	4
	G2		3	Morula	23
	G3		2.5	Morula	26
	G4		3	Blastocyst	47
H	H1	42	2	Blastocyst	50
	H2		2	Blastocyst	67
	H3		2	Morula	48
	H4		2.5	Blastocyst	41
	H5		2.5	Arrested	12
	H6		2.5	Morula	9

Table 6.2 present patients ID (as A to H), embryo number belong to each patient, maternal age, embryo day 3 morphology scoring (given by embryologists, 1 is best and 3 is the poorest quality), embryo day 5 morphology (given by embryologists) and number of cells found in each embryo.

For all 42 embryos, successful sequential hybridisation results were obtained (as described in material and methods section 2.2.3) and strong signals were usually seen in all layers. However due to occasional fluorescent debris, a small proportion of the cells were not scored for a particular layer. In other words, results were counted in this study if at least 3 of 4 layers did not have fluorescent debris obscuring the preparation.

Using these criteria, the number of blastomeres that produced successful FISH signals for at least 3 layers was 1399 cells out of 1543 (92%). Table 6.2 shows the number of cells counted per embryo and the FISH efficiency.

Table 6.3: FISH efficiency of embryos analysed.

Patient number	Embryo ID	Number of cells	Number of cells counted in this study	FISH efficiency % i.e. proportion of cells with clear signals in at least 3 hybridization layers
A	1	50	49	98
	2	38	32	84
	3	60	58	97
	4	62	52	84
	5	60	58	97
	6	25	22	88
B	1	0	0	N/A
	2	19	11	58
	3	63	49	78
	4	17	17	100
	5	29	29	100
	6	10	10	100
C	1	23	23	100
	2	12	12	100
	3	23	23	100
	4	25	25	100
	5	28	28	100
D	1	60	54	90
	2	61	39	64
	3	56	52	93

	4	53	45	85
	5	31	14	45
	6	8	7	88
E	1	22	18	82
	2	68	67	99
	3	49	44	90
	4	43	40	93
F	1	44	44	100
	2	101	97	96
	3	20	19	95
	4	40	33	83
	5	16	15	94
G	1	4	4	100
	2	23	23	100
	3	26	25	96
	4	47	47	100
H	1	50	49	98
	2	67	56	84
	3	48	48	100
	4	41	40	98
	5	12	12	100
	6	9	9	100
TOTAL		1543	1399	92%

FISH efficiency for each embryo; the proportion of cells with clear signals in at least 3 hybridization layers

Figure 6.1 shows examples of normal female blastomere (A), blastomere with minor abnormalities (B), triploid blastomere with XXY (C), tetraploid blastomere (D) and normal female embryo metaphase (E).

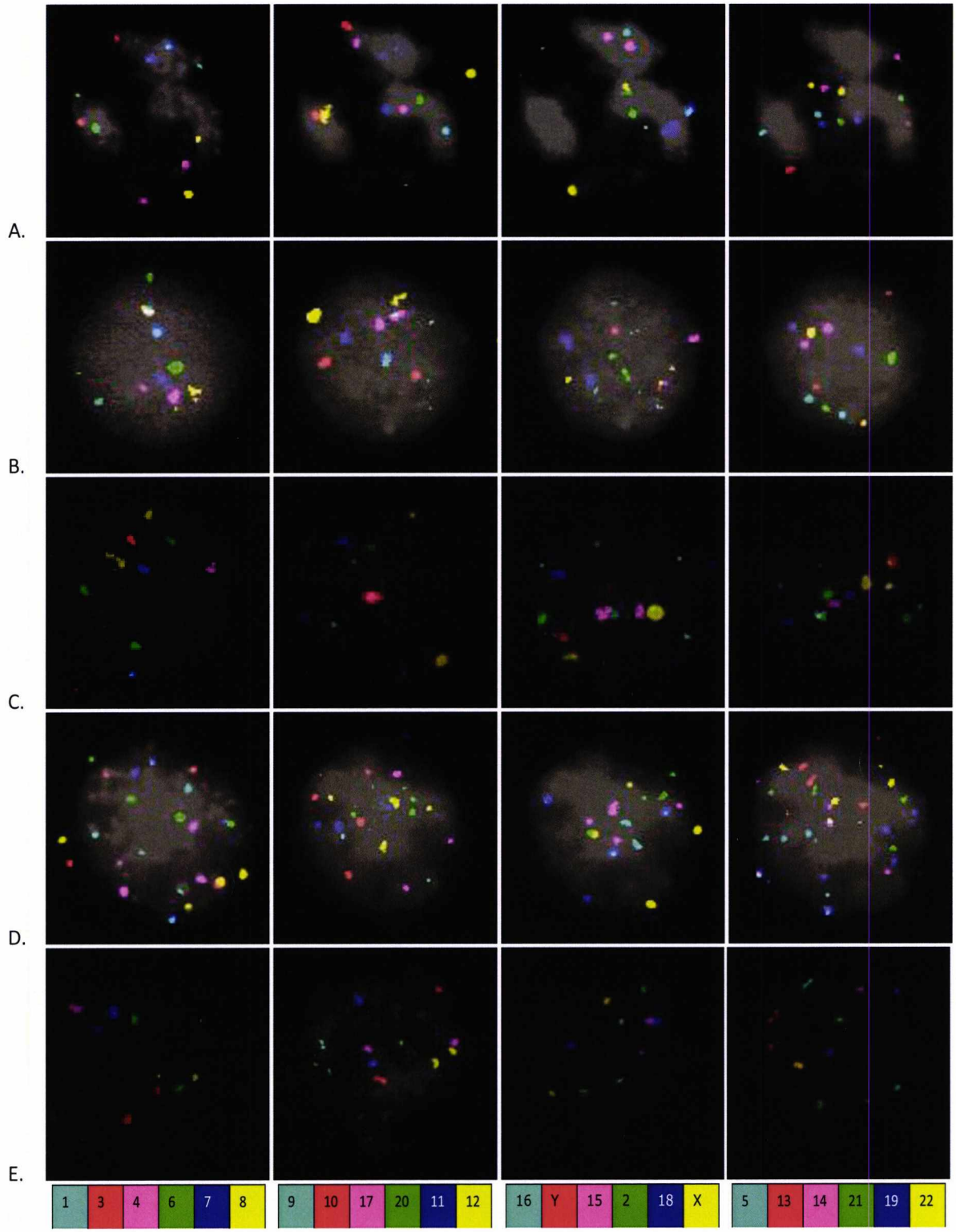


Figure 6.1: 24 FISH in normal female blastomere (A), minor abnormal (B), triploid blastomere with XXY (C), and tetraploid blastomere (D) and normal female embryo metaphase (E). Four images in each row represent each FISH layer performed. Chromosomes detected in each layer are showen in colour coded boxes.

6.3.2. To test the hypothesis that certain chromosomes are more prone to aneuploidy than others and that chromosome loss is more common than chromosome gain as suggested by previous studies.

The overall observation was that aneuploidy was present in every whole embryo, and in majority of individual cells. Of nearly 1400 cells from embryos were analysed, monosomy rates per chromosome ranged from 8.4% to 36.4% with a mean of 21.3% Trisomy rates ranged from 1.2% to 25.8% with a mean of 8.5% On the whole therefore, monosomy was approximately 2.5 times more common than trisomy with nullisomy the least common. The results also support the hypothesis that some chromosomes are more prone to error than others, with chromosomes 1, 4 (nullisomy) 8, 20, 7, 6, 3 (monosomy) 2, 19, 22 (trisomy). Chromosome 1 appeared the least likely to show two signals followed by chromosome 4 and 20 with chromosomes 14, 12 and sex chromosomes the most prone to showing the normal 2 copies. These results are summarised in table 6.4 and figure 6.2. Table 6.5 shows the data presented in table 6.4 but in descending order of frequency. In the electronic appendix 'specific aim 4' section, results from cell by cell analysis for all chromosomes for all of the embryos are given.

Table 6.4: number of normal, monosomy, trisomy, nullisomy and more than 3 signals for all chromosomes in 42 embryos analysed.

Chromosome number	Normal		Monosomy		Trisomy		Other		Nullisomy		Number of blastomeres
	No	%	No	%	No	%	No	%	No	%	
1	399	28.9	413	29.9	17	1.2	4	0.3	546	39.6	1380
2	781	56.1	153	11.0	359	25.8	42	3.0	55	3.9	1393
3	532	38.6	451	32.7	68	4.9	10	0.7	318	23.0	1380
4	427	30.9	424	30.7	23	1.7	7	0.5	498	36.1	1380
5	940	69.7	148	11.0	135	10.0	17	1.3	98	7.3	1348
6	608	44.1	457	33.1	66	4.8	7	0.5	237	17.2	1380
7	694	50.3	472	34.2	42	3.0	7	0.5	162	11.7	1380
8	629	45.6	506	36.7	65	4.7	14	1.0	161	11.7	1380
9	702	50.3	380	27.2	88	6.3	15	1.1	206	14.8	1395
10	970	69.6	210	15.1	143	10.3	23	1.6	34	2.4	1394
11	638	45.8	374	26.8	58	4.2	11	0.8	310	22.2	1394
12	988	70.8	272	19.5	91	6.5	23	1.6	14	1.0	1395
13	906	67.1	187	13.8	144	10.7	25	1.9	78	5.8	1351
14	981	72.6	160	11.8	113	8.4	20	1.5	66	4.9	1351
15	730	52.5	236	17.0	137	9.9	19	1.4	264	19.0	1390
16	666	47.8	306	22.0	134	9.6	22	1.6	262	18.8	1393
17	766	54.9	359	25.7	67	4.8	11	0.8	186	13.3	1395
18	843	60.5	245	17.6	112	8.0	28	2.0	160	11.5	1393
19	769	56.9	114	8.4	259	19.2	39	2.9	155	11.5	1351
20	435	31.2	497	35.6	50	3.6	9	0.6	401	28.7	1395
21	956	70.8	176	13.0	112	8.3	20	1.5	74	5.5	1351
22	906	67.1	161	11.9	225	16.7	21	1.6	20	1.5	1351
X&Y	1033	73.8	57	4.1	166	11.9	143	10.2	0	0	1399
Mean		54.6		21.3		8.5		1.7		13.5	

Table 6.4 present the incidence of normal, monosomy, trisomy, nullisomy, and other (more than 3 signals) chromosomes as whole numbers and percentages for each chromosome.

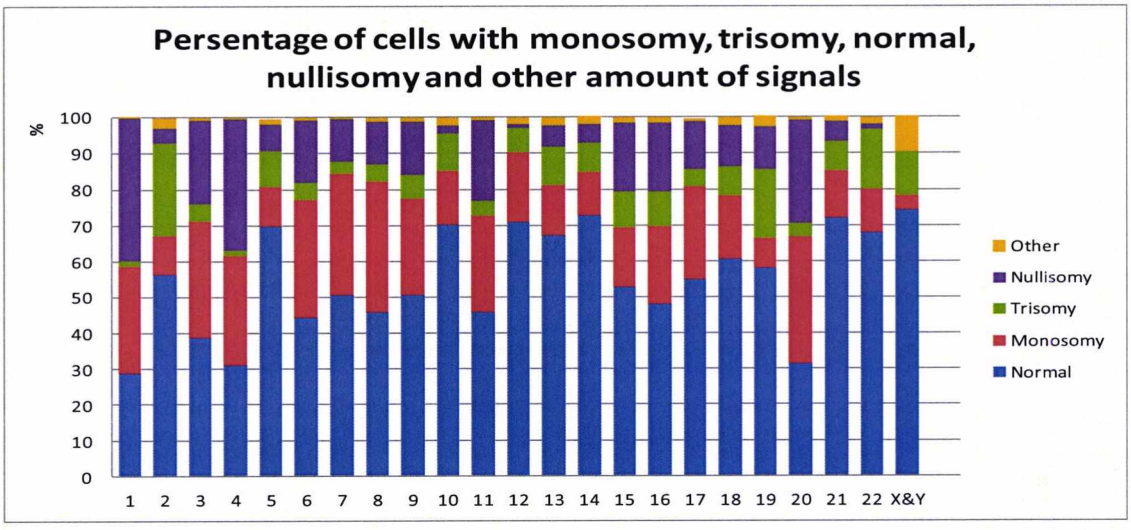


Figure 6.2: number of cells with monosomy, trisomy, normal, nullisomy and other amount of signals for all 24 chromosomes. X and Y chromosomes were analysed together.

Table 6.5: chromosomes with percentage of normal, monosomy, trisomy and nullisomy signal in descending order.

Chromosome number	Normal		Chromosome number	Monosomy		Chromosome number	Trisomy		Chromosome number	Nullisomy
	%			%			%			%
X&Y	73.8		8	36.7		2	25.8		1	39.6
14	72.6		20	35.6		19	19.2		4	36.1
12	70.8		7	34.2		22	16.7		20	28.7
21	70.8		6	33.1		X&Y	11.9		3	23.0
5	69.7		3	32.7		13	10.7		11	22.2
10	69.6		4	30.7		10	10.3		15	19.0
13	67.1		1	29.9		5	10.0		16	18.8
22	67.1		9	27.2		15	9.9		6	17.2
18	60.5		11	26.8		16	9.6		9	14.8
19	56.9		17	25.7		14	8.4		17	13.3
2	56.1		16	22.0		21	8.3		7	11.7
17	54.9		12	19.5		18	8.0		8	11.7
15	52.5		18	17.6		12	6.5		18	11.5
9	50.3		15	17.0		9	6.3		19	11.5
7	50.3		10	15.1		3	4.9		5	7.3
16	47.8		13	13.8		17	4.8		13	5.8
11	45.8		21	13.0		6	4.8		21	5.5
8	45.6		22	11.9		8	4.7		14	4.9
6	44.1		14	11.8		11	4.2		2	3.9
3	38.6		2	11.0		20	3.6		10	2.4
20	31.2		5	11.0		7	3.0		22	1.5
4	30.9		19	8.4		4	1.7		12	1.0
1	28.9		X&Y	4.1		1	1.2		X&Y	0

Table 6.5 present the incidence of normal, monosomy, trisomy, nullisomy, and other (more than 3 signals) in decending order. Chromosomes gives highest incidence for each category (normal or aneuploid) listed in the top and lowest listed in the bottom row.

Even though sex chromosome abnormalities were less common in pre implantation embryos our results showed some evidence for presence of sex chromosome abnormalities and mosaicism. The following table (table 6.6) is a breakdown of the sex chromosome patterns observed.

Table 6.6: ***Sex chromosome abnormalities***

Patient Number	Embryo Number	XX	XY	XO	XXY	XYY	XXX	XXXY	YO	Other	Total
A	1	44	0	0	2	0	2	0	0	1	49
	2	1	13	2	10	1	1	2	0	2	32
	3	0	55	0	2	0	0	0	1	0	58
	4	14	1	0	0	0	35	1	0	1	52
	5	0	47	1	1	2	0	0	0	7	58
	6	0	15	1	3	0	0	0	0	3	22
B	2	11	0	0	0	0	0	0	0	0	11
	3	40	0	1	1	0	1	0	0	6	49
	4	2	1	0	11	0	0	1	0	2	17
	5	25	0	2	0	0	0	0	0	2	29
	6	0	4	1	0	0	0	0	1	4	10
C	1	0	19	0	2	2	0	0	0	0	23
	2	9	0	1	0	0	2	0	0	0	12
	3	15	0	2	2	0	0	0	0	4	23
	4	0	2	0	13	0	0	7	0	3	25
	5	1	2	0	5	0	0	8	2	10	28
D	1	7	25	12	8	1	0	0	0	1	54
	2	31	0	2	1	0	3	0	0	2	39
	3	50	0	0	0	0	2	0	0	0	52
	4	40	0	3	0	0	2	0	0	0	45
	5	13	0	0	0	0	0	0	0	1	14
	6	1	0	6	0	0	0	0	0	0	7
E	1	0	9	0	8	0	0	0	0	1	18
	2	0	49	1	3	1	0	1	0	12	67
	3	36	0	1	1	0	2	0	0	4	44
	4	30	0	1	0	0	4	1	0	4	40
F	1	38	0	3	0	0	2	0	0	1	44
	2	1	74	1	2	6	0	0	0	13	97
	3	0	15	1	1	1	0	0	0	1	19
	4	28	0	0	0	0	1	0	0	4	33
	5	0	14	1	0	0	0	0	0	0	15
G	1	4	0	0	0	0	0	0	0	0	4
	2	20	0	2	0	0	1	0	0	0	23
	3	0	14	1	4	0	0	0	0	6	25
	4	34	1	1	4	0	1	0	0	6	47
H	1	39	0	1	3	0	0	1	0	5	49
	2	47	1	3	0	0	1	0	0	4	56
	3	38	0	1	1	0	3	1	0	4	48
	4	38	0	1	0	0	0	0	0	1	40
	5	0	10	0	1	0	0	0	0	1	12
	6	0	5	0	0	0	0	0	0	4	9

Different sex chromosome combinations in each embryo; normal XX and XY as well as X and Y monosomies and trisomies. Other refers to rare XY combinations seen in the data set.

6.3.3. To assess the level of chromosome mosaicism in human preimplantation development (at least for the embryo cohort studied here) for all chromosomes

Table 6.7 below shows the main results from the embryo mosaicism analysis. Abnormalities present in more than 50% of cells and chromosomes normal for more than 50% of the nuclei were listed in the table however the full summary is given in the appendix section 10.3.1. According to the previously published criteria, if a specific abnormality is found in more than 90% of cells the abnormality was categorised as meiotic error. Unlike in the previous chapter the terms such as "major" and "minor" mosaic were considered unfit for purpose because of the larger numbers of chromosome pairs analysed. No specific patterns were outstanding and thus the raw data is summarised below and presented in the appendix 10.3.1 in full.

Table 6.7: Analysis of mosaicism in each embryo. Meiotic errors are inferred if more than 90% of the cells have the same abnormality.

Embryo number	Maternal age	Day 3 morphological quality	Day 5 Morphology	Follow up results		Mosaicism
				Aneuploidies	Normal	
A1	32	2	Cells	53% monosomy 7, 57% monosomy 20	15 chromosomes normal for > 50%	Mitotic error in chromosome 7 and 20
A2		2	Blastocyst	50% trisomy 2, 53% monosomy 9 56% monosomy 11, 50% monosomy 18 56% monosomy 20, 88% normal for 21 88% normal for 22	11 chromosomes normal for > 50%	Mitotic errors Extensive chromosome loss
A3		2	Cells	52% monosomy 1, 50% monosomy 8, 52% trisomy 2	16 chromosomes normal for > 50%	Mitotic errors for chromosome 1,2 and 8
A4		2.5	Blastocyst	60% trisomy 2, 65% trisomy 5 62% trisomy 13, 81% trisomy 14 60% trisomy 15, 42% trisomy 16 56% trisomy 18, 67% trisomy 19 69% trisomy 21, 77% trisomy 22 69% trisomy X	5 chromosomes normal for > 50%	Evidence for triploid embryo
A5		2.5	Blastocyst	53% trisomy 2, 50% monosomy 6 72% trisomy 22	16 chromosomes normal for > 50%	Mitotic errors
A6		3	Cells	55% monosomy 4, 55% monosomy 8 50% monosomy 17	14 chromosomes normal for > 50%	Mitotic errors in chromosome 4, 8 and 17.
B1	36		Morula	No result		
B2		2	Cells	38% trisomy 18, 55% normal 18 55% mono 21, 27% normal	18 chromosomes normal for > 50%	Mitotic errors
B3		2	Blastocyst	96% mono 22	14 chromosomes	Meiotic error for chromosome 22

					normal for > 50%	
B4		2	Cells	82% trisomy 2, 65% trisomy 5 76% trisomy 10, 65% trisomy12 76% trisomy13, 65% trisomy 14 71% trisomy 15, 65% trisomy 17 76% trisomy 18, 82% trisomy 21 88% trisomy 22, 76% XXY	4 chromosomes normal for > 50%	Evidence for triploid embryo
B5		2	Morula	59% normal 15,	9 chromosomes normal for > 50%	Final layer of FISH did not work
B6		3	Cells	83% monosomy 6, 50% monosomy 8 50% monosomy 9, 60% monosomy 11 60% monosomy 15 50% monosomy 20	12 chromosomes normal for > 50%	Extensive chromosome loss
C1	38	3	Blastocyst	61% monosomy 4, 61% monosomy 6 57% monosomy 7, 52% monosomy 8 70% monosomy 20	13 chromosomes normal for > 50%	Extensive chromosome loss
C2		3	Morula	50% monosomy 1, 50% monosomy 6 67% monosomy 7, 50% monosomy 9 50% monosomy 15, 50% monosomy 16 67% monosomy 20	15 chromosomes normal for > 50%	Extensive chromosome loss
C3		3	Morula	57% monosomy 4, 52% monosomy 6 52% monosomy 8, 52% trisomy 22 22% trisomy 21, 48% monosomy 21	12 chromosomes normal for > 50%	
C4		2	Blastocyst	100% normal for 15, 100% monosomy 16	16 chromosomes normal for > 50%	Meiotic error for chromosome 16.
C5		2.5	Morula	54% monosomy 6, 65% normal for	10 chromosomes	Mitotic errors

				15 75% monosomy 16, 54% monosomy 20	normal for > 50%	
D1	40	2	Hatching Blastocyst	50% nullisomy 16, 15% monosomy 16	13 chromosomes normal for > 50%	Mitotic errors
D2		2	Blastocyst	51% monosomy 8, 51% monosomy 11 51% monosomy 20	12 chromosomes normal for > 50%	Chromosome losses
D3		2	Blastocyst	58% trisomy 2, 50% monosomy 8 33% nullisomy 16, 12% monosomy 16 17% trisomy 16, 38% normal	17 chromosomes normal for > 50%	Mitotic errors
D4		2.5	Blastocyst	53% monosomy 14	9 chromosomes normal for > 50%	
D5		2.5	Morula	57% monosomy 6, 57% monosomy 7 64% monosomy 8, 50% monosomy 15 50% monosomy 16	7 chromosomes normal for > 50%	Extensive chromosome loss
D6		3	Morula	86% monosomy 1, 86% monosomy 2 86% monosomy 3,100% monosomy 4 67% monosomy 5, 100% monosomy 6 71% monosomy 7, 86% monosomy 8 100% monosomy 9, 86% monosomy 10 86% monosomy 12,100% monosomy13 71% monosomy 14, 57% monosomy 15 86% monosomy 17, 71% monosomy	0 chromosomes normal for > 50%	Evidence for haploid embryo

				18 71% monosomy 19, 57% monosomy 20 86% monosomy 21, 86% monosomy 22 86% monosomy X		
E1	39	2	Morula	67% monosomy 22, 78% monosomy 10 56% monosomy 14, 94% monosomy 15 67% monosomy 22	11 chromosomes normal for > 50%	Extensive chromosome loss
E2		1.5	Hatching Blastocyst	No aneuploidy present more than 50% of cells	15 chromosomes normal for > 50%	Evidence for overall normal embryo
E3		2	Hatching Blastocyst	56% monosomy 7, 95% monosomy 22	13 chromosomes normal for > 50%	Meiotic errors for chromosome 22
E4		2.5	Morula	73% monosomy 16	10 chromosomes normal for > 50%	Extensive loss for chromosome 16; could be due to meiotic errors.
F1	33	1	Morula	57% monosomy 8, 80% normal 16	16 chromosomes normal for > 50%	Mitotic errors
F2		1	Blastocyst	No aneuploidy present more than 50% of cells	20 chromosomes normal for > 50%	Evidence for overall normal
F3		2	Cells	58% monosomy 3, 58% monosomy 4 63% monosomy 6	18 chromosomes normal for > 50%	Mitotic error for chromosome 3, 4 and 6
F4		2.5	Morula	No aneuploidy present more than 50% of cells	17 chromosomes normal for > 50%	Evidence for overall normal
F5		2.5	Morula	87% monosomy 16	20 chromosomes normal for > 50%	Extensive loss for chromosome 16; could be due to meiotic errors.
G1	37	3	Degenerate	75% normal 17, 50% monosomy 9 50% monosomy 11, 50% monosomy 13 50% monosomy 14, 50% monosomy	20 chromosomes normal for > 50%	Mitotic errors

				22		
G2		3	Morula	No aneuploidy present more than 50% of cells	23 chromosomes normal for > 50%	Overall normal
G3		2.5	Morula	24 % monosomy 16, 56 % normal 16 56% monosomy 13	11 chromosomes normal for > 50%	Mitotic errors
G4		3	Blastocyst	77% trisomy 22	20 chromosomes normal for > 50%	Extensive gain for chromosome 22; could be due to meiotic errors.
H1	42	2	Blastocyst	24% trisomy 17, 39% normal 17 10% monosomy 17, 61% monosomy 20	15 chromosomes normal for > 50%	Mitotic errors
H2		2	Blastocyst	79% trisomy 22	13 chromosomes normal for > 50%	Extensive gain for chromosome 22; could be due to meiotic errors.
H3		2	Morula	15% monosomy 13, 19% trisomy 13 60% normal 13, 88% monosomy 7 67% trisomy 10, 73% monosomy 12	14 chromosomes normal for > 50%	Extensive loss for chromosome 7 and 12; could be due to meiotic errors.
H4		2.5	Blastocyst	80% normal 18, 18% monosomy 18 66% monosomy 6, 55% monosomy 8 53% monosomy 11, 75% trisomy 19	15 chromosomes normal for > 50%	Extensive gain for chromosome 19; could be due to meiotic errors.
H5		2.5	Cells	42% monosomy 15, 42% normal 15 8% trisomy 15, 92% trisomy 2 50% monosomy 8, 75% monosomy 17 92% monosomy19	17 chromosomes normal for > 50%	Meiotic errors for chromosome 2, and 19
H6		2.5	Morula	56% monosomy 11	9 chromosomes normal for > 50%	Highly mosaic

Summury of follow up results for each embryo. For each embryo, abnormalities present in more than 50% of cells and chromosomes normal for more than 50% of the nuclei were listed. Majority of emrbryos show mosaic and chaotic patterns.

6.3.4. To test the hypothesis that chromosome abnormality in human embryos is related to maternal age

The table 6.8 below shows the overall disomy and aneuploidy levels in 3 maternal age groups. Results suggest that although the proportion of disomic chromosomes was higher in the younger age group ($p<0.05$ for <35 vs the rest), a maternal age effect for trisomy could not be detected. Overall monosomy and nullisomy were however slightly higher in the older age groups (differences not statistically significant). These results were different to those in the previous chapter. However when individual chromosomes were analysed, certain chromosomes gave statistically significant differences. Significant results are highlighted and presened in table 6.9 below.

Table 6.8 Relative levels of disomy and aneuploidy in three age groups

	≤35	36-39	≥40
Normal	58.78	53.42	52.01
Monosomy	19.62	21.70	22.96
Trisomy	10.05	8.062	7.20
Nullisomy	9.95	14.05	17.26
Others	1.60	2.771	0.57
Total abnormalities	41.22	46.58	47.99

List of normal, monosomy, trisomy, nullisomy and other abnormalities seen in different age groups as a percentage.

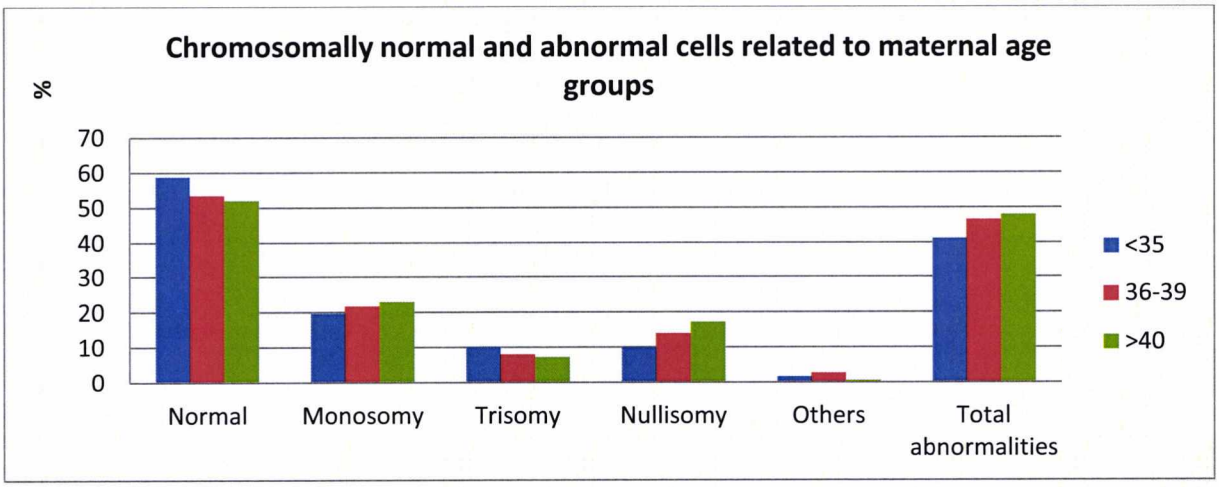


Figure 6.3 Relative levels of disomy and aneuploidy in three age groups.
Normal, monosomy, trisomy, nullisomy, other and total abnormalited seen with different age groups are presented as presentages.

Table 6.9: Percentage of disomy and aneuploidy per chromosome – highlighted numbers indicate significant differences at p<0.1

	Percentages																						
Chromosome	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**X,Y**
Normal																							
≤35	28	54	38	28	73	43	50	44	57	75	57	75	77	76	65	56	64	69	66	28	80	75	75
36-39	32	62	43	37	66	48	52	48	46	70	42	70	62	70	56	43	53	59	55	36	62	50	66
≥40	26	53	34	27	72	41	49	45	48	66	37	69	63	74	35	44	47	54	51	30	72	80	81
Monosomy																							
≤35	34	7	38	37	8	36	35	41	26	14	25	16	9	7	8	17	26	16	5	36	6	3	2
36-39	29	13	32	28	12	28	31	32	28	16	26	17	13	14	19	31	23	18	8	33	18	27	3
≥40	27	12	28	28	13	36	38	38	28	15	29	27	20	16	25	18	29	20	13	38	16	6	7
Trisomy																							
≤35	1	35	5	1	13	3	2	4	10	8	6	8	10	12	15	14	4	10	19	5	11	19	15
36-39	2	18	6	2	8	6	4	6	5	9	3	9	12	7	11	9	5	9	14	3	7	17	14
≥40	1	24	3	2	9	5	3	5	4	14	3	3	10	6	3	6	6	5	26	3	7	13	6
Nullisomy																							
≤35	37	1	19	34	4	17	12	11	6	2	10	1	3	3	11	11	5	2	7	32	2	0	0
36-39	37	3	17	32	12	17	13	13	19	2	27	1	9	7	11	15	17	12	19	26	10	4	0
≥40	46	8	35	43	5	18	9	11	19	4	31	1	7	5	37	32	18	22	9	29	5	0	0
Others																							
≤35	0	3	0	0	2	0	1	1	1	2	1	1	1	2	1	2	1	3	3	0	2	2	8
36-39	0	4	2	1	2	1	1	2	2	3	1	3	4	2	2	2	1	2	4	2	3	3	17
≥40	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	6

Percentages of normal, monosomy, trisomy, nullisomy and other abnormalities related to 3 age groups for all 24 chromosomes. For each chromosome normal and different abnormalities were analysed accrding to different maternal ages with chi squared test. Resusts were considerd significant when P≤0.1 (at 90% confidence). Significant results are highlighted in yellow.

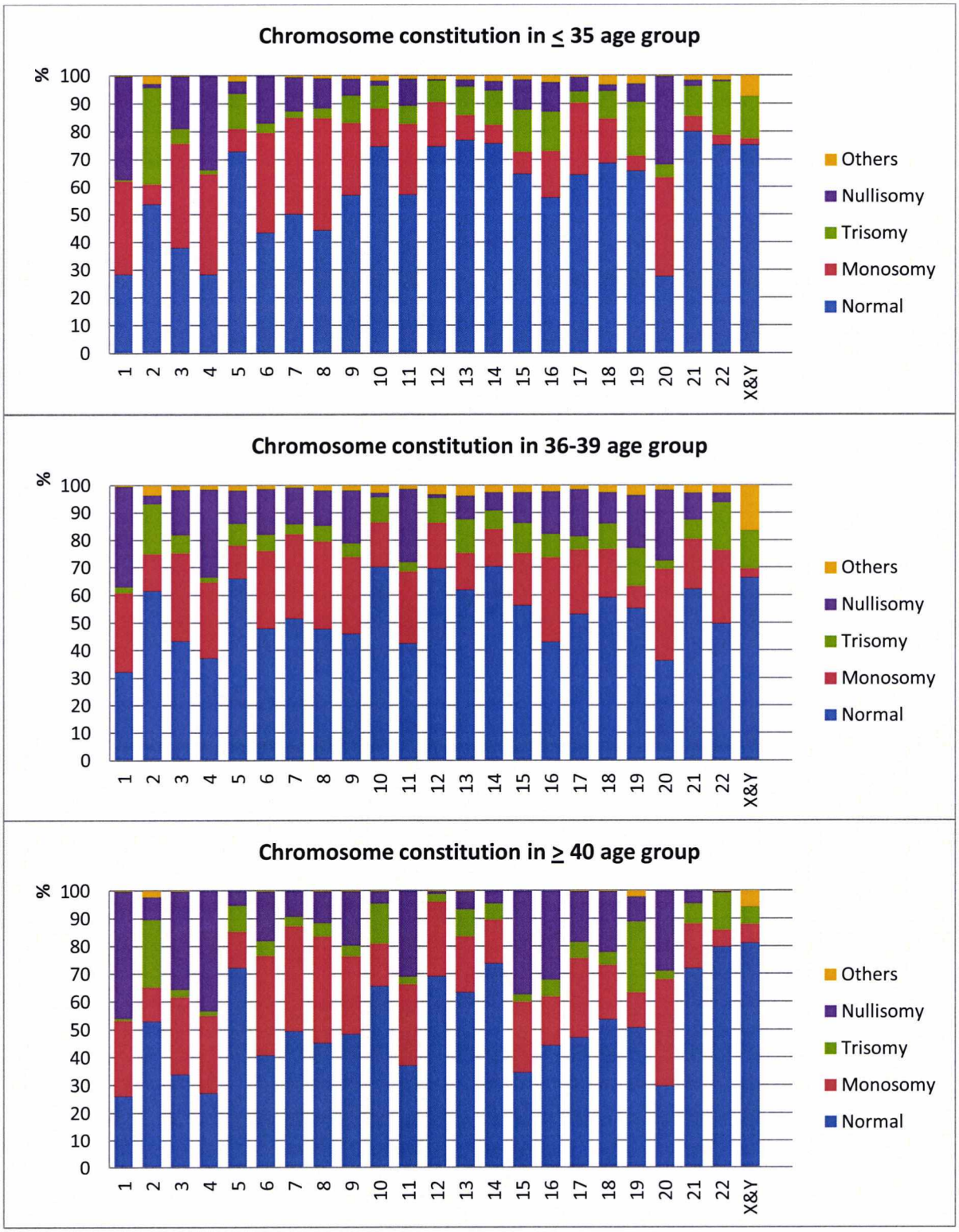


Figure 6.4: Percentages of aneuploidy and disomy per chromosome
Normal, monosomy, trisomy, nullisomy, and other abnormalites seen with different age groups are presented as presentages for each chromosome.

6.3.5. To test the hypothesis that the morphological quality of the embryo is a reasonably accurate indicator of chromosome abnormality

The table 6.10 below shows the overall disomy and aneuploidy levels related to 4 embryo morphology criteria at day 3 (where 1 is the best and 3 is the worst). Results suggest that the total level of abnormalities was relatively higher in the poorest quality embryos but not statistically significat. However when individual chromosomes were analysed, certain chromosomes gave statistically significant differences related to embryo day 3 morphology which presened in table 6.11 below. Significant results were highlighted.

Table 6.10: Relative levels of disomy and aneuploidy and its relationship to embryo morphology.

%	1-1.5	2.5	2	3
Disomic cells	64.48	50.59	53.44	56.27
Monosomic cells	19.22	20.44	21.49	24.73
Trisomic cells	6.79	11.00	7.86	7.23
Nullisomic cells	8.15	15.11	15.61	9.12
Other abnormalities	1.37	2.64	1.14	2.65
Total abnormalities	35.52	46.11	49.19	43.73

List of normal, monosomy, trisomy, nullisomy and other abnormalities seen in different day 3 morphology groups as a percentage. Embryos scores as 1 are the best and 3 are the worst.

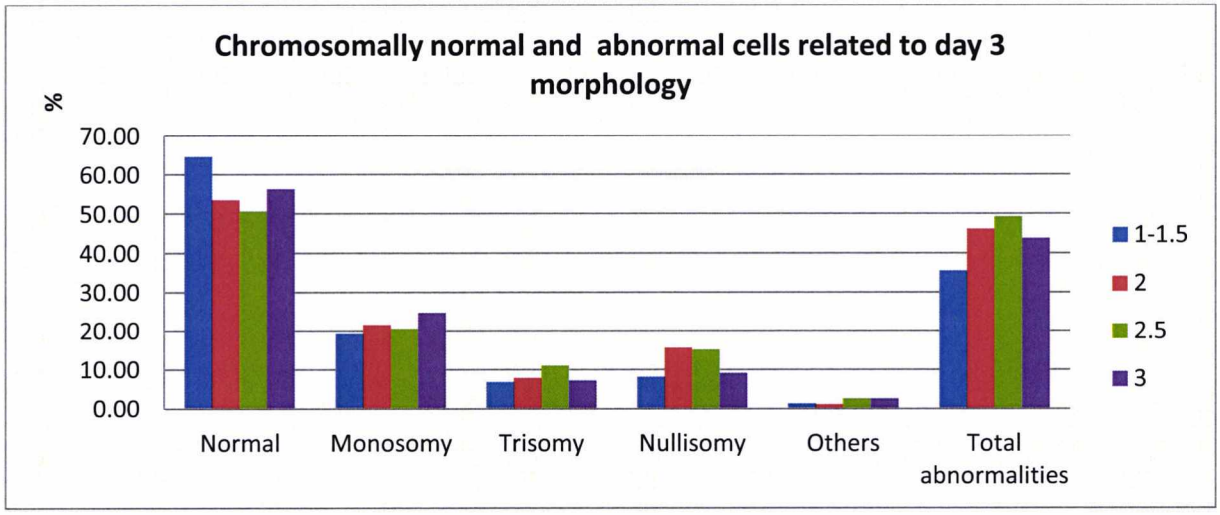


Figure 6.5 Relative levels of disomy and aneuploidy in three age groups.
Normal, monosomy, trisomy, nullisomy, other and total abnormalited seen with different day 3 morphology groups are presented as presentages. Embryos scores as 1 are the best and 3 are the worst.

Table 6.11: Percentage of disomy and aneuploidy per chromosome related to embryo day 3 morphology– highlighted numbers indicate significant differences at p<0.1

Percentages																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X&Y
Normal																							
3	42	61	52	53	70	44	52	50	46	67	42	67	65	73	62	54	47	70	63	27	61	52	71
2.5	30	57	39	31	61	37	50	43	41	68	44	69	60	61	53	43	45	59	45	29	65	66	69
2	26	52	36	27	70	45	48	47	50	66	44	67	69	75	45	46	55	57	59	32	72	64	76
1-1.5	25	61	36	24	84	53	56	42	71	86	59	89	79	88	66	57	80	66	67	36	86	92	78
Monosomy																							
3	32	16	31	34	11	40	31	37	33	22	36	25	15	16	19	23	35	19	11	44	21	9	9
2.5	29	9	31	29	14	36	29	37	22	13	21	16	17	14	17	29	31	17	11	27	13	4	3
2	28	11	31	29	10	31	37	34	30	17	28	25	12	12	19	18	24	18	6	38	13	20	4
1-1.5	35	10	42	38	9	28	37	45	23	8	26	5	12	4	9	18	14	17	8	38	8	5	2
Trisomy																							
3	2	18	5	1	6	5	2	5	6	8	3	5	11	5	9	6	2	2	11	3	5	35	10
2.5	2	25	8	3	13	5	2	4	12	13	6	11	14	15	13	10	6	10	23	6	14	24	15
2	1	29	3	2	11	4	4	5	4	11	3	5	10	7	5	9	6	6	20	3	7	13	12
1-1.5	1	26	5	0	5	6	2	5	2	4	4	4	6	2	20	14	1	15	18	3	4	2	7
Nullisomy																							
3	23	1	10	11	11	8	13	5	14	1	18	1	6	4	6	15	13	4	13	25	9	2	0
2.5	39	4	21	37	9	21	17	15	23	2	27	1	7	6	15	15	17	9	15	37	6	3	0
2	45	6	30	42	8	19	11	13	14	3	24	1	7	5	30	25	14	19	11	27	6	1	0
1-1.5	39	0	17	37	1	13	5	8	2	2	10	0	1	5	4	9	4	1	6	22	1	0	0
Others																							
3	1	4	2	2	2	2	2	2	2	2	1	3	2	2	4	2	2	5	2	1	3	3	10
2.5	1	4	1	0	3	1	1	1	2	4	2	2	3	3	2	2	1	4	5	1	2	3	13
2	0	3	0	1	1	0	0	1	0	1	0	1	2	1	0	1	0	0	4	0	1	1	8
1-1.5	0	2	0	0	0	0	0	0	1	0	0	1	1	1	1	2	0	0	1	1	1	1	13

Percentages of normal, monosomy, trisomy, nullisomy and other abnormalities related to 4 morphology groups for all 24 chromosomes. Embryos scores as 1 are the best and 3 are the worst. For each chromosome normal and different abnormalities were analysed according to different morphology with chi squared test. Resusts were considerd significant when P≤0.1 (at 90% confidence). Significant results are highlighted in yellow.

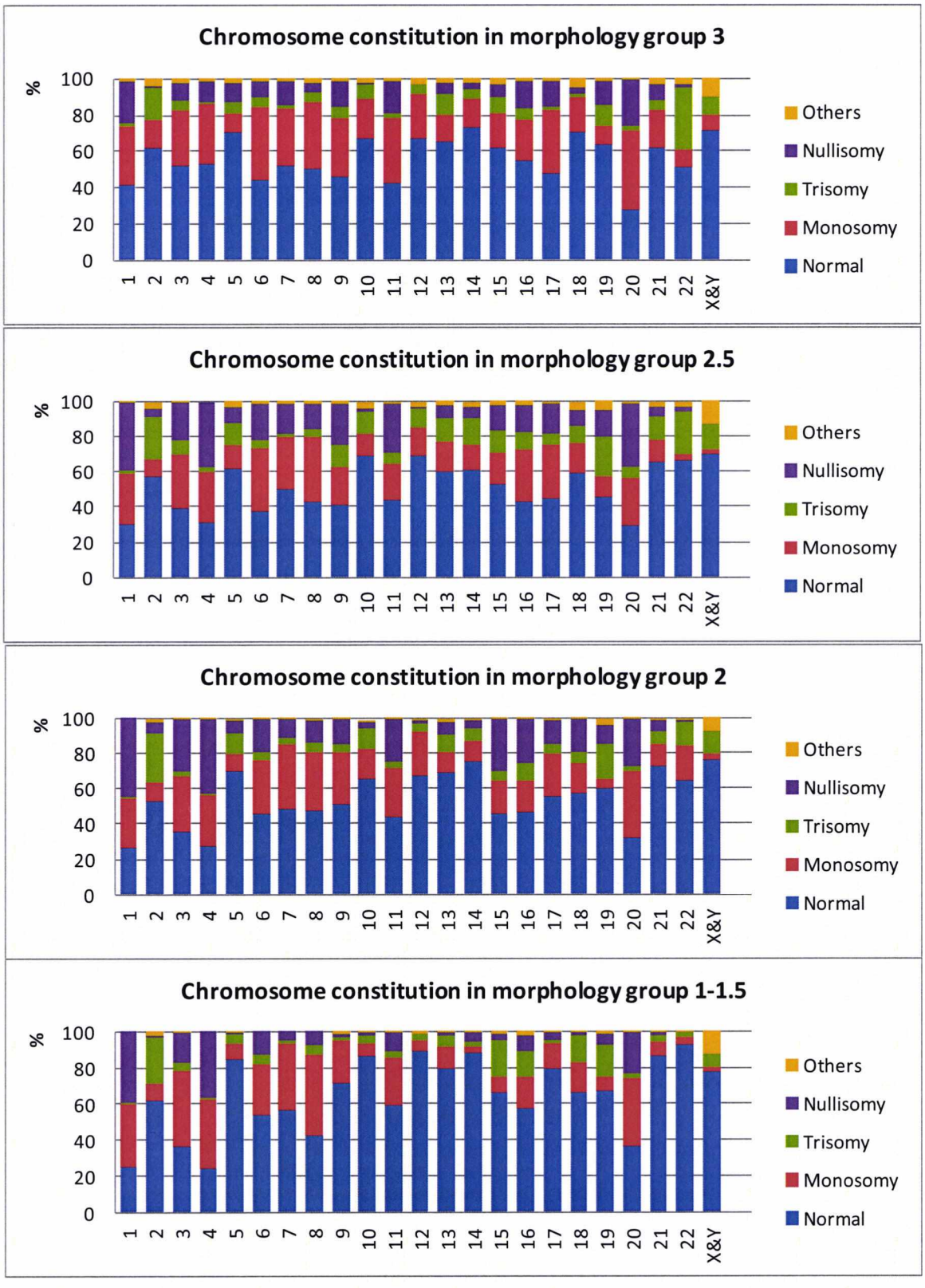


Figure 6.5: Percentages of aneuploidy and disomy per chromosome related to day 3 embryo morphology Normal, monosomy, trisomy, nullisomy, and other abnormalites seen with different morphology groups are presented as presentages for each chromosome. Embryos scores as 1 are the best and 3 are the worst.

6.3.6. To test the hypothesis that chromosomal abnormalities are less in number, in more advanced developmental stages (i.e. that there is some mechanism of "self-correction" or differential survival of euploid lines)

The table 6.12 and figure 6.7 below shows analysis of overall disomic and aneuploid cells related to four embryo morphology criteria at day 5. Results suggest that the incidence of chromosomal normality and abnormality are not different according to these day 5 morphology criteria. This was studied according the specific chromosome and found that certain chromosomes demonstared statistical significant differences according to embryo day 5 morphology which presened in table 6.13 below. Significant results were highlighted. However no chromosome specific patterns were seen.

Table 6.12: Relative levels of disomy and aneuploidy compared to embryo day 5 morphology

	Arrested		Morula		Blastocyst		Hatching blastocyst	
	No	%	No	%	No	%	No	%
Normal	111	56.10	198	53.80	341	55.07	89	53.64
Monosomy	43	21.79	83	22.61	121	19.50	42	25.24
Trisomy	18	9.14	23	6.34	61	9.90	13	7.77
Nullisomy	23	11.69	56	15.33	85	13.77	19	11.58
Others	3	1.30	7	1.92	11	1.76	3	1.77

List of normal, monosomy, trisomy, nullisomy and other abnormalities seen in different day 5 morphology groups as whole numbers and percentages.

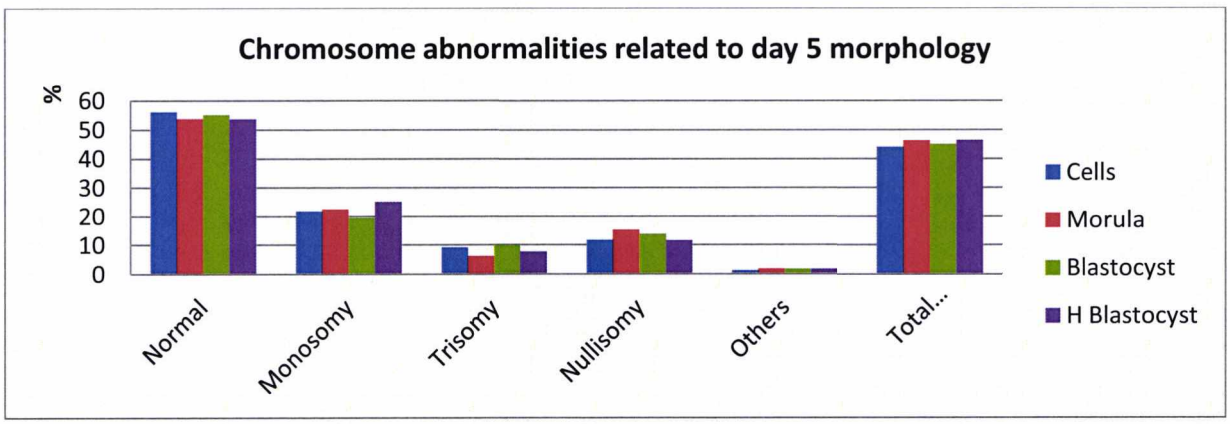


Figure 6.6: Relative levels of disomy and aneuploidy compared to embryo day 5 morphology
Normal, monosomy, trisomy, nullisomy, other and total abnormalited seen with different day 5 morphology groups are presented as presentages.

Table 6.13: Percentage of disomy and aneuploidy per chromosome related to embryo day 5 morphology– highlighted numbers indicate significant differences at p<0.1

Percentages																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X&Y
Normal																							
Cells	30	49	36	33	71	30	41	38	53	68	53	65	75	78	62	64	49	72	64	25	72	82	79
Morula	25	68	49	36	67	48	46	49	52	65	38	65	59	67	56	40	57	62	52	25	65	74	71
Blastocyst	29	50	35	28	73	47	57	48	49	72	48	76	71	73	47	46	55	58	62	33	74	60	74
H Blastocyst	37	51	25	24	60	43	44	45	56	75	50	71	62	76	46	45	64	49	56	50	72	63	71
Monosomy																							
Cells	39	6	37	34	7	43	34	41	28	18	31	25	12	11	16	11	34	13	10	37	10	5	3
Morula	30	12	30	28	15	28	37	33	24	18	23	25	18	19	20	37	22	16	11	35	23	11	6
Blastocyst	26	13	31	28	9	32	30	36	27	14	25	15	11	8	16	19	25	18	7	37	9	10	2
H Blastocyst	33	10	43	41	19	33	46	38	30	17	33	21	22	15	19	18	23	24	9	30	15	33	8
Trisomy																							
Cells	1	42	4	2	12	8	5	3	6	12	3	10	9	8	12	13	8	11	11	2	9	11	12
Morula	2.5	13	7.1	4.4	5.3	5.5	3.3	6.1	4.1	14	3	4.7	9	6.2	4.1	2.7	3.9	5.8	15	3.8	4.3	8.3	9
Blastocyst	1	28	3	1	13	3	3	5	9	9	5	8	13	12	12	13	5	8	21	4	11	28	14
H Blastocyst	1	32	6	0	10	7	2	6	4	5	5	5	8	3	17	10	3	13	19	6	4	2	10
Nullisomy																							
Cells	30	1	22	31	10	18	19	18	12	2	13	1	3	2	8	13	8	3	13	35	7	1	0
Morula	42	4.9	13	30	10	18	13	9.9	19	1.1	36	3	12	6.8	19	20	15	14	19	36	6.5	4.3	0
Blastocyst	44	5	30	42	5	17	10	10	14	3	21	0	4	5	24	20	14	13	7	26	4	0	0
H Blastocyst	28	3	25	35	10	17	7	11	8	2	12	1	5	4	17	23	9	13	14	13	8	1	0
Other																							
Cells	0	3	1	0	1	1	1	1	1	1	1	1	2	2	2	0	1	2	3	1	2	2	7
Morula	0.3	1.9	1.4	1.1	1.9	0.6	0	1.4	1.1	1.7	0.5	1.9	2.5	1.9	1.4	0.8	1.1	1.7	2.8	0.3	2.2	2.2	14
Blastocyst	0	4	0	0	1	1	1	1	1	2	1	2	2	1	1	2	1	3	3	0	1	1	9
H Blastocyst	0	4	1	0	1	0	0	0	2	1	1	2	2	2	2	5	1	1	2	2	1	1	11

Percentages of normal, monosomy, trisomy, nullisomy and other abnormalities related to 4 morphology (day 5) groups for all 24 chromosomes. For each chromosome normal and different abnormalities were analysed according to different morphology with chi squared test. Resusts were considerd significant when P≤0.1 (at 90% confidence). Significant results are highlighted in yellow.

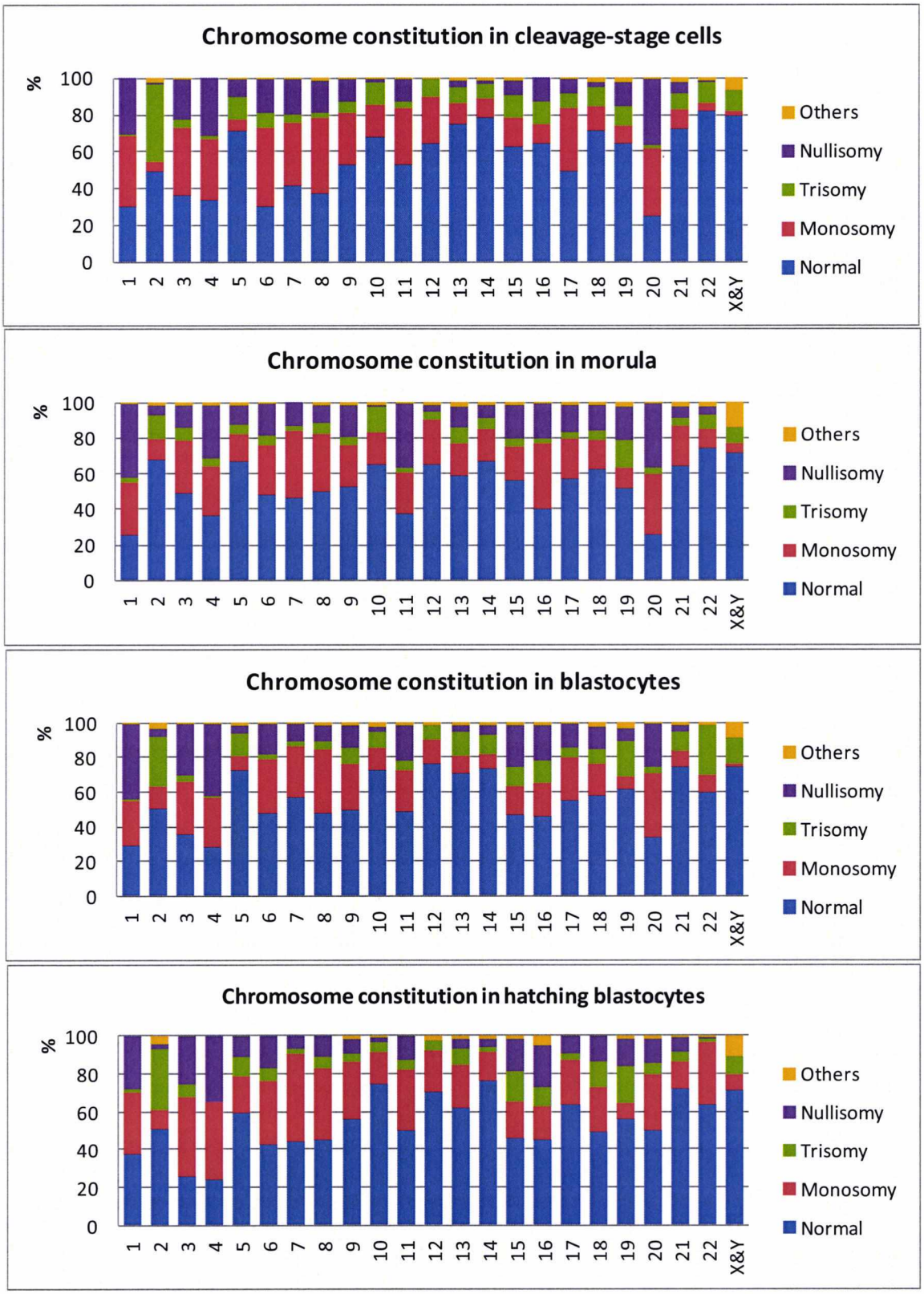


Figure 6.7: Percentages of aneuploidy and disomy per chromosome related to day 5 embryo morphology Normal, monosomy, trisomy, nullisomy, and other abnormalites seen with different day 5 morphology groups are presented as presentages for each chromosome.

6.3.7. To compare the results in embryos fixed in 2 different fixative methods to ask if chromosomal losses are indeed biological or due to technical reasons.

In this part of the study, embryos fixed in 2 different fixative methods were compared interms of number of cells gave normal, monosomies, trisomies, nullisomies, other abnormalities. Results below (table 6.14, figure 6.9) clearly indicate that the apparent incidence of chromosome loss was significantly less (T test, P=0.014) in this study than previously reported (Ioannou *et al.*, 2012). This provides evidence that the new fixation approach is likely to produce less technical artefact than that previously reported. That is, with the possible exception of layer A, the incidence of single signals (which could be attributed to either hybridization failure or monosomy) was much less in this study.

Table 6.14: Comparison of Tween HCL/ Methanol: acetic acid combination methodand Tween HCL method for embryo fixing

	Embryo fixed with Tween HCL/ Methanol: acetic acid combination method					Embryo fixed with Tween HCL method				
	Normal	Monosomy	Trisomy	Nullisomy	Others	Normal	Monosomy	Trisomy	Nullisomy	Others
A	888	587	74	700	31	692	607	70	107	24
B	1153	495	166	403	63	679	617	84	97	23
C	1376	240	212	399	51	684	362	97	64	38
D	1250	287	325	168	76	711	367	62	123	38

Summary of FISH results from comparing embryo fixed with Tween HCL/ Methanol: acetic acid combination method (this study) vs. Embryo fixed with Tween HCL method (Ioannou et al 2011). A, B, C and D represent the four hybridization layers each with a 6 different probes. For each method number of cells gave normal, monosomies, trisomies, nullisomies, other abnormalities were presented.

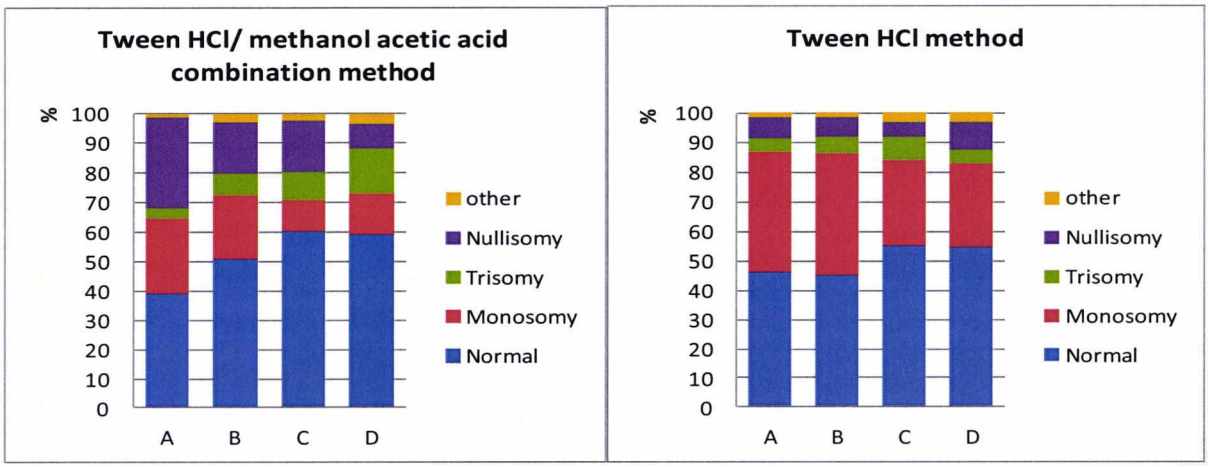


Figure 6.8: Summary of FISH results from comparing Embryo fixed with Tween HCL/ Methanol: acetic acid combination method (left) vs. Embryo fixed with Tween HCL method (right).
Results are presented for layer A, B, C and D; normal, monosomy, trisomy, nullisomy, and other abnormalities as percentages.

The results seem to suggest an increasing efficiency in subsequent layers, in particular layers C and D where levels of trisomy and monosomy were similar. Perhaps then previous assertions of the relative levels of monsomy vs trisomy may have been somewhat premature.

6.3.8. To test the hypothesis that PGS diagnosis (for 8 chromosomes) is an accurate predictor of the ploidy status of the rest of the embryo with regards to embryo fixing.

All embryos used in this study, were from clinical PGS cases. These embryos were not transferred for implantation due to abnormalities in day 3 PGS diagnosis or in one case, poor morphological quality even though it was diagnosed as normal in day 3. Table 6.15 and figure 6.10 below summaries the day 3 diagnosis and day 5 diagnosis results and any additional abnormalities found (when that specific abnormality exceed 50% of cells in the follow up study.

Table 6.15: Day 3 vs. day 5 diagnosis for embryos.

Embryo number	Day 3 diagnosis	Follow up results		
		Aneuploidies	Normal	PGS confirmed?
A1	complex	53% monosomy 7, 57% monosomy 20	15 chromosomes normal for > 50%	Yes
A2	Monos 21,22	50% trisomy 2, 53% monosomy 9 56% monosomy 11, 50% monosomy 18 56% monosomy 20, 88% normal for 21 88% normal for 22	11 chromosomes normal for > 50%	No
A3	complex	52% monosomy 1, 50% monosomy 8, 52% trisomy 2	16 chromosomes normal for > 50%	No
A4	Tris-16,18	60% trisomy 2, 65% trisomy 5 62% trisomy 13, 81% trisomy 14 60% trisomy 15, 42% trisomy 16 56% trisomy 18, 67% trisomy 19 69% trisomy 21, 77% trisomy 22 69% trisomy X. Probably partial triploid	5 chromosomes normal for > 50%	Partially
A5	Tris-22	53% trisomy 2, 50% monosomy 6 72% trisomy 22	16 chromosomes normal for > 50%	Yes
A6	NORMAL	55% monosomy 4, 55% monosomy 8 50% monosomy 17	14 chromosomes normal for > 50% including 13, 16, 18, 21, 22, XY	Yes
B2	Tris-18,21	38% trisomy 18, 55% normal 18 55% mono 21, 27% normal	18 chromosomes normal for > 50%	No
B3	Monos-22	96% mono 22	14 chromosomes normal for > 50%	Yes
B4	Triploid	82% trisomy 2, 65% trisomy 5 76% trisomy 10, 65% trisomy12 76% trisomy13, 65% trisomy 14 71% trisomy 15, 65% trisomy 17 76% trisomy 18, 82% trisomy 21 88% trisomy 22, 76% XXY	6 chromosomes normal for > 50%	Yes
B5	Tris-15,21	59% normal 15,	9 chromosomes normal for > 50%	No
B6	complex	83% monosomy 6, 50% monosomy	10	Partially

		8 50% monosomy 9, 60% monosomy 11 60% monosomy 15 50% monosomy 20	chromosomes normal for > 50%	
C1	complex	61% monosomy 4, 61% monosomy 6 57% monosomy 7, 52% monosomy 8 70% monosomy 20	13 chromosomes normal for > 50%	No
C3	Tris-21	57% monosomy 4, 52% monosomy 6 52% monosomy 8, 52% trisomy 22 22% trisomy 21, 48% monosomy 21	12 chromosomes normal for > 50%	No
C4	Tris 15,16	100% normal for 15, 100% monosomy 16	16 chromosomes normal for > 50%	No
C5	Monos 15,16	54% monosomy 6, 65% normal for 15 75% monosomy 16, 54% monosomy 20	10 chromosomes normal for > 50%	Partially
D1	Monos-16	50% nullisomy 16, 15% monosomy 16	13 chromosomes normal for > 50%	No
D3	Tris-16	58% trisomy 2, 50% monosomy 8 33% nullisomy 16, 12% monosomy 16 17% trisomy 16, 38% normal	17 chromosomes normal for > 50%	No
D6	Haploid	86% monosomy 1, 86% monosomy 2 86% monosomy 3,100% monosomy 4 67% monosomy 5, 100% monosomy 6 71% monosomy 7, 86% monosomy 8 100% monosomy 9, 86% monosomy 10 86% monosomy 12,100% monosomy13 71% monosomy 14, 57% monosomy 15 86% monosomy 17, 71% monosomy 18 71% monosomy 19, 57% monosomy 20 86% monosomy 21, 86% monosomy 22 86% monosomy X	0 chromosomes normal for > 50%	Yes

E1	Monos 22	67% monosomy 22, 78% monosomy 10 56% monosomy 14, 94% monosomy 15 67% monosomy 22	11 chromosomes normal for > 50%	Yes
E2	Complex	No aneuploidy present more than 50% of cells	15 chromosomes normal for > 50%	No
E3	Monos 22	56% monosomy 7, 95% monosomy 22	13 chromosomes normal for > 50%	Yes
F1	Monos 16	57% monosomy 8, 80% normal 16	16 chromosomes normal for > 50%	No
F2	Haploid	No aneuploidy present more than 50% of cells	20 chromosomes normal for > 50%	No
F3	complex	58% monosomy 3, 58% monosomy 4 63% monosomy 6	18 chromosomes normal for > 50%	No
F4	complex	No aneuploidy present more than 50% of cells	17 chromosomes normal for > 50%	No
F5	complex	87% monosomy 16	20 chromosomes normal for > 50%	No
G1	Tris-17	75% normal 17, 50% monosomy 9 50% monosomy 11, 50% monosomy 13 50% monosomy 14, 50% monosomy 22	20 chromosomes normal for > 50%	No
G2	Complex	No aneuploidy present more than 50% of cells	23 chromosomes normal for > 50%	No
G3	Monos-16	24 % monosomy 16, 56 % normal 16 56% monosomy 13	11 chromosomes normal for > 50%	No
G4	Complex	77% trisomy 22	20 chromosomes normal for > 50%	No
H1	Tris 17	24% trisomy 17, 39% normal 17	15	No

		10% monosomy 17, 61% monosomy 20	chromosomes normal for > 50%	
H2	Tris 22	79% trisomy 22	13 chromosomes normal for > 50%	Yes
H3	Mon 13	15% monosomy 13, 19% trisomy 13 60% normal 13, 88% monosomy 7 67% trisomy 10, 73% monosomy 12	14 chromosomes normal for > 50%	No
H4	Mon18	80% normal 18, 18% monosomy 18 66% monosomy 6, 55% monosomy 8 53% monosomy 11, 75% trisomy 19	15 chromosomes normal for > 50%	No
H5	Mon 15	42% monosomy 15, 42% normal 15 8% trisomy 15, 92% trisomy 2 50% monosomy 8, 75% monosomy 17 92% monosomy19	17 chromosomes normal for > 50%	Partially

Table present day 3 PGS results and follow up results for each embryo. For follow up results additional abnormalities are mentioned when that specific abnormality exceeds 50% of cells in the follow up embryo. Concordance between PGS and and follow up study also presented.

The results therefore show that the diagnosis was mostly accurate in 9, partially in 4 and not accurate in 22 cases. The other 7 embryos did not have day 3 results.

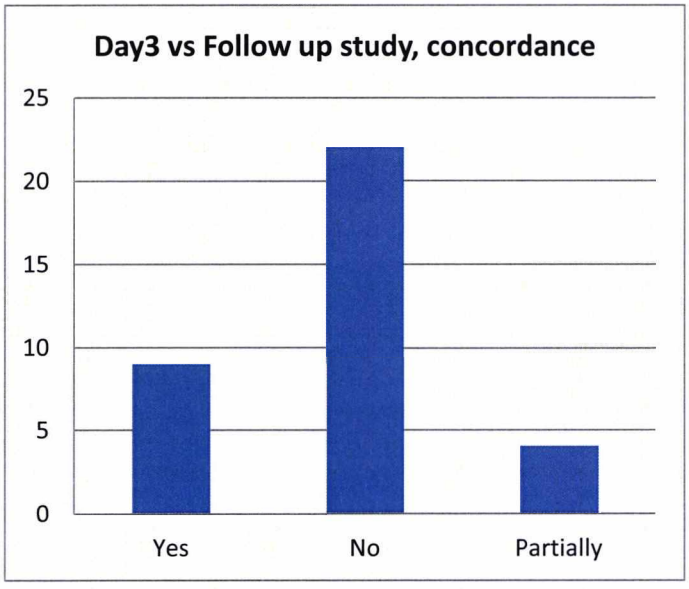


Figure 6.9: Concordance day 3 vs. follow up embryo results as whole numbers

6.4. Conclusions

In conclusion this study shows that 24 chromosome FISH has the potential for use in follow up studies post PGS. The ability to assay every chromosome on a cell by cell basis is extremely attractive for the assessment of mosaicism and overall abnormality. The correlations between maternal age and embryo morphology are less clear cut than in the previous chapter (specific aim 3), despite, by and large, there being bright and punctuate signals throughout. Overall, all of the embryos had some sort of chromosomally abnormal cells and indeed only a single nucleus showed 24/24 signals. To put this into perspective, if each probe were working at 97-98% efficiency then, even a known 100% diploid cell population would only display 24/24 signals in approximately 40-60% of nuclei. This could easily be controlled for however and observed values adjusted accordingly. The significant "unknown" however is whether the control values for lymphocytes given in table 6.1 represent the true hybridization efficiencies in blastomeres. Certainly the blastomere signals were brighter than those of the lymphocytes however the two cell types were, by necessity, prepared somewhat differently. Moreover, the data clearly show an improvement in the signal efficiency compared to a previous study. The evidence suggested that some chromosomes are more likely to be abnormal than others in human preimplantation development warrants further investigation. Complete in depth discussion for all the results of this chapter is given in section 8.4. The overall conclusion from this work might therefore be that, despite my best efforts to clarify issues raised in the previous chapter through development of a 24 chromosome FISH assay, more questions have been raised than answered.

7. To apply the 24 chromosome FISH strategy to investigate nuclear organisation in human embryos

7.1. Background

Previous studies examining nuclear organisation in human preimplantation embryos have investigated the nuclear address of specific chromosome loci in chromosomally normal and abnormal human blastomeres. In these studies, embryo cells were classified as normal based on ploidy from a subset of chromosomes. This was 5 chromosomes (13, 18, 21, X, Y) in study by McKenzie et al. 2004, 7 chromosomes (13, 16, 18, 21, 22, X, Y) in (Diblik et al. 2007) and 8 chromosomes (13, 15, 16, 18, 21, 22, X, Y) (Finch et al. 2008a). These studies also used basic homemade template with five rings which placed on the 2D embryo image and count number of signals in each ring to measure the chromosome position.

More recently work in our lab (Dimitris Ioannou, PhD thesis, 2010) and (Ioannou *et al.*, 2012) investigated the nuclear address of all 24 chromosomes using a methodology based on the "mainstream" nuclear organisation literature to analyse radial position (as described in method section). This allowed more accurate assessments of relative nuclear positions in blastomeres through analysis of the nuclear addresses of all 24 chromosomes in 17 embryos (255 cells) fixed by the HCl, tween method. However possibly due to the poor quality and/or due to specific spreading and fixing method used, embryos were found to have huge numbers of chromosomal losses (see previous chapter – raw data is the same). The current study looked at 42 embryos (1399 cells) fixed with an HCl tween and methanol: acetic acid combination method with the hope to find more normal cells and investigate if there are any alteration of nuclear organisation between normal and abnormal groups related to various

parameters. In this part of the study, captured images of embryos from the previous chapter (Specific aim 4) were utilised. As described in the section 2.5.1 in the material and methods, images were analysed for chromosome positions by using bespoke macro written for Image J and calculations were performed according to DAPI normalisation model.

7.2.Aims and hypotheses

The purpose of chapter was to assess the nuclear organisation of specific chromosomal loci from 24 chromosomes in human preimplantation embryos through the pursuit of following specific aims:

1. To test the hypothesis that there is a non-random nuclear organisation detectable in early human development by determining the nuclear address of the loci probed for aneuploidy screening in the previous chapter

2. To test the hypothesis that centromeric loci occupy a more central nuclear address i.e. that human embryos, like mouse, show a "chromocentre" pattern

3. To test the hypothesis that gross chromosomal abnormality adversely affects patterns of nuclear organisation
    a. When whole embryos are compared to one another
    b. When nuclei are compared cell by cell

4. To test the hypothesis that other factors adversely affect nuclear organisation, e.g.
    a. Maternal age
    b. Embryo day 3 morphology
    c. Embryo day 5 morphology

5. To assess whether nuclear organisation is affected by the spreading tequniques by comparing the current and previous data (Ioannou *et al.*, 2012).

7.3. Results

7.3.1. To test the hypothesis that there is a non-random nuclear organisation detectable in early human development

In order to obtain reliable values for nuclear organisation per embryo at least 40 cells should be analysed. To the best of my knowledge this is the first time this has been achieved in a study of preimplantation human development. Whole embryo analysis for 24 chromosomes was done in 42 embryos in total. 504 graphs were produced and presented in the electronic appendix 'specific aim 5' section. Results are presented in the table 7.1. Embryos with more than 40 cells analysed (18 embryos in total) were included in thie part and embryos that had less than 40 cells were shaded in grey.

With regard to nuclear position analysis chi squared test was used; the nuclear address of each chromosomal locus was considered as non random when $p<0.05$ (4d.f.), otherwise the distribution was considered as random or Not Discernable from a Random Distribution Pattern (NDRP) and thus assigned the status "R." Depending on the preferential nuclear address, signals were classified as follows; Peripheral (P) – Shell 1 or 1/2, Peripheral/Medial (PM) – Shell 2 or 1-3, Medial (M) – Shell 3, 2/3, or 3/4, Central/Medial (CM) – Shell 4 or 3-5, Central (C) – Shell 5 or 4/5. Table 7.1 shows the preferential location of each chromosomal locus for each embryo and table 2 shows the median positions assigned for each shell (1-5) for whole embryos.

Table 7.1: preferential localisation of chromosome for each embryo.

Patient	Embryo	Cells	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
A	1	49	C	CM	R	R	R	R	C	C	C	R	C	R	CM	CM	C	C	C	C	C	C	C	C	R	
	2	32	C	R	1,5 BI	P	R	P	C	R	C	R	C	R	R	R	C	C	R	R	P	C	R	C	R	R
	3	58	C	C	R	R	R	CM	C	R	C	R	C	R	R	C	C	C	C	C	C	C	C	C	R	R
	4	52	C	R	R	R	R	C	C	C	C	C	C	R	CM	CM	C	C	C	C	R	C	C	C	R	
	5	58	R	R	P	R	R	M	R	R	C	R	R	R	R	R	C	C	C	R	R	C	C	C	R	C
	6	22	C	R	R	R	R	P	C	R	C	C	C	R	R	R	R	R	C	R	R	C	CM	C	R	R
B	2	11	C	R	R	R	R	R	C	R	C	R	R	R	R	C	R	R	C	R	C	R	R	C	R	
	3	49	C	C	R	R	CM	R	C	R	C	C	C	R	C	C	C	C	C	C	C	C	C	C	R	
	4	17	C	R	R	R	R	R	C	CM	R	C	C	R	R	R	C	R	R	R	R	C	R	C	R	R
	5	29	CM	R	R	M		R	CM	M	C	CM	CM	R			R	C	C	R		CM			R	
	6	10	C	C	R	R	R	M	R	R	R	R	R	R	R	M	R	R	R	R	R	R	R	C	R	R
C	1	23	R	3,5 BI	R	R	R	R	R	C	CM	C	C	R	R	C	C	C	CM	C	C	R	C	C	CM	R
	2	12	R	PM	R	R	R	R	M	R	R	R	R	R	R	R	R	P	R	R	R	C	R	C	R	
	3	23	R	R	R	R	R	R	C	R	C	R	C	C	R	C	R	C	R	R	C	R	C	C	R	
	4	25	C	C	R	R	R	CM	R	R	C	CM	R	R	R	CM	C	C	C	C	C	CM	C	C	R	C
	5	28	R	CM	R	R	R	R	R	R	R	3,5 BI	R	R	C	C	C	P	C	C	C	C	C	C	R	R
D	1	54	C	C	1,3 BI	R	C	R	C	C	C	C	C	R	R	C	1,5 BI	P	C	P	C	C	C	C	R	P
	2	39	R	R	P	R	R	R	R	C	C	R	C	R	M	CM	R	R	C	R	C	C	R	C	R	
	3	52	R	M	R	R	PM	PM	R	M	C	R	C	R	R	R	145 BI	P	C	R	C	C	C	C	R	
	4	44	C	C	R	1,5 BI	CM	CM	C	R	C	R	C	R	C	C	C	R	C	R	C	C	CM	C	M	
	5	14	C	R	R	R	R	R	R	R	C	C	C	R	R	C	R	C	R	R	R	C	C	C	R	
	6	7	R	R	R	R	R	R	R	M	M	R	R	R	R	R	R	P	R	R	R	R	C	C	C	
E	1	18	CM	C	P	R	C	R	R	R	C	R	C	R	C	C	CM	C	R	R	C	R	C	C	C	R
	2	67	C	R	R	R	R	R	R	CM	C	C	C	R	C	C	C	C	C	C	C	C	C	C	R	C

	3	44	C	C	P	R	R	R	C	R	C	CM	C	R	C	C	C	C	C	C	C	C	C	C	R	
	4	40	C	C	CM	C	C	C	C	R	C	CM	C	C	CM	C	C	C	C	C	C	C	C	C	C	
F	1	44	CM	R	P	R	R	P	R	R	CM	R	CM	M	R	CM	C	C	CM	R	C	C	CM	C	R	
	2	97	C	CM	R	R	PM	R	C	C	CM	C	C	C	R	C	C	C	C	C	C	C	C	C	C	C
	3	19	R	C	P	R	R	P	R	R	R	M	C	R	R	M	C	C	CM	C	R	P	R	R	C	R
	4	33	C	CM	R	R	R	C	C	M	C	C	C	CM	C	C	C	C	C	R	C	C	C	C	CM	
	5	15	R	P	R	C	R	R	C	R	R	R	C	R	R	CM	R	C	R	C	C	R	R	C	C	C
G	1	4	C	R	R	R	R	R	R	R	R	R	C	R	R	R	R	R	C	R	R	R	R	R	R	
	2	23	C	R	R	R	R	R	R	R	C	R	CM	R	R	R	3,5 BI	R	C	R	R	C	CM	C	R	
	3	25	C	C	R	C	R	C	C	C	C	C	C	C	R	R	C	R	C	C	C	C	C	C	R	R
	4	47	C	C	R	C	R	C	C	R	C	C	C	R	R	M	C	C	C	C	R	C	C	C	R	
H	1	49	C	C	R	R	PM	R	C	R	C	R	C	C	R	C	C	C	C	C	C	C	C	C	CM	
	2	56	C	C	R	R	R	R	R	R	C	R	C	R	R	C	C	C	C	R	C	C	C	C	R	
	3	48	C	C	R	R	R	R	C	R	C	CM	C	C	C	C	C	C	C	C	C	C	C	C	CM	
	4	40	C	CM	R	R	R	R	C	M	C	C	C	C	C	C	C	C	C	C	C	C	C	C	CM	
	5	12	R	R	R	R	PM	R	R	M	R	R	R	R	R	CM	R	R	C	R	R	P	C	CM	R	R
	6	9	R	R	R	R	R	R	R	R	R	R	R	R	R	CM	R	R	C	R	R	C	3,5 BI	R	R	R
TOT AL			C	C	R	R	PM	R	C	CM	C	C	C	C	C	C	C	C	C	C	C	C	C	C	CM	C

Nuclear organisation of individual embryos for all 24 chromosomes; for each embryo all the available cells analysed. Number of cells analysed per each embryo is presented in column 3. Embryos had less than 40 cells were shaded in gray. Random (R), Peripheral (P) – Shell 1 or 1/2, Peripheral/Medial (PM) – Shell 2 or 1-3, Medial (M) – Shell 3, 2/3, or 3/4, Central/Medial (CM) – Shell 4 or 3-5, Central (C) – Shell 5 or 4/5.

The above results support the hypothesis that there is a non-random pattern of nuclear organisation in preimplantation human development with the majority of probes showing non-random patterns for the majority of chromosomes (21/24).

7.3.2. To test the hypothesis that centromeric loci occupy a more central nuclear address i.e. "chromocentre" pattern

This study also attempted to find any evidence for chromocentre in human preimplantation embryos. Most probes used in this study were centromeric probes other than probes for chromosome 5, 13, 14, 19, 21 and 22. Of these 19, 21 and 22 locate near to the centromere thus might be expected to have central position if the hypothesis is correct. A chromosome 5 locus was the most distal from the centromere (being sub-telomeric on the q arm) and 13 are second most far away from the centromere (being half way down the q arm).

The pooled results suggest that other than chromosome 3, 4 and 6 all centromeres have central localisation. The probe for chromosome 5 had the most peripheral location consistent with the notion of a chromocentre. However other probe for chromosome 13 had central localisation.

7.3.3. To test the hypothesis that gross chromosomal abnormality adversely affects patterns of nuclear organisation

- **When whole embryos (pooled) are compared with one another**
- **When nuclei are compared cell by cell**

Only one embryo was diagnosed as normal by PGS at day 3. However follow up (day 5) results showed that embryo was mosaic with monosomies and trisomies present in certain blastomeres and had approximately 60% normal cells. On the other hand some embryos that were categorised as abnormal in day 3 appeared to be relatively normal with some chromosomal losses.

7.3.3.1. When whole embryos (pooled) are compared with one another

To address this question, individual nuclei embryos were divided in to 2 categories depending on the overall level of their abnormality. The embryos roughly equally around the median gave 800 normal and 600 abnormal cells in each group to analyse.

Table 7.2 presents the preferential localisation and median position for all chromosomes in the "relatively normal" and "grossly abnormal" embryos. Results showed no significant difference in the 2 groups for majority of chromosomes, however for chromosome 4, 5 and 8, the preferential localisation is different in the 2 categories (table 7.2). Apendix 10.4.1 present graphs for each chromosome in normal (left) and abnormal (right) categories..

Table 7.2: pooled preferential location and median position of chromosomes in normal and abnormal embryos.

Chromosome	Relatively normal		Grossly abnormal	
	Position	Median	Position	Median
1	C	4.00	C	4.02
2	C	3.59	C	3.76
3	R	3.00	R	2.99
4	R	3.00	C	3.01
5	PM	2.94	C	3.10
6	R	3.05	R	3.06
7	C	3.58	C	4.00
8	M	3.17	C	3.27

9	C	4.02	C	4.00
10	C	3.41	C	3.48
11	C	4.02	C	4.20
12	C	3.37	C	3.38
13	C	3.45	C	3.49
14	C	3.69	C	3.87
15	C	4.03	C	4.00
16	C	3.77	C	3.59
17	C	4.05	C	4.08
18	C	3.66	C	3.55
19	C	3.79	C	3.78
20	C	4.13	C	4.08
21	C	3.86	C	4.00
22	C	4.11	C	4.05
X	CM	3.32	CM	3.32
Y	C	3.56	C	3.08

Nuclear organisation was analysed embryo by embryo basis with 800 relatively normal and 600 abnormal cells for all 24 chromosomes. Images of embryo cells run through a macro program which analyse the nuclear positions of FISH signals. Software divides the each embryo into 5 concentric shells and output percentage of signals in each shell. Positions of signals were analysed with chi squared test and when p value is less than 0.05 results consideres as significant. Depending on the shell number (1to 5) majority of signals located, distribution is catogorised as peripheral, medial or central. Criteria used to to allocate preferantial locations as follows. Peripheral (P) – Shell 1 or 1/2, Peripheral/Medial (PM) – Shell 2 or 1-3, Medial (M) – Shell 3, 2/3, or 3/4, Central/Medial (CM) – Shell 4 or 3-5, Central (C) – Shell 5 or 4/5. When p value is higher than 0.05 results were considered as random (R). Median refers to overall median position of 100 signal analysed.

7.3.3.2. When nuclei are compared blastomere by blastomere

In this case, analysis was similar to above but, this time, classifying each individual nucleus as either "relatively normal (by virtue of the fact that it had 65% or more of chromosomes normal) or "grossly abnormal (clear trisomies and less 65% of chromosomes normal). Again, the groups were roughly equal in size. Graphs are presented in appendix 10.4.2.

Table 7.3: pooled preferential location and median position of chromosomes in normal and abnormal blastomeres from cell by cell analysis

Chromosome	Relatively normal		Abnormal	
	Position	Median	Position	Median
1	C	4.00	C	4.00
2	C	3.64	C	3.68
3	R	2.97	PM	3.00
4	R	3.00	C	3.04
5	PM	2.98	C	3.06
6	R	3.03	R	3.06
7	C	3.65	C	3.84
8	CM	3.20	CM	3.21
9	C	4.01	C	4.00
10	C	3.39	C	3.52
11	C	4.05	C	4.12
12	C	3.33	C	3.37
13	C	3.43	C	3.54
14	C	3.71	C	3.38
15	C	4.00	C	4.00
16	C	3.73	C	3.65
17	C	4.05	C	4.05
18	C	3.57	C	3.66
19	C	3.80	C	3.77
20	C	4.12	C	4.10
21	C	3.83	C	4.00
22	C	4.13	C	4.03
X	CM	3.32	CM	3.30
Y	C	3.37	C	3.41

Nuclear organisation was analysed cell by cell basis with equal size normal and abnormal cells for all 24 chromosomes. Images of embryo cells run through a macro program which analyse the nuclear positions of FISH signals. Software divides the each embryo into 5 concentric shells and output percentage of signals in each shell. Positions of signals were analysed with chi squared test and when p value is less than 0.05 results consideres as significant. Depending on the shell number (1to 5) majority of signals located, distribution is catogorised as peripheral, medial or central. Criteria used to to allocate preferantial locations as follows. Peripheral (P) – Shell 1 or 1/2, Peripheral/Medial (PM) – Shell 2 or 1-3, Medial (M) – Shell 3, 2/3, or 3/4, Central/Medial (CM) – Shell 4 or 3-5, Central (C) – Shell 5 or 4/5. When p value is higher than 0.05 results were considered as random (R). Median refers to overall median position of 100 signal analysed.

As with the previous analysis, only 3 chromosomes showed alterations in nuclear address, i.e. 3,4 and 5 and two of these (4 and 5) were in common with the previous analysis.

The results therefore support the hypothesis that subtle changes at the level of individual loci may alter if the embryo or cell is chromosomally abnormal however the overall nuclear organisation largely remains unaltered.

7.3.4. To test the hypothesis that other factors adversely affect nuclear organisation, e.g.

- **Maternal age**
- **Embryo day 3 morphology**
- **Embryo day 5 morphology**

7.3.4.1. Maternal age

As with previous analyses, only subtle differences were seen. It is noteworthy however that, common loci keep recurring as changed, in this case chromosomes 5 and 8 (table 7.4). The overall picture however suggests that nuclear organisation is not grossly changed in response to maternal age. Graphs presented in electronic appendix 'specific aim 5' section.

Table 7.4: Position for all chromosomes at different maternal age

Chromosome	<35	36-39	>40
1	Central	Central	Central
2	Central	Central	Central
3	Random	Random	Random
4	Random	Central	Random
5	Peripheral medial	Central	Peripheral medial
6	Central medial	Random	Random
7	Central	Central	Central
8	Central medial	Central medial	Medial
9	Central	Central	Central
10	Central	Central	Central
11	Central	Central	Central
12	Central medial	Central	Central
13	Central	Central	Central
14	Central	Central	Central
15	Central	Central	Central
16	Central	Central	Central
17	Central	Central	Central
18	Central	Central	Central
19	Central	Central	Central
20	Central	Central	Central
21	Central	Central	Central
22	Central	Central	Central
X	Central medial	Central medial	Central medial
Y	Central	Central	Central

Nuclear organisation was analysed for embryo catogorised into 3 groups according to maternal age. Images of embryo cells run through a macro program which analyse the nuclear positions of FISH signals. Software divides the each embryo into 5 concentric shells and output percentage of signals in each shell. Positions of signals were analysed with chi squared test and when p value is less than 0.05 results consideres as significant. Depending on the shell number (1to 5) majority of signals located, distribution is catogorised as peripheral, medial or central. Criteria used to to allocate preferantial locations as follows. Peripheral (P) – Shell 1 or 1/2, Peripheral/Medial (PM) – Shell 2 or 1-3, Medial (M) – Shell 3, 2/3, or 3/4, Central/Medial (CM) – Shell 4 or 3-5, Central (C) – Shell 5 or 4/5. When p value is higher than 0.05 results were considered as random (R).

7.3.4.2. Embryo day 3 morphology

In day 3, prior to PGS, embryos were assigned embryological "scores" according to their morphology. If embryo morphology was reliable indicator of chromosome abnormalities it could be expected to see alteration of nuclear address of chromosomes in embryos with poor

morphological quality. Table 7.5 present the median chromosome positions at different morphology scorings. Graphs presented in electronic appendix 'specific aim 5' section

Table 7.5: Position for all chromosomes at different morphology scoring

Chromosome	Positions at different morphology scoring			
	1 (best)	2	2.5	3 (poorest)
1	Central	Central	Central	Central
2	Central	Central	Central	Central
3	Peripheral	Peripheral	Random	Random
4	Random	Random	Central	Peripheral medial
5	Peripheral medial	Peripheral medial	Medial	Peripheral medial
6	Random	Random	Central	Random
7	Central	Central	Central	Central
8	Central medial	Central medial	Central medial	Central medial
9	Central	Central	Central	Central
10	Central	Central	Central	Central
11	Central	Central	Central	Central
12	Central medial	Central	Central	Central
13	Central medial	Central	Central	Medial
14	Central	Central	Central	Central
15	Central	Central	Central	Central
16	Central	Central	Central	Central
17	Central	Central	Central	Central
18	Central	Central	Central	Central
19	Central	Central	Central	Central
20	Central	Central	Central	Central
21	Central	Central	Central	Central
22	Central	Central	Central	Central
X	Central medial	Central medial	Central medial	Central medial
Y	Central	Central	Central	Peripheral medial

Nuclear organisation was analysed for embryo catogorised into 4 groups according to embryo day3 morphology. Images of embryo cells run through a macro program which analyse the nuclear positions of FISH signals. Software divides the each embryo into 5 concentric shells and output percentage of signals in each shell. Positions of signals were analysed with chi squared test and when p value is less than 0.05 results consideres as significant. Depending on the shell number (1to 5) majority of signals located, distribution is catogorised as peripheral, medial or central. Criteria used to to allocate preferantial locations as follows. Peripheral (P) – Shell 1 or 1/2, Peripheral/Medial (PM) – Shell 2 or 1-3, Medial (M) – Shell 3, 2/3, or 3/4, Central/Medial (CM) – Shell 4 or 3-5, Central (C) – Shell 5 or 4/5. When p value is higher than 0.05 results were considered as random (R).

Similar to the previous analyses, subtle differences were seen for chromosome 3, 4, 5, 6, 12, 13, and Y. The overall picture however suggests that nuclear organisation is not grossly changed in relation to day 3 morphology.

7.3.4.3. Embryo day 5 morphology

Again similar to previous sections, subtle differences were seen for chromosome 3, 4, 5, 6, 8, 13, and 14. However it seems that nuclear organisation is not grossly changed in relation to day 5 morphology. Graphs presented in electronic appendix 'specific aim 5' section

Table 7.6: Position for all chromosomes at different day 5 morphology scoring

Chromosome	Cells	Morula	Blastocyst	Hatching blastocyst
1	Central	Central	Central	Central
2	Central	Central	Central	Central
3	Peripheral	Random	Random	Peripheral
4	Peripheral	Central	Central	Peripheral medial
5	Medial	Central	Peripheral medial	Central
6	Random	Random	Central	Central
7	Central	Central	Central	Central
8	Central	Medial	Central medial	Central
9	Central	Central	Central	Central
10	Central	Central	Central	Central
11	Central	Central	Central	Central
12	Random	Central	Central	Random
13	Central medial	Central	Central	Central
14	Central media	Central	Central	Central
15	Central	Central	Central	Central
16	Central	Central	Central	Central
17	Central	Central	Central	Central
18	Central	Central	Central	Central
19	Central	Central	Central	Central
20	Central	Central	Central	Central
21	Central	Central	Central	Central
22	Central	Central	Central	Central
X	Central medial	Central medial	Central medial	Central medial
Y	Central	Central	Central	Central

Nuclear organisation was analysed for embryo catogorised into 4 groups according to embryo day5 morphology. Images of embryo cells run through a macro program which analyse the nuclear positions of FISH signals. Software divides the each embryo into 5 concentric shells and output percentage of signals in each shell. Positions of signals were analysed with chi squared test and when p value is less than 0.05 results consideres as significant. Depending on the shell number (1to 5) majority of signals located, distribution is catogorised as peripheral, medial or central. Criteria used to to allocate preferantial locations as follows. Peripheral (P) – Shell 1 or 1/2, Peripheral/Medial (PM) – Shell 2 or 1-3, Medial (M) – Shell 3, 2/3, or 3/4, Central/Medial (CM) – Shell 4 or 3-5, Central (C) – Shell 5 or 4/5. When p value is higher than 0.05 results were considered as random (R).

7.3.5. To assess whether nuclear organisation is affected by the spreading techniques by comparing current and previous data.

Similar comparisons were performed to ask whether there were any significant differences between the results generated by (Ioannou *et al.*, 2012) where the same probe set was used on a different set of embryos using a different spreading technique and the results generated here. Despite noticeably different results for the levels of aneuploidy, the nuclear organisation results were near identical in the two data sets. A table of result summarising this is presented in the appendix 10.4.3.

7.4. Concluding remarks

The results of this chapter suggest that there is some evidence for a chromocentre in human preimplantation embryos with most of the centromeres located in the nuclear centre and at least on locus further away from the centromere locating close to the periphery. When nuclear organisation was compared related to maternal age, day 3 morphology and day 5 morphology, certain chromosome loci demonstrated subtle alterations. A complete discussion of the results presented in this chapter is given in section 8.5. In conclusion the suggestion therefore is that, although the gross organisation of the nucleus remains largely the same regardless of chromosome abnormality, maternal age or embryo quality, loci on certain chromosomal loci are more prone to alterations in their nuclear address than others.

8. Discussion

This thesis was largely successful in the fulfilment of proposed aims, by providing insight into the nuclear organisation of sperm and embryos in relation to various criteria.

More specifically:

- The nuclear organisation of telomeres for all chromosomes and subtelomere loci of 11 chromosomes were investigated in the nuclei of sperm from control men with normal semen parameters compared to OAT patients. Results suggested that the organisation of sub-telomeric loci is dynamic, and ranges from the nuclear periphery to central regions. Even though we could not find a difference comparing individual patient data, pooled results of median positions suggested that there was a significant difference between patients and controls for certain sub-telomere loci.

- In the control males, telomeric sequences appear to locate preferentially towards the periphery of the nucleus by visual inspection of the majority of captured images. However when data was analysed using our computer program a random pattern seemed to be generated; the reasons for this apparent discrepancy will be the subject of future studies . Localisation of the pan telomeric probe in OAT patients however, appeared to indicate a more central localisation suggesting an association between altered nuclear organisation and compromised spermatogenesis.

- The nuclear organisation of 5 chromosomal loci (2 centromeric and 3 q arm specific loci) in sperm nuclei were assessed in patients with testicular cancer or Hodgkin's lymphoma, before and after chemotherapy, and compared with men with normal

semen parameters. Results suggested that, in certain testicular cancer patients, the nuclear organisation of some chromosomes is altered compared to controls.

- FISH based PGS clinical data from The London Bridge Fertility Centre was analysed to investigate the incidences of aneuploidy, PGS accuracy, concordance, false negative and false positive results by comparing single cell PGS and "follow up" embryos related to different indications and age groups. Results suggested that the overall levels of monosomy were significantly higher than trisomies in single cells from day 3, and in follow up embryos from day 5. Also, evidence is provided that PGS is a reasonable, though not entirely accurate, predictor of the karyotype of the rest of the embryo, that a maternal age effect could be detected and that there were significant differences in the RIF referral category compared to the others (mostly AMA).

- A 24 chromosome FISH assay was further developed following original work in the lab. The level of aneuploidy in all 24 chromosome specific loci were assessed in human embryos fixed with a novel approach i.e. a Tween HCl or methanol: acetic acid combination. In this case only a weak maternal age effect was detected as was an association with embryo quality. Again monosomy appeared more frequent than trisomy and this new approach appeared overall to give a greater FISH efficiency than a prior one in the laboratory. Results suggested very high levels of abnormality overall with most cells appearing abnormal for at least one chromosome. This approach may have some applicability for follow up analysis, but not for PGS.

- Nuclear organisation in human embryos using 24 chromosome FISH was assessed in relation to chromosome abnormalities, maternal age, embryo morphology, and stage of embryo development. Comparisons were made between the individual blastomeres of each whole embryo, and between different whole embryos. Differences between groups with multiple abnormalities and with relatively fewer abnormalities were revealed and specific centromeric loci seemed to be the ones consistently most prone to altered nuclear organisation in the various comparisons.

8.1.Specific aim 1: To test the hypothesis that nuclear organisation is altered in men with severely compromised semen parameters by assaying telomere and sub-telomere loci for 11 chromosomes

As discussed in the introduction section 1.3.7, it is reasonable to hypothesise altered nuclear organisation in the sperm head is linked with reduced fertility. The link between nuclear organisation and male fertility is still to be established however and, so far, any association between radial chromosome position and male infertility has only been published from our lab (Finch *et al.*, 2008b; Ioannou and Griffin, 2010). Both studies assessed the nuclear organisation of OAT patients vs. controls using the positions of centromeres, the first suggesting an association for the sex chromosomes, the second suggesting a remarkable stability of the chromocentre, despite severely compromised spermatogenesis. However the positions of telomeres and sub-telomeric regions were not analysed and therefore this study was the first to focus on the telomere and sub-telomere organisation of the sperm head from OAT patients and control donors.

8.1.1. Organisation of telomeres and sub-telomeres in the sperm of men with normal semen parameters

Previous literature reports that, in the sperm head, telomeres interact with each other to form dimers, and that these dimers are located towards the nuclear periphery, close to the nuclear envelop (Mudrak *et al.*, 2005; Zalenskaya and Zalensky, 2002, , 2004; Zalensky and Zalenskaya, 2007; Zalensky *et al.*, 1993; Zalensky *et al.*, 1995). Our results on sub-telomeres clearly showed that localisation of sub-telomere regions is peripheral to medial, depending on the specific chromosome probe and the individual (Specific aim 1.3.3.1). However pan telomeric probes from control males showed a more peripheral localisation in captured images, but when they were analysed via our automated method, results suggested that the localisation of pan telomere regions were random in this group (Specific aim 1.3.3.1).

In this study, analysis was based on extrapolating 3D information from 2D images as published by (Boyle *et al.*, 2001; Croft *et al.*, 1999) in order to correct the errors that occur due to this extrapolation. 2 different mathematical models were used and data was independently analysed with each model. However in contrast to previous findings, our results suggested that telomeres and sub-telomeres have a more relaxed and dynamic organisation, having located anywhere between the periphery to centre of the sperm head. The localisation of telomeres is indeed more peripheral compared to the centromere regions of the same chromosome, however they do not have distinct peripheral localisation as previously published. With regard to individual results, telomere and sub-telomere positions are different in each individual for each chromosome.

The fact that telomeres and sub-telomeres studied in this project did not always have distinct peripheral localisation may suggest that telomeres for all chromosomes may not be peripheral

in every sperm cell. When visualised under the microscope, some cells showed peripheral telomere signals i.e. a halo-like effect (figure 3.1a). However, other subsets of cells showed multiple signals throughout the cell, even in the centre (figure 3.1b). The number of cells with signals on the periphery compared to the number of cells with random patterns was very varied and was dependent on the individual case.

Other criticisms of this work could be that when analysing pan telomeric signals, the program analysed clumps of signals representing telomere dimers together as one signal. This would bias the results towards a random distribution of signals, when in fact the majority of signals may well be present at the periphery. When images are observed as a whole it becomes obvious that the number of telomere signals close to periphery is relatively high. Therefore the fact that I did not always obtain a peripheral pattern following analysis could be due to technical issues in thresholding and inaccuracies of the algorithm when trying to read multiple overlapped signals. Thus before making final conclusions regarding this study, manual assessment of telomere signal positions should be carried out. A final interpretation is that the pre-FISH preparation approaches used in this study may have altered the nuclear organisation and thus our inability to detect a consistent peripheral pattern may be a technical phenomenon.

Using sub-telomeric probes for p and q arms, positions of 11 chromosomes were analysed. Similarly to the case in telomeres, the positions of sub-telomeres were different in each patient for each chromosome arm. In contrast to previous publications, p and q arms were not close together in all cases. Some sperm cells showed p and q arm signals located on the opposite sides of the sperm head. This also suggests the dynamic nature of telomeres in the sperm head.

However, this observation could, as discussed for pan telomeric probes, be due to technical reasons. The study by (Zalensky *et al.*, 1993) used extremely mild procedures to swell and purify human sperm nuclei to retain the characteristic shape and constituent proteins. They used non ionic detergent to destroy the cell membrane (Gusse *et al.*, 1986). However the current study used pepsin to digest membranes, and this could be digesting the telomere anchoring proteins in the sperm nuclei resulting in disturbance to the real peripheral localisation of telomeres. Also swelling with DTT might affect the positions due to swollen nuclei.

Furthermore, studies previously showing peripheral telomere patterns used 3D techniques and did not look at 100s of cells. Therefore results obtained might not be a common phenomenon to all sperm cells and it is possible that previous 3D studies may have inadvertently selected those cells with peripheral signals. The current study however, uses a 2D technique. In reality, signals actually in the centre should always be represented in the centre of 2D images whereas signals at the periphery could be seen as either in the periphery or in the centre if the sperm cell has rolled over. So in our telomere images some of the signals we see in the centre of the nuclei could be actually located in the periphery. In order to correct for this phenomenon, 2 mathematical models were used. The DAPI density model was the standard one that had been previously published with sperm nuclear organisation analysis in our lab (Ioannou and Griffin, 2010; Skinner *et al.*, 2009). The new volumetric model also corrects the errors of 3D extrapolation of 2D data by considering the pressure the cell has been subjected to when the cell was flattened. Using both models, signals located in the periphery get a relatively higher score compared to signals located in the centre, thereby correcting for the actual number of cells with peripheral signals. Comparing 2 mathematical

models, the volumetric model gives slightly more peripheral distributions overall, therefore the number of cells with peripheral results is increased compared to the DAPI density model.

The methodology used in this thesis to assess the radial position is more robust compared to that used in (Finch *et al.*, 2008b) as it used a computer program to determine the proportion of signals in each portion of the nuclei. The computer software recognises the nuclear periphery and divides the sperm head into 5 concentric shells of equal area. Therefore this way of analysis directly represents nucleus shape and size through the "pixel translation" of the DAPI counterstain into the borders of the nucleus. Thus any surface perturbation in the shape of the sperm head (e.g. the pointed edge close to the tail) can be better represented. Similarly, when signals are scored, they are measured through the intensity of the pixels, providing a better representation of the signal in terms of the size, and in which portion of the nucleus the signal resides.

8.1.2. Organisation of telomeres and sub-telomeres in the sperm of OAT patients compared to controls donors

Similar to the control sperm samples, in sperm cells of the OAT patients, a preferential localisation of telomere and sub-telomeric loci was varied depending on the patient (Specific aim 1.3.3.2). Preferential positions ranged from the periphery to the centre, and even random patterns were apparent. When examined collectively, only subtle differences could be seen by comparing the peak positions of the graphs. Therefore a T test was used to compare the average positions between patients and controls (Tables 3.11 and 3.12). T test results suggested that the median positions were statistically significant between patients and controls for sub-telomere loci 1pq, 2pq 3pq 4pq and 8pq. Significantly different median

positions were also obtained for telomere specific regions in OAT patients compared to controls.

The general message by comparing telomere positions of OAT patients and controls therefore was that telomeres in the sperm head have more "relaxed" organisation compared to centromeres (where the chromocentre appears intact, regardless of the state of infertility – Ioannou et al. 2010). This organisation varies between each individual control and patient sample. This could be due to biological reasons like telomere shortening; for example patients with longer telomeres may give more peripheral telomere and sub-telomere localisations. By comparing median positions it has become evident that the localisation of certain loci tended to be significantly different to controls. Thus this could be due to a breakdown in the nuclear organisation associated with OAT.

The position of the telomeric arrays has not been studied in relation to infertility before. To my knowledge only 2 studies have looked at the radial nuclear organisation in sperm of men with impaired semen parameters and both of them were from our laboratory (Finch *et al.*, 2008b; Ioannou and Griffin, 2010). Both of these studies however looked at the organisation of centromeres. Overall results from both studies suggested that in control males chromosome centromere loci occupy a distinct position providing evidence for the chromo-centric model, and that differences between patients and control groups were subtle. A similar study that compared the longitudinal position of centromeres reported that there are some alterations in the position of chromosomes in infertile men (Olszewska *et al.*, 2008). By contrast a study that looked at 3D images of sperm from a fertile donor using a suspension FISH however, suggested a chromosome size and gene density related arrangement in the sperm head (Manvelyan *et al.*, 2008). If developed further therefore an assay for telomere

position may ultimately become part of a screening test for certain types of fertility in humans.

8.1.3. Future developments

In the current study only the radial nuclear organisation of telomeres and sub-telomeres were assessed. It will also be interesting to know the longitudinal positions of telomeres and sub-telomere loci related to infertile phenotypes. Also it will be interesting to assess the relative positions of the p and q arms of telomere dimers in relation to male infertility. The disadvantage of these types of studies is that this work requires capturing images of thousands of nuclei, which is extremely time consuming and labour intensive. Until recently automated capturing was not popular in 2D image capturing systems (Perry *et al.*, 2007). However automated and more advanced image capturing system such as suspension FISH will be required to perform this kind of analysis in the future (Steinhaeuser *et al.*, 2002).

8.2.Specific aim 2: To test the hypothesis that nuclear organisation is altered in men had cancer and chemotherapy

Altered chromosome position has been seen in several cell disorders including epilepsy, certain types of laminopathies, cancer and in some infertile phenotypes (Borden and Manuelidis, 1988; Finch *et al.*, 2008b; Meaburn *et al.*, 2005; Misteli, 2004). To the best of my knowledge this is the first time that nuclear organisation of sperm has been studied in relation to certain forms of cancers and treatments associated with potential human reproductive issues.

8.2.1. Nuclear organisation in control men over time

My results in control men (section 4.3.1) provide evidence for a chromo-centric pattern of nuclear organisation in the sperm head, as previously shown by numerous research groups (Finch *et al.*, 2008b; Ioannou and Griffin, 2010; Zalenskaya and Zalensky, 2004; Zalensky and Zalenskaya, 2007; Zalensky *et al.*, 1993; Zalensky *et al.*, 1995). These results demonstrate that all three centromeric chromosomal loci occupy the centre of the nuclei of all of the control males (table 4.1). In addition our results demonstrate a central medial location of the 13q locus and a subsequent medial location of 21q (table 4.2). Overall, longitudinal positions were not different between centromeric and non centromeric chromosome loci (tables 4.3 and 4.4). My findings show that this central position of the centromeric loci and medial central positions of non centromeric loci are maintained over time in healthy control males. Centromeric loci always appear to maintain the chromo-centre with slight alteration in the medial positions. However non centromeric probes are more dynamic and position alters to a slightly higher level between the central and medial positions. Longitudinal positions for both centromere and non centromere probes are also maintained over the time in control sperm samples (tables 4.3 and 4.4).

8.2.2. Nuclear organisation in TC and HD patients before and after treatments

In this study, data was analysed both individually and collectively for radial and longitudinal positions. The results on individual analysis provide some preliminary evidence in support of the hypothesis that, at least in certain men, significantly altered nuclear organisation is associated with TC. More specifically, a chromosome locus 21q was significantly different in one of the five testicular cancer patients having a randomly organised position (table 4.2).

Other TC patients and all HD patients show significantly non random patterns for all chromosomal loci tested. The effect of chemotherapy on nuclear organisation has also been studied. One TC patient showed a significantly altered nuclear organisation 12 months after chemotherapy treatments, however other TC and HD patients at all time points after chemotherapy, did not show significantly altered patterns following chemotherapy.

When data was pooled and compared using a T test, several differences in the positions of certain chromosomes at various time points were observed (table 4.5): The position of chromosome 21 was significantly different between the control and TC group at 12 months after chemotherapy, and between the control and HD group 18-24 months after chemotherapy. Similarly, the position of chromosomes 1 and Y were also significantly different between controls and TC patients before cancer treatments, and 6 months after treatments.

Furthermore, individual data on the longitudinal organisation of chromosomes did not significantly differ in any of the cancer patients compared to the controls (tables 4.3 and 4.4). However when data was analysed collectively (table 4.6), some significant differences between patients and controls became apparent. For example the longitudinal position for chromosomes X and 21 was different between TC and HD groups straight after cancer diagnosis compared to controls. At the time point 6 months after cancer treatments, chromosome 13 was significantly different between TC and control groups, and TC and HD groups. 12 months after chemotherapy, chromosome 21, 1 and chromosome X positions were significantly different in TC and control groups. At 18-24months after chemotherapy the position of chromosome 13 and 1 was significantly different between TC and control groups, and the position of chromosome X was significantly different between HD and control

groups. It is noteworthy however that, although individual significant differences could be seen, there was no overt pattern suggesting a fundamental difference between controls, patients and treatment groups. The results at best therefore point to the need to perform many future studies to establish whether general patterns emerge.

Previous studies that have looked into the radial positions of chromosomes (18 and 19) revealed a partial loss of gene density related chromatin localisation in cancer cell lines (Cremer *et al.*, 2003a). Similarly a more recent study by Marella and colleagues 2009 reported a significant difference in the pair wise association of chromosome territories in a human breast cancer cell line compared to its original epithelial cell equivalent, proposing a significantly altered nuclear organisation in cancer cells (Marella *et al.*, 2009a). Our results on at least one germinal TC patient having a significantly altered nuclear organisation for 21q loci, agrees with this previously published data i.e. tumour cells have significantly altered nuclear organisation compared to it's original cell lineage. The reason behind alterations in sperm nuclear organisation in TC could be because germinal testicular tumours directly affect the spermatogenesis process. Also chemotherapy drugs such as Cisplatin directly target germinal tumour cells, therefore the possibility of targeting non tumour germinal stem cells also possible.

A recent study by Guffei and colleagues revealed that nuclear remodelling is associated with a Hodgkin's lymphoma cell line, proposing the relevance of nuclear architecture in cancer (Guffei *et al.*, 2010). This study looked at inter nuclear DNA bridges, consisting of chromatids and chromosomes in mononucleated Hodgkin (H) cells, and multi-nucleated Reed-Sternberg (RS) cells. The study revealed the presence of inter-nuclear DNA bridges in RS cells but not in H cells, indicating that the complexity of chromosomal rearrangements

increased with tumour progression. On the other hand however, the present study provides evidence that nuclear organisation in the sperm of HD patients was not significantly different compared to control sperm cells. This could be because HD is a tumour in lymphocytes rather than having a germinal cell origin, therefore nuclear organisation changes could be expected more in lymphocytes rather than in sperm cells.

When analysed individually, certain TC and all HD patients after chemotherapy did not have a significant alteration in nuclear organisation for any of the chromosomal loci tested. As a result of chemotherapy, patients tend to have low sperm counts, with severe oligozoospermia and azoospermia. However the sperm that has survived could be those that have originated from more robust stem cells which were more resistant to the treatments, therefore we could expect them to have fewer changes in nuclear architecture.

Even though altered nuclear organisation is believed to be associated with increased levels of sperm aneuploidy (Finch *et al.*, 2008b), the results of the second chapter show no evidence for such an association. Increased sperm aneuploidy is associated with compromised semen parameters, this was first reported by (Pang *et al.*, 1999) and recently reviewed by (Tempest and Griffin, 2004; Tempest *et al.*, 2004). Indeed cancer and chemotherapy are known to be linked with severely compromised parameters and alterations of frequencies of aneuploidy, thus it is reasonable to hypothesise alterations in nuclear organisation as well. In a previous study Tempest and colleagues looked at the frequencies of aneuploidy of the same patients and controls used in the current study, and demonstrated that both testicular cancer and Hodgkin's lymphoma patients had elevated frequencies of aneuploid sperm for chromosomes 13, 21 and the sex chromosomes after chemotherapy (Tempest *et al.*, 2008). The study also revealed that there were significantly increased aneuploidy levels at 6 months after the

initiation of chemotherapy and that these aneuploidy levels tend to reduce to pre treatment levels approximately 18 months after the treatments. Furthermore, Tempest and colleagues compared the levels of aneuploidy between each of the cancer groups and with controls, and reported that some pre treatment aneuploidy frequencies in testicular cancer and Hodgkin's lymphoma patients were increased compared to controls. Interestingly Hodgkin's lymphoma patients had higher levels of aneuploidy for all chromosomes compared with both testicular cancer patients and controls throughout treatment. The current study tested the hypothesis that time points with high levels of aneuploidy in these groups is associated with alterations in the positions of chromosomes. However, we failed to find any evidence for this. One criticism of our study could be that we have only included chromosomally normal cells to analyse for the nuclear organisation related to aneuploidy levels. However by analysing chromosomally normal cells we cannot expect to have alterations of the position, as in this way we are selecting for the normal cells in an elevated population of abnormal cells. Therefore it will also be interesting to see nuclear organisation of aneuploid cells individually. In addition, closer examination of the positions of more chromosomes and chromosomal loci, the relationship between sperm aneuploidy and compromised semen parameters in a larger group of patients, may allow us to determine the effect of cancer and chemotherapy we have yet to see.

8.2.3. The effect of cancer and chemotherapy on DNA integrity in the sperm head

Various adverse effects of cancer on sperm count and quality are believed to be a result of primary gonadal damage, (Viviani *et al.*, 1991) and involve cytokines (Rueffer *et al.*, 2001). Although sperm from patients who have suffered with cancer and received chemotherapy treatment have severely compromised semen parameters and much higher aneuploidy

frequencies, in the present study we did not observe alterations of nuclear organisation as a result of cancer (other than in one TC patient) or chemotherapy (other than in one TC patient 12 months after treatments) in individual patient data. This could be due to number of reasons. Firstly we cannot forget about the intra individual variance or unknown individual genetic factors or susceptibilities that cause patients to react differently to the cancer and treatment. Also it is difficult to compare patients to each other because the dosage of treatment received may vary between patients.

More recently, studies by several research groups have looked at DNA integrity and fragmentation levels in cancer patients before and after treatments. Romerius and colleagues found an increased DNA fragmentation index in childhood cancer survivors (Romerius *et al.*, 2010) and suggested that these DNA damages were a result of the disease rather than the treatments. Another study by Smit and colleagues looked more specifically at the sperm DNA fragmentation index in TC and HD cancer patients before and after treatment, in order to see the affect on sperm DNA integrity by cancer itself, or its treatment. Results suggested that cancer does not negatively impact on the sperm DNA integrity of germinal TC and HD patients (Smit *et al.*, 2010). In contrast, a similar study by O'Flaherty and colleagues reported that even before chemotherapy, both cancer groups had high sperm DNA damage than in controls (O'Flaherty *et al.*, 2008; O'Flaherty *et al.*, 2010). This damage was increased further at 6 months and remained elevated 24 months after treatment (O'Flaherty *et al.*, 2010). This study also reported that significantly higher FSH hormone levels were associated in the cancer group compared with controls at 6 to 12 months after chemotherapy. This result is consistent with another study by (Sieniawski *et al.*, 2008) that reported significantly different post treatment FSH levels in patients with azoospermia as a result of Hodgkin's lymphoma.

This study proposed a possibility of using FSH levels as surrogate parameter for male fertility in future.

Even though aneuploidy levels and DNA fragmentation levels are altered as a result of cancer and chemotherapy, our study did not provide evidence of obvious changes in chromosome position in the sperm head as a result of cancer or chemotherapy in individual patients. In some ways this is surprising given the circumstantial evidence that there would be an association. Nonetheless statistically significant median positions of pooled data indicated that there might be some effect on nuclear organisation in patients compared to healthy controls. Until the effect of cancers and treatments have been studied in terms of all other semen parameters, the mechanisms of, and their heritable consequences, will remain the subject of future studies.

8.3.Specific aim 3: Assessment of FISH based PGS outcomes of clinical cases in terms of sensitivity, specificity and accuracy

Preimplantation genetic screening (PGS) is a method of selecting embryos against chromosomal abnormalities in order to increase pregnancy rates (Munne *et al.*, 1993a) (Gianaroli *et al.*, 1997a; Munne *et al.*, 1994; Munne *et al.*, 2007a). However the use of PGS has been highly debated over the last few years, as some studies suggested that PGS does not improve the pregnancy rate; in fact it reduces the chances of implantation (Blockeel *et al.*, 2008; Debrock *et al.*, 2009, , 2010; Hardarson *et al.*, 2008; Mastenbroek *et al.*, 2007; Mersereau *et al.*, 2008a; Meyer *et al.*, 2009; Schoolcraft *et al.*, 2009; Staessen *et al.*, 2004; Staessen *et al.*, 2008).

Specific aim 3 of my thesis was to provide some insight into the PGS outcome in terms of sensitivity, specificity and accuracy. The first part of this study aimed to analyse case cancelations and inconclusive results in the whole data set according to maternal age and indication groups. Secondly incidence of chromosomal abnormalities was assessed both in day 3 and follow up embryos. Thirdly, concordance between day 3 and follow up data was analysed. Fourthly incidence of chromosomal abnormalities was analysed according to maternal age and finally according to indication groups. However due to insufficient sample size only recurrent implantation failure was analysed compared to rest of the groups.

8.3.1. PGS cancelations and inconclusive results

Table 5.1 in the specific aim 3 section suggests maternal age effect on case cancelations. In general terms, the association between maternal age and case cancellation is hardly surprising. Increased age has an adverse effect on a range of obstetric issues including the incidence of aneuploidy. Indeed, even when considering aneuploidy alone, several mechanisms are thought to contribute to what, overall, is termed "the maternal age effect."

8.3.2. Chromosome abnormalities in day 3 blastomeres

In this data set, the incidences of monosomies was generally higher (in most cases double) compared to the incidence of trisomies (table 5.4). This phenomenon was clear in all indication and age groups other than the 35 and younger age group, which had more normal cells than abnormal cells in day 3 embryos. Overall, in the whole data set we had a total of 51% aneuploid cells (table 5.2), which comparable to a study by Munne and colleagues who analysed abnormalities in day 3 embryos (Munne *et al.*, 1995b). Similar results were also reported in a study by Li and colleagues who report that from 660 embryos 367 (55.6%) were

normal and 281 (42.6%) were aneuploid (Li *et al.*, 2005). Furthermore another study that used comparative genomic hybridisation to assess day 3 embryo chromosome abnormalities, found that 57% were aneuploid (17 out of 30 embryos).

In addition several other studies looked at day 3 chromosomal abnormalities in human embryos related to various other parameters. A study by (Rabinowitz *et al.*, 2012) investigated chromosomal error types and parental origin of aneuploidy in cleavage stage embryos. This study reported that maternal meiotic trisomy are more common and rose significantly with age; however other trisomies did not show any relationship with age. In terms of monosomies both paternal and maternal origin was roughly equal. (Voullaire *et al.*, 2000) performed a study to analyse chromosome abnormalities in human blastomeres with CGH technique and provide information about various chromosomal abnormalities in human embryos. Similarly study by (Laverge *et al.*, 1997) also investigated chromosome abnormalities in day 3 human embryos and found the existence of diploid, aneuploid, haploid, triploid, tetraploid, moaic and chaotic embryos. Trussler and colleagues also studied chromosomal abnormalities in human embryos using CGH techniques and conclude that chromosomal abnormalities in embryos may arise as a result of cultural artefact or inadequate cell cycle surveillance, rather than meiotic error(Trussler *et al.*, 2004). Our results on 24 FISH in the next chapter agree with this and are discussed in more detail.

The main difference between the current study and most of previous studies is that day 3 results from the current study were obtained from a single cell, rather than from the whole embryo. Therefore it is not possible to make a direct comparison between these studies. However next part of the study looked at the whole embryos at day 5.

8.3.3. Chromosome abnormalities in day 5 embryos

In day 5 follow up studies, the overall rate of aneuploidy was 36% (table 5.3), which was considerably lower than in other published studies that reported 50-82% (Donoso *et al.*, 2007b; Mantzouratou *et al.*, 2007; Mantzouratou and Delhanty, 2011). Similar to day 3 blastomeres, follow up embryos had a high incidence of monosomies compared to trisomies (table 5.5). Several groups have performed number of investigations to analyse chromosomal abnormalities in follow up embryos. For example studies by (Daphnis *et al.*, 2005; Harper *et al.*, 1995; Jamieson *et al.*, 1994) investigated the chromosome constitution of human preimplantation embryos and found various abnormalities in human embryos and the most common of which was mosaicism.

Levels of mosaicism in the current study including major and minor was 25% (table 5.3), which was within the expected range (Barbash-Hazan *et al.*, 2009; Munne and Cohen, 1998; Munne *et al.*, 2005). In human embryos mosaicism has been extensively studied by several group using FISH (Bielanska *et al.*, 2000; Magli *et al.*, 2000) CGH (Voullaire *et al.*, 2000; Wells and Delhanty, 2000) and array CGH (Vanneste *et al.*, 2009). In our data levels of mosaicism in the whole data set were 25%; 13% major mosaic and 12% minor mosaic (table 5.3). We also obtained 8% chaotic embryos (table 5.3). Mosaicism is believed to arise through chromosome loss followed by gain (Delhanty, 2005). Alternatively some studies have reported that mosaicism occurs due to errors in post zygotic divisions; possibly due to spindle abnormalities which lead to chromosome gains and losses (Chatzimeletiou *et al.*, 2005).

8.3.4. Concordance and PGS accuracy

When compared, day 3 single cell and follow up results, current study had a higher confirmation rate. Within the whole data set 33% of embryos had more or less the same diagnosis as in day 3 embryos, and 49% had some abnormalities that were confirmed, or had more abnormalities compared to day 3 embryo analysis (table 5.6 and figure 5.3). With regard to the concordance, the whole data set had 82% of concordance (full or partial match), which was similar to previous published data (Emiliani *et al.*, 2004; Gianaroli *et al.*, 2001; Magli *et al.*, 2000; Sandalinas *et al.*, 2001; Staessen *et al.*, 2004). For example, a Study by Magli and colleagues assessed the chromosome mosaicism in day 3 aneuploid embryos that develop to morphologically normal blastocysts in vitro using FISH (Magli *et al.*, 2000). Authors reported a higher concordance between day 3 and follow up aneuploidy diagnosis was observed with trisomies (97%), and multiple complex chromosome numerical abnormalities (100%) and a lower concordance for monosomy (65%) and haploid (18%) embryos. Another example study by (Daphnis *et al.*, 2008) used CGH to analyse 1-2 cells biopsies on day 3 and the rest of the embryo was cultured until Day 5 and analysed with FISH. Depending on CGH results embryos were categorised as normal or abnormal. When day 5 analysis was performed, the majority of the embryos categorised as normal in day 3 were found to be mosaic with various abnormalities. Also most of the embryos categorised as abnormal in day 3 were confirmed as mosaic or chaotic in day 5. (Baart *et al.*, 2004) also performed FISH analysis to investigate the chromosome abnormalities in day 3 and day 5 human embryos and found that in only 6 of 17 cases cytogenetic analyses were concordant. Current study also witness for similar patterns for concordance between day 3 and follow up results (table 5.6 and figure 5.3).

In the current study overall misdiagnosis rate was therefore 18% with false positive rate of 17% (table 5.7). False positive results indicate the number of embryos diagnosed as abnormal in day 3 embryos but end up being normal in day 5 embryos. Published data provides evidence for a wide range of false positive rates, from <1% to 17% (Cooper *et al.*, 2006; DeUgarte *et al.*, 2008; Hanson *et al.*, 2009; Li *et al.*, 2005; Mantzouratou *et al.*, 2007; Staessen *et al.*, 2004). Our results fall within the higher margin of this range. In our data the false negative rate was 1% for the whole data set. This is because most of the embryos diagnosed as normal were either transferred or cryopreserved, therefore the number of normal embryos available to analyse in the follow up study is considerably lower. As if further evidence were needed therefore, this and other studies provide evidence that PGS by FISH, although perhaps not as disastrous as the initial studies may have led us to believe, has "had its day" and should be superseded by more advanced approaches.

8.3.5. Embryo aneuploidy related to maternal age

Among age groups, the highest aneuploidy levels were in the 40 and older group reflecting the maternal age effect (table 5.9 and 5.10). This is similar to the study by (Munne and Cohen, 1998) and (Marquez *et al.*, 2000). Another study by (Benadiva *et al.*, 1996) reported that aneuploidy 16 in human embryos increases significantly with maternal age. In this study embryos were categorised in to 3 different maternal age groups; < 34 years, 35 – 39 and > 40 years and found significantly increased level of chromosome aneuploidies with maternal age. However a study by (Baart *et al.*, 2006) saw similar levels of abnormalities in older and younger patients. Surprisingly in the current study, among age groups, the highest levels of major and minor mosaic and chaotic embryos were found in the 35 and younger group (table 5.11). In addition some randomised clinical trials by (Debrock *et al.*, 2009) and (Schoolcraft

et al., 2009) reported that PGS results in improved reproductive outcome in patients with AMA.

8.3.6. Embryo aneuploidy related recurrent implantation failure

The current study did not find any difference in chromosomal abnormalities among RIF and non RIF groups (table 5.14 and figure 5.6). Our study initially intended to analyse chromosome abnormalities related to all indication groups, however due to a lack of sufficient samples in all groups only recurrent implantation failure (RIF) group were studied individually compared to the rest of the others. A more complete study by (Mantzouratou *et al.*, 2007) looked at chromosome abnormalities in PGS embryos from patients with advanced maternal age, recurrent miscarriage or repeated implantation failure and identified significant differences between referral groups for chromosome aneuploidies. However the current study failed to find such a difference between RIF and non RIF groups. Studies by two other groups also suggested that RIF patients had a decreased level of meiotic aneuploidy, in disagreement with our study (Mantzouratou *et al.*, 2007; Voullaire *et al.*, 2002).

More studies in literature have reported chromosome abnormalities related to RIF. For example Kahraman and colleagus reported that there was no significant difference between AMA, RIF and repeated early spontaneous abortion (RSA) groups and this is similar to results obtained in the current study (Kahraman *et al.*, 2004). Useing more advanced techniques than FISH some groups also provide evidence of chromosome abnormalities associated with RIF patients. For example (Voullaire *et al.*, 2002) analysed chromosome abnormalities by comparative genomic hybridisation in embryos from RIF patients and found the presence of chromosome aneuploidy for one or two chromosomes and complex chromosomal abnormality is 54%. The current study however found 45% of chromosome abnormalities in day 3 and 75% abnormalities in follow up embryos form RIF patients (table

5.14 and 5.16). (Voullaire *et al.*, 2007) also studied incidence of complex chromosome abnormality in cleavage embryos from AMA and RIF patients using aCGH. Results suggested that the chromosome abnormality in cleavage embryos is independent from maternal age but is related to recurrent implantation failure which is opposite to the observations in the current study.

Several studies have also explored the impact of PGS on IVF outcome of RIF patients. For example (Pehlivan *et al.*, 2003) suggested that use of PGS improves IVF outcome of RIF patients. Further, (Gianaroli *et al.*, 1999) suggest that PGS has an immediate impact on the ongoing implantation rate of RIF patients and (Pagidas *et al.*, 2008) also had similar findings. Due to lack of information available on pregnancy rates, the current study did not look at the IVF outcome of RIF patients. In addition study which analysed gametes (first polar body and partners sperm) from couples with repeated implantation failure found out that aneuploidy rate is increased in both members (Vialard *et al.*, 2008). Authors suggested that implantation failure has a heterogeneous origin, which gamete chromosome abnormality rate is one of the major contributing factors.

8.3.7. The future of PGS and follow up studies

Embryo follow up studies in order to confirm single cell diagnosis is recommended as a part of an internal quality control procedure (Thornhill *et al.*, 2005). PGS inaccuracies due to biological causes such as mosaicism cannot be not totally prevented. However technical aspects of causing inaccuracies such as quality of the FISH and the number of chromosomes tested can be improved; our approaches to solve some of these issues were presented in specific aim 4 and 5. In future, screening of all chromosomes through more advanced high resolution techniques such as array CGH or SNP arrays and sequencing will increase the

accuracy of PGS in single cells and will provide better information regarding chromosome abnormalities in whole embryos.

8.3.7.1. Array CGH

Array comparative genomic hybridization (aCGH) is a high resolution technique that has the ability to analyse copy number variation using WGA amplified test DNA which has already been used for the detection of aneuploidy following PGS by large number of groups (Fishel *et al.*, 2010; Fragouli *et al.*, 2010; Le Caignec *et al.*, 2006; Schoolcraft *et al.*, 2010; Traversa *et al.*, 2011; Vanneste *et al.*, 2009), in some case with successful live birth (Fishel *et al.*, 2010). Currently array CGH is being used on polar bodies (Geraedts *et al.*, 2011) cleavage stage embryos (Hellani *et al.*, 2008) and trophectoderm cells (Yang *et al.*, 2012) also reviewed in (Harper and Harton, 2010; Harper and Sengupta, 2012). Array CGH analysis is fully automated, it allows PGS procedure to be performed with 24 hour window and embryo transfer on day 5 (Hellani *et al.*, 2008). Array CGH has also used in polar bodies and blastocysts in patients with repeated implantation failure and showed significant improvement of implantation and pregnancy rates (Fragouli *et al.*, 2010). Currently, randomised trials performed on array CGH based PGD around the world in order to see the effectiveness on this method (Harper and Sengupta, 2012; Harper *et al.*, 2012).

8.3.7.2. SNP arrays

SNP arrays allow the most comprehensive screening of IVF embryos as they provide information regarding chromosomal abnormalities, single gene defects as well as distinguish the parent and phase that the abnormality originated from (Handyside, 2011). Several groups have also reported the application of SNP arrays clinically for PGD and PGS (Handyside *et*

al., 2009; Johnson *et al.*, 2010; Northrop *et al.*, 2010; Treff *et al.*, 2010a; Treff *et al.*, 2010b; Vanneste *et al.*, 2009) and it has been reported successful ongoing pregnancy results (Brezina *et al.*, 2011). SNP array produce vast amount of information regarding the embryos tested. In addition to information of aneuploidies and specific genetic disorders it can potentially produce information regarding chance to have common diseases, physical characteristics and late onset disorders (Harper and Sengupta, 2012). A number of groups around the world are now utilising SNP arrays and different analysing software for clinical PGS cases (Harper and Sengupta, 2012). One of the well known program was Karyomapping which was developed by professor Alan Handyside and colleagues (Handyside *et al.*, 2009).

8.3.7.3. Karyomapping

Karyomapping provides a universal linkage based methodology for preimplantation genetic diagnosis. This method is based on Mendilian analysis of SNP data from parents and or other appropriate family member to identify informative loci of haplotypes across the chromosome and map the inheritance of them to embryos (Handyside *et al.*, 2009). In this way karyomapping can screen all 24 chromosomes and detect monosomies, trisomies, deletions, duplications, uniparental disomies, translocations and monogenic disorders simultaneously. It can also distinguish the parental origin and meiotic phase of the aneuploidy.

8.3.7.4. Sequencing

Full genetic sequencing is also playing a significant role in the in future PGD and PGS applications. Various groups around the world have utilised sequencing based methods to perform PGD for number of genetically inherited disorders (Chen *et al.*, 2011; Hellani *et al.*, 2009; Sanchez-Garcia *et al.*, 2006; Tomashov-Matar *et al.*, 2012; Wu *et al.*, 2010).

8.4.Specific aim 4: To apply the 24 chromosome FISH strategy to human blastomeres and assay the level of chromosome abnormalities and assess the efficacy of PGS

PGS by FISH generally tests for abnormalities in a small subset of chromosomes (Delhanty *et al.*, 1997; Munne *et al.*, 1995b; Munne *et al.*, 2004b). In 2010 Dimitris Ioannou from this laboratory developed a FISH technique for all 24 chromosomes on a single cell (blastomeres and lymphocytes) (Ioannou *et al.*, 2011). Using 24 FISH he assessed chromosome copy number and nuclear organisation of sperm and embryos. However this study examined only 250 blastomeres from 17 embryos that were fixed using the Tween HCl method. Perhaps due to poor quality embryos and/or the method used to fix those embryos (Velilla *et al.*, 2002) extensive chromosome losses were observed. The current study continued on from this previous work and aimed to analyse approximately 1400 blastomeres from 42 embryos fixed in Tween HCl, and methanol: acetic acid combination methods which was, it was assumed, would give a lower incidence of artefactual apparent chromosome less. This study also analysed the chromosome aneuploidy and mosaicism in relation to advanced maternal age, day 3 and day 5 morphological quality, and asked to what extent day 3 PGS results represented the ploidy of whole embryo.

8.4.1. Assessment of chromosome copy number in human embryos to find out if certain chromosomes are more prone to aneuploidy and if chromosome loss is more common than gain

A total of 42 embryos (1399 blastomeres) were analysed in this study for chromosome copy number. The main observation was that chromosomal abnormalities appeared extremely high in human preimplantation embryos: In fact only *one single nucleus* out of 1399 blastomeres appeared completely normal for the full karyotype. There are many possible explanations for this observation. First of all, this study used IVF embryos that were rejected for implantation either due to aneuploidy (41 embryos) or poor morphology (1 embryo). Therefore it is reasonable to hypothesise that the levels of abnormalities in the embryos would be relatively high. Also a study by Delhanty 2005 reported that more than 50% of embryos generated via IVF have a high degree of abnormalities (Delhanty, 2005). Additionally, failure of hybridisation (which was common in the first layer but improved in the subsequent layers) could be a side effect of the spreading method (the combination methods is still as not good as the methanol : acetic acid method in terms of producing nuclei with better signals with less overlapping (Velilla *et al.*, 2002)). Finally Ioannou 2011 reported a reprobing efficiency of only 60% in lymphocyte controls (Ioannou *et al.*, 2011). What is interesting in this set of data was that unlike the study by Ioannou, the first layers tended to give poor quality signals with multiple chromosome losses, and layer 4 gave the best results out of all layers, with very clear signals. However, reprobing with the same probe combination seemed to solve this problem. Therefore it can be said that 24 FISH is potentially a good and relatively inexpensive method to analyse follow up embryos. However, methodological and biological challenges of using the 24 chromosome FISH approach in a PGS setting still require further analysis.

Apart from chromosomes 1, 3, 4, 6, 8, 11, 16 and 20, all other chromosomes had two signals in more than 50% of the blastomeres analysed and the sex chromosomes were normal in about 90% of cases (table 6.4 and 6.5). Out of all the chromosomal abnormalities, generally

monosomies were more common compared to trisomies; this was also showed by (Munne *et al.*, 2004b). Higher levels of monosomies were seen in chromosomes 7, 8 and 20 (about 35% of blastomeres). This was consistent with the previous study by Ioannou, who reported an increased frequency of monosomy for chromosomes 20 (60%), 3, 7, 4, 17 and 9 (all above 40%). However in the current study, the maximum frequency of monosomy was only 36.7%. When monosomy results obtain for an embryo the obvious suspicion is if that is a real monosomy or due to failure to hybridise FISH probes and in the current study with 24 FISH we could not address that issue. However in the literature there are various studies address this issue such as study by (Cooper *et al.*, 2006) which looked at embryos diagnosed with monosomy by FISH. In this study embryos had monosomies in PGS results were reanalysed using the same set of probes. Results suggested that false positive rate for diagnosis of monosomies id 3.8% and majority of time PGS diagnoses represents true monosomy or mosaicism in human embryos.

In the current study the highest levels of trisomies (table 6.4 and 6.5) were seen in chromosomes 2, 19 and 22 (more than 15% of blastomeres). For chromosomes 10, 13, 15, 5, and 16 trisomy was found in about 10% of cases. This is consistent with Ioannou's study, which reported 10% trisomy in chromosome 15. In the current study, the highest levels of nullisomies were seen in chromosomes 1 and 4 (table 6.4 and 6.5): In Ioannou's study, chromosome 4 also had high levels of monosomies. Furthermore, Hassold and Hunt (2001) reported that in spontaneous abortions trisomy 16, 21 and 22 contribute to 50% of all trisomies (Hassold and Hunt, 2001). In the current study the frequencies of trisomy 16, 21 and 22 were 10%, 8% and 16% respectively; altogether these contribute to 34% of all trisomies. One of the difficulties in gleaning biological meaning from this data however was distinguishing the actual level of abnormality from the technical issue of probe hybridization

failure. When working well, control lymphocyte preparation gave good signals with all probes working at 90% + efficiency and some greater. The blastomeres however gave consistently stronger and brighter signals and thus the question arose as to how useful the controls were. In future studies, a search for a known diploid control cell type that better provides a baseline for these blastomere studies is a priority.

8.4.2. Mosaicism in day 5 human embryos

Due to the nature of experiment (FISH based, reprobing and involvement of all 24 chromosomes), previously described classifications to describe mosaicism (e.g. normal, minor mosaic, major mosaic, chaotic) (Daphnis *et al.*, 2005; Delhanty *et al.*, 1997; Munne *et al.*, 1994) would not represent the real nature of the embryos. However here we attempted to perform our classification adhering to the standard procedure as previously published (table 6.7). In this way our study with 42 embryos had 3 uniform abnormal embryos due to most probably meiotic errors. Our data also consist with 3 triploid and 1 haploid embryos according to follow up analysis. The rest of the embryos (35) were mosaic. The current results in mosaic embryos demonstrated more of chaotic mosaicism pattern which was in contrast to the study by (Munne *et al.*, 1994), which reported aneuploid mosaicism being the most common type of mosaicism seen in preimplantation embryos. It has also been reported that aneuploid mosaicism occur within the first few cleavage divisions due to errors of cell cycle checkpoints (Delhanty and Handyside, 1995; Munne *et al.*, 1994) causing mitotic non-disjunction allow chromosome loss or gain in the daughter cells. Also chromosome duplication and anaphase lag as well reported as possible mechanisms (Daphnis *et al.*, 2005). Chaotic mosaicism arises due to chromosome loss and gains through no specific mechanism to explain and characterised by nuclei showing randomly different chromosome complements. However, as the current study looked at all 24 chromosomes simultaneously,

different chromosomes indicate different pattern of mosaicism, may be also to do with errors in hybridisation, fluorescent fading etc. It will be interesting to investigate if there are any chromosome specific mechanisms for mosaicism.

In accordance with previous studies (Munne *et al.*, 2004a; Munne *et al.*, 2004b), and the previous results chapter, the current data also observed high levels of monosomies (21.3%) compared to trisomies (8.5%), (table 6.4, 6.5 and figure 6.2) indicating chromosome loss could be predominant mechanism for this type of mosaicism which has been widely reported in FISH studies (Daphnis *et al.*, 2005; Delhanty *et al.*, 1993; Delhanty *et al.*, 1997; Delhanty, 2005; Harper *et al.*, 1995). However, existence of both monosomy and trisomy for the same chromosome in an embryo also a common phenomenon, which leads us to believe that there is a role of chromosome non disjunction in embryo mosaicism.

8.4.3. Correlations of chromosome aneuploidy with maternal age, day 3 embryo morphology and day 5 embryo morphology

Chromosome abnormalities in human embryos related to maternal age, embryo day 3 morphology and development rate was studied by various groups. For example (Marquez *et al.*, 2000) studied cleavage-stage human embryos related to maternal age, embryo morphology and development rate and suggested firstly, that aneuploidy increased with maternal age, secondly, aneuploidy is more common in embryos with good morphology and development rate and thirdly, slowly developing or arrested embryos with poor morphology had significantly increased levels of polyploidy and are highly mosaic than normal embryos (Marquez *et al.*, 2000). Another study investigated if embryo morphology, developmental rates, and maternal age are correlated with chromosome abnormalities (Munne *et al.*, 1995a) using 3 or 5 chromosome FISH. 524 cleavage-stage human embryos were allocated into three

groups according to morphological and developmental characteristics and analysed according to maternal age. Results demonstrated that morphologically poor embryos had higher rates of polyploidy and diploid mosaicism. Authors also suggested that in morphologically and developmentally normal human embryos, cleavage stage aneuploidy significantly increases with maternal age.

Overall correlation between maternal age and chromosome aneuploidy was not significant in the current study (table 6.8). However certain chromosomes showed significantly different distribution in different maternal age groups which is presented in the (table 6.9). In the current study, clinical data analysis chapter showed significant association between maternal age and chromosome aneuploidy as previously published by many studies. The fact that 24 FISH related method do not show such a correlation cast doubt on the efficacy of the current protocol.

Using 24 FISH, this study attempted to find the link between embryo day 3 morphology and chromosomal aneuploidy. Our results gave evidence for some association between certain chromosomes and embryo morphology which is presented in the table 6.11. There are other studies have looked the embryo morphology in human embryos related to various factors. For example one study by Moayeri and colleagues have looked at the embryo morphology related to maternal age and found out that embryo morphology predict normal embryos in the AMA group, but not in younger patients (Moayeri *et al.*, 2008). Also study by (Ziebe *et al.*, 2003) reported that uniformity of blastomere size, degree of fragmentation and cleavage kinetics have implication in the correct chromosome copy number in embryos. In addition study by (Magli et al., 2007) also suggested that embryo morphology is associated in chromosome aneuploidy.

Current study also attempted to find the correlation between aneuploidy and embryo day 5 morphology and found out that certain chromosomes have linked between aneuploidy and day 5 morphology as presented in table 6.13. Similar study has reported in the literature by (Kroener *et al.*, 2012). This study compared the relationship between aneuploidy and timing of blastocyst formation. Authors suggested that day 5 morula that develop to blastocysts on day 6 were significantly less likely to involve in aneuploidy (79.8%) than day 5 morula that did not develop to blastocysts (92.9%) (Kroener *et al.*, 2012). (Magli *et al.*, 2007) also investigated if development stage dependent on the chromosomal complement and found out that the incidence of higher aneuploidy rates in arrested, slow cleaving embryos and rapidly cleaving embryos compared to embryos with eight cells at 62 hours after insemination.

8.4.4. Comparison of two embryo fixation methods

Different fixation techniques and their advantages and limitations were described by (Velilla *et al.*, 2002). This study assessed the number of cells lost, the number of informative cells, the levels of signal overlaps and FISH errors following both fixation techniques. Results suggested (table 6.14 and figure 6.9) that the Tween HCL technique gives the poorest results in terms of nuclear quality, with more cytoplasm present, a higher number of overlaps, and a higher frequency of FISH errors. On the other hand, the methanol: acetic acid/ HCl Tween combination method provides reasonably good nuclear quality. For this reason, the methanol: acetic acid/ HCl Tween combination method was the one used in this study. This approach gave more normal and trisomy signals compared to the previous study which used the HCL tween method. Therefore it is reasonable to hypothesise that high level of

chromosome losses seen previously could be due to technical issues rather than biological reasons and that the fixation method used in this thesis should be the one going forward.

8.4.5. The efficacy of PGS

In the final part of this chapter, the level of accuracy between day 3 embryos vs follow up analysis was assessed. 9 (26%) embryos gave exact match, 4 (11%) embryos gave partial match, 22 (63%) embryos did not match at all (table 6.15 and figure 6.10). There was only one embryo diagnosed to be normal on day 3. When follow up results were analysed it became evident that this particular embryo was normal for 14 chromosomes (for more than 50% of the cells in the embryo) and monosomy for 3 chromosomes. The rest of the chromosomes had mosaic patterns. In this study among all embryos, the most normal we observed was 50% or more cells being normal for 16 chromosomes and 4 monosomies. However that specific embryo was originally diagnosed as trisomy 15 and 16 in day 3. This observation was consistent with a study by Deugarte and colleagues (2008), who reported that 17% of embryos were misdiagnosed in day 3 as abnormal (DeUgarte *et al.*, 2008).

In the current study, clinical data analysis chapter (specific aim 3) witness for a higher concordance between day 3 and follow-up analysis. In contrast the concordance of this chapter is extremely poor with 37% for total of both fully and partial confirmation. Similar study by (Magli *et al.*, 2000) reported that higher concordance between day 3 and follow up aneuploidy diagnosis was observed with trisomies (97%), and multiple complex chromosome numerical abnormalities (100%) and a lower concordance for monosomies (65%) and haploidy (18%) which is somewhat similar to what we have obtained in this study. In the current study hapiloidies were conformed in 50% of cases. Interestingly abnormalities

involved in chromosome 22 tend to confirm in most of cases due to strong bright signals in both PGS single cells and in follow up embryos.

In most cases of our day 3 vs. follow up analysis by 24 FISH, more abnormalities compared to the initial diagnosis became apparent. This reinforces the need of high resolution improved diagnosis methods such as SNP arrays to perform PGD in a real clinical diagnostic setting (Colls *et al.*, 2009; Munne *et al.*, 2010).

8.4.6. The future of chromosomal diagnosis with 24 FISH in PGS and follow up studies

Use of FISH in PGS was highly debated and still remains controversial. Previous specific aim 3 and 4 chapters attempted to provide an insight into the chromosomal abnormalities in day 3 and follow up embryos; first using a few chromosomes in a larger study group and then using a FISH based 24 chromosome assay in a smaller study group. Both studies have seen extensive chromosome loss compared to chromosome gain so it is still remain unknown that if this is actually a biological or technical related issue. Both studies have witnessed poor concordance between day 3 and day 5 diagnosis; the mosaicism and technical issues will still play a role in related to errors in PGS outcome.

The main issue regarding FISH based PGS has traditionally been FISH could only screen for limited number of chromosomes. To solve this, various studies suggested the use of FISH through the screening of more chromosomes in more hybridization layers (Colls *et al.*, 2009; DeUgarte *et al.*, 2008; Munne *et al.*, 2010) which could allow to identify more abnormalities. Here we initially attempted to introduce 24 FISH to PGS which could be performed within 24 hour window for single cells allowing the transferring of embryos still in cleavage stage.

However it has became apparent that this may even complicate the result and effect the decision making process. From my study and from our previous study (Ioannou *et al.*, 2012) it became clear that 24 FISH probes react different to the embryo spreading; HCL/ tween method provide best signals in the first layers and combination method provide it best signals towards the last layers. Thus use of 24 FISH in clinical PGS cases would not be practical, however it could be still of use in research, or for follow up studies as a way to analyse chromosomal abnormalities in cell by cell basis. The future of PGS has now moved towards higher resolution techniques such as array CGH and SNP genotyping (Geraedts *et al.*, 2010).

24 FISH of human embryos were initially performed with the intention of using in the clinical follow up cases to get more comprehensive information regarding all 24 chromosomes by a relatively inexpensive method. However current protocol still have considerable amount of limitations such as failure to hybridisation, fluorescent signal fading, signal overlapping and appearing under different filters. Also it became clear that FISH probes act different to certain embryo fixative methods. So before using 24 FISH for follow up analysis in embryos these issues should be addressed and both probe preparation and FISH protocols should be optimised accordingly.

8.5.Specific aim 5: Investigation of nuclear organisation in human embryos

The final results chapter in my thesis aimed to assess the organisation of all chromosome loci in human preimplantation embryos. Previous work by Dimitris Ioannou in my laboratory assessed the nuclear organisation of all 24 chromosomes in human embryos fixed using the

HCl Tween method (Ioannou *et al.*, 2012). However, literature suggests that this specific method for embryo fixing causes inaccuracies in PGS outcome. This chapter more specifically aimed to assess the organisation of all chromosomal loci in human preimplantation embryos fixed with HCl tween and methanol: acetic acid combination methods.

8.5.1. To assess the positions all 24 chromosomes in individual embryos

For the first time, this thesis analysed the nuclear organisation in individual embryos. In this study 8 embryos with more than 50 cells (100 signals) (which are usually recognised as the minimum number of cells for nuclear organisation analysis) were studied (table 7.1). Some embryos had a central location of chromosome centromeres, and others had random organisation. This could be due to different developmental stages of the cells in the same embryo. Also out of these 8 embryos, some were abnormal with high levels of trisomies and some were relatively less abnormal. With regard to individual embryos, populations of both chromosomally normal (relatively normal, no trisomies) and abnormal cells (trisomies, extensive chromosome loss, poliploidies, chaotic) were seen; and each embryo was made up of different proportions of normal and abnormal cells. In addition the fact that some chromosomes did not show evidence of a chromocentre may indicate undeveloped chromatin structure in the early development stage (Martin *et al.*, 2006a), which is evident in a lack of defined positions. In order to test for the chromocentre it will also be interesting to see how telomeres are organised in human embryos.

8.5.2. To test the hypothesis that centromeric loci occupy a more central nuclear address i.e. "chromocentre" pattern

When data was pooled together all centromeric probes gave central localisation other than chromosome 3, 4 and 6 (table 7.1). Locus specific probes for chromosome 5 gave the most peripheral localisation, however LSI 13, 14, 19, 21 and 22 gave central localisation. LSI 5 probe binds to the region most away from the centromere, i.e. region closest to the telomere. Therefore the LSI 5 probe displaying a peripheral-medial position was consistent with the presence of a chromocentre in human embryos.

8.5.3. To test the hypothesis that gross chromosomal abnormality adversely affects patterns of nuclear organisation when whole embryos are compared with one another

In this section embryos were categorised into normal and abnormal groups, and their chromosome positions were analysed (table 7.2). Embryos that are relatively normal (with no clear trisomies) were considered as normal and others were considered as abnormal and in this way there were 20 normal and 22 abnormal embryos. In normal cells most chromosomes showed a central localisation except for chromosomes 4 and 6 (random), 5 (peripheral medial), and 8 and X (central medial). In the abnormal group chromosomes 3 and 6 had random patterns, and chromosomes 8 and X had central medial patterns. The positions for chromosomes 4 and 5 were central in the abnormal group. These positions were significantly different compared to the normal group. In this part of study I thus had clear evidence for the chromocentre as most of the centromere signals appeared in the centre in both abnormal and normal groups of embryos. Differences in nuclear organisation in normal and abnormal groups were very subtle. Similar study to this was previously performed in our lab by

(Ioannou *et al.*, 2012) using embryos fixed with HCl tween medium. However that study did not find a significant difference between 2 groups analysed.

8.5.4. To assess the pooled nuclear position of 24 chromosomal loci in embryos and to test the hypothesis that chromosomally normal cells display a different pattern to those that are chromosomally abnormal.

Here, I present the most comprehensive appraisal of nuclear organisation in human preimplantation development to date (table 7.3). Preliminary results pertaining to nuclear organisation in human preimplantation embryo nuclei indicate a different pattern from lymphocytes and the presence of a "chromocentre" with the centromeric probes occupying the nuclear centre (similar to that seen in sperm heads – (Ioannou *et al.*, 2011). A chromocentre has been demonstrated in mouse day 1-2 cells that persists to the blastocyst stage and involves changes in pericentric chromatin as well as activation of replication and chromatin structure (Martin *et al.*, 2006a). Chromocentre formation has been related to the onset of zygote transcription thus implicating a functional significance for the regulation of gene expression (Martin *et al.*, 2006a; Martin *et al.*, 2006b; Martin, 2006).

The apparent "chromocentre" pattern seen may have its roots in spermiogenesis. Spermiogenesis is accompanied by a significant alteration of the nuclear address of the chromosomal loci, specifically repositioning of the centromeres to the nuclear centre. According to the results of this study, a similar pattern may persist in the human preimplantation embryo. The reasons for the association between tight nuclear packaging and the reorganisation of the chromosome territories warrants further investigation in the context of this study as it has been suggested that efficient packaging is essential to facilitate proper

delivery of the paternal genome to the resultant preimplantation embryo (reviewed in (Miller *et al.*, 2010). Ward (2010) however argued that the cysteine residues of protamines confer extra stability in the sperm chromatin through intermolecular disulphide cross-linking and therefore sperm chromatin rearrangement functions to ensure proper fertilization as a protective agent of the paternal genome, not for future embryonic development (Ward, 2010). Further studies of the nuclear organisation of human embryos may yet reveal processes fundamental to these earlier stages of our development.

To the best of my knowledge, up until now only 5 studies (summarised in table 8.1 below) (including the current study) have assessed the radial nuclear organisation in human blastomeres (Diblik *et al.*, 2007; Finch *et al.*, 2008a; Ioannou *et al.*, 2012; McKenzie *et al.*, 2004). The current study used a similar method to that by Finch and colleagues, where the nucleus was divided into 5 concentric shells with equal area representing equal volume in the 3D nucleus. In order to compensate for the errors that occur due to 3D extrapolation of 2D data, signals located in the periphery were given higher scores.

However, in the current study and the study by Ioannou, a computer program was used to do the shell analysis as described in the methods section. In contrast McKenzie et al. 2004 used a 5 concentric ring with increasing diameter sizes in order to lower the probability of signals being located in the centre. A study by Diblik et al. 2007 used a 9 concentric ring model. A summary of results for all studies is presented in the table below.

Table 8.1: Positons of chromosomes 13, 15, 16, 18, 21, 22, X and Y in human blastomeres according to 5 different studies

	McKenzie et al.2004		Diblik et al. 2007		Finch et al. 2008		Ioannou *et al.* 2012		Current study	
Locus	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
13	C	P	R	R	R	P	R	C	C	C
15	N/A	N/A	N/A	N/A	R	C	C	C	C	C
16	P	P	R	R	R	C	CM	C	C	C
18	C	P	C	R	R	PM	C	C	C	C
21	C	P	R	R	R	C	C	C	C	CM
22	P	P	R	R	R	C	C	C	C	C
X	C	P	R	R	R	R	C	CM	CM	CM
Y	P	P	R	R	R	R	C	C	C	C

5 different studies assessed nuclear organisation using slightly different methods. Table 8.1 shows similarities and differentces of results for same chromosomes obtained by various research groups.

My results for both normal and abnormal blastomeres agree with the study by Ioannou except for 3 occasions. My results for normal embryos agree with some of the results by McKenzie et al.2004. My results also agree with some of Finch et al. 2008 results in abnormal embryos.

8.5.5. Nuclear organisation in relation to maternal age, day 3 embryo morphology, day 5 embryo morphology

To the best of my knowledge, this is the first time presentation of data for nuclear organisation related to maternal age embryo day 3 and day 5 morphology in human embryo cells (table 7.4, 7.5 and 7.6). This study also provides some evidence for subtle alterations of nuclear organisation in certain chromosome loci related to above parameters.

The study of nuclear organisation is widespread in the chromosomal literature. Indeed, the arrangement of chromosome territories in the interphase nucleus is thought to be fundamental to a nuclear network in which particular functions occur within specific nuclear compartments. Such studies suggest a dynamic plasticity of chromatin, demonstrating that, while chromosomes reside in specific domains in the nucleus, the movement of chromatin within these domains has considerable flexibility. Each nucleus therefore has an optimal "steady state" that is thought to occur normally but this can be disrupted e.g. in disease or

altered developmental potential. In other words, the dynamic spatial-temporal organisation within interphase nuclei, (nuclear organisation) correlates with functional status within a "healthy" nucleus and alterations in this organisation are commonly seen when nuclear function is altered. The fact that alterations of similar loci were reported (e.g. the same centromeres recurred in several of the comparisons made) related to maternal age, embryo day 3 and day 5 morphology suggested that subtle changes in the positions of the chromosome domains in which they reside may have a functional significance hitherto undiscovered. The dynamics of nuclear organisation and its relationship to gene expression in the early human embryo clearly thus warrant further investigation, in particular if there are patterns that may indicate future development potential.

8.5.6. To assess whether nuclear organisation is affected by spreading technique by comparing current and previous data.

In this study, embryo spreading was performed using the Tween HCl methanol: acetic acid combination method. One clear observation was that embryo quality remains the same over the layers and that FISH quality improved after each layer. In almost all occasions embryos maintained their shape over the layers, and cell swelling did not occur, as reported by Ioannou with the Tween HCl method (Ioannou *et al.*, 2012).

In the final part of this study I compared the medial position of all chromosomes spread using the Tween HCl method (data from Ioannou thesis), with embryos spread using the combination method (table presented in appendix 10.4.3). Nuclear organisation did not significantly alter due to the different spreading methods. In the literature it has been reported that the methanol: acetic acid method gives the best results with large cell diameters, resulting in better signals with relatively less overlapping (Velilla *et al.*, 2002). It will be

interesting to see if information we obtain by analysing chromosome positions is improved when the embryos are fixed using the methanol: acetic acid method.

8.5.7. Future of embryo nuclear organisation

To sum up this study provide evidence for existence of the chromocentre in human embryos. Although the gross organisation of the nucleus remains largely the same regardless of chromosome abnormality, maternal age or embryo quality, loci on certain chromosomal loci are more likely to alterations in their nuclear address than others. This study only focuses of the organisation of centromere regions in human embryos. In future it would be interesting to see the positions of telomeres and sub telomeres in human embryo cells.

8.6. Role of FISH in future cytogenetics

With advanced and higher resolution techniques, the use of FISH in PGD is extremely doubtful. However in favour to FISH, a recent study by Fragouli et al (2012) provided some evidence for higher concordance of FISH and other high resolution techniques. This study analyse human blastocysts using FISH, CGH and array CGH and report that there is a good concordance between all three methods, i.e. concordance between CGH and FISH is 94% and between array CGH and FISH is 100%. Using FISH, this study tested for Chromosomes 13, 15, 16, 17, 18, 21, 22, X and Y along with any other chromosomes that had given an abnormal CGH and/or aCGH result including chromosome 3, 4, 6, 7, 8, 9, 11, 12 and 20(Fragouli *et al.*, 2011). Another study by Munne and colleagues, used 12-chromosome screen to compare screening efficiency between FISH, CGH, aCGH and SNP microarrays (Munne *et al.*, 2010) and suggested that using a 10 and 12 probe panel the efficiency of detecting aneuploid blastocysts was 89 and 91%. Study by Treff and colleagues preformed

randomized, blinded and pair wise comparison between microarray and FISH-based aneuploidy screening and reported that SNP based 24 chromosome aneuploidy screening provides more complete and consistent results than FISH (Treff *et al.*, 2010a). Authors also suggested that FISH may overestimate the chromosomal mosaicism (Treff *et al.*, 2010a).

Results from this thesis also agree the fact that FISH over estimate chromosome mosaicism. In addition to the previously described issues associated with FISH such as failure to hybridising, split and diffuse signals, probe and fluorochrome related issues (not been visible to human eye etc.) 24 FISH even more complicate the diagnosis by providing more information which can be more doubtful. Nowadays, especially with the presence of such high resolution molecular biology techniques, the survival of 24 FISH in clinical PGS cases are not realistic.

However in research, the use of FISH is still crucial. Therefore it will be important to validate the accuracy of 24 FISH perhaps with aid of array CGH and or with Karyomapping to ensure the results we getting are not due to technical issues. As FISH is a relatively economical technique and it will allow performing cell by cell analysis in follow up embryos, it may still prove to find the issues related to mosaicism still are not totally revealed. The other most important application is to find the relative nuclear addresses of chromosomes in interphase nuclei which have to be based on molecular cytogenetic technique. Nuclear organisation is a major interest in our laboratory. This thesis as well examined the nuclear organisation in sperm (specific aim 1 and 2) and embryos (specific aim 5). Also in the future it will be interesting to establish chromosome copy number and nuclear organisation in embryonic stem cells.

8.7. Concluding Remarks

This thesis attempted to provide an insight into nuclear organisation and aneuploidy of human sperm and embryos in relation to various criteria and medical conditions such as infertility, cancer, PGS indications, maternal age, and various parameters measuring embryo quality in the clinical setting. Small differences in certain chromosome positions related to the above variables were identified, however larger studies with much bigger sample size will be needed before final conclusions can be identified. For efficient and more accurate results, in the area of nuclear organisation, 2D FISH and microscopes should be replaced by automated and more advanced techniques such as suspension FISH, allowing large studies to be carried out in the future. Assessment of chromosome aneuploidy has already moved towards novel and high resolution techniques, such as array CGH and Karyomapping for diagnostic purposes. Perhaps the most disappointing aspect of this thesis was, despite developing a usable 24 chromosome FISH assay, it is still not entirely clear the extent to which the results obtained represent true biological phenomena of technical errors. Once array based approaches are used to validate the FISH method however and spreading/hybridization methods are optimised my belief is that FISH still has it place in reproductive medicine. As a screening/diagnostic approach is clearly has "had its day" but, as a means of determining the chromosome copy number of human embryos on a cell by cell basis and as a starting point for investigating the complexities of nuclear organisation in early human development, it has great potential. Such potential should not be ignored just because newer technologies (which, in truth would struggle to achieve this) happen to be available.

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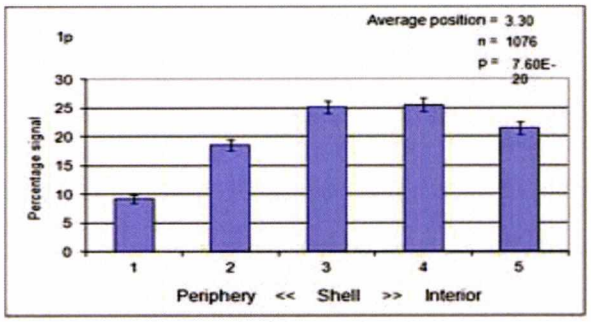
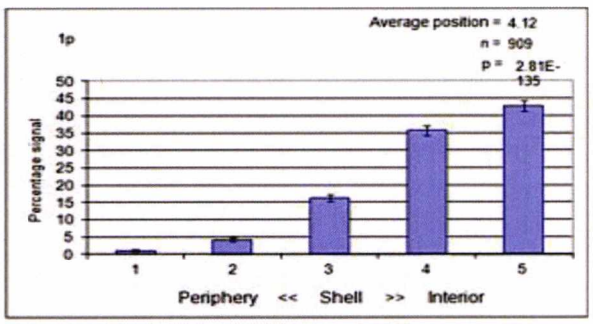
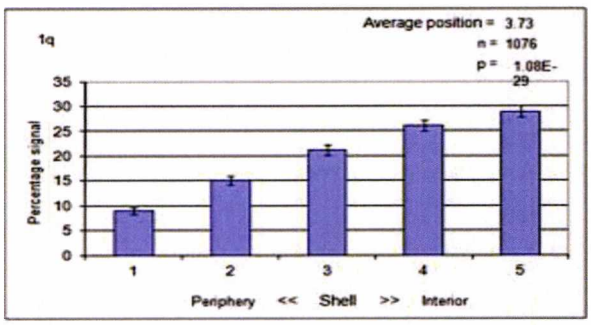
10. Appendix

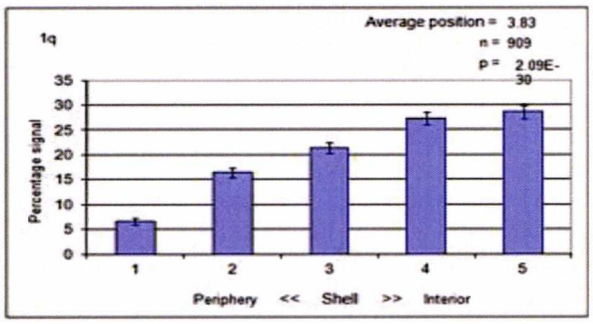
10.1. Specific aim 1

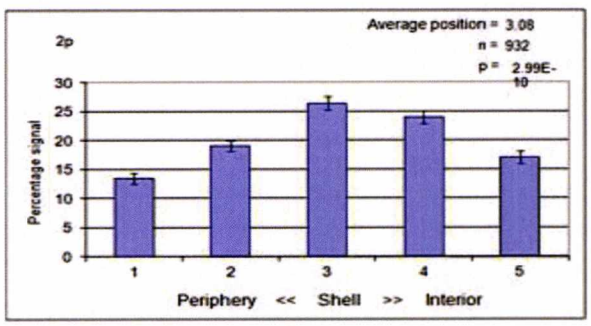
10.1.1.Pooled nuclear organisation of telomeres and sub telomeres (DAPI density model) for controls and OAT patients

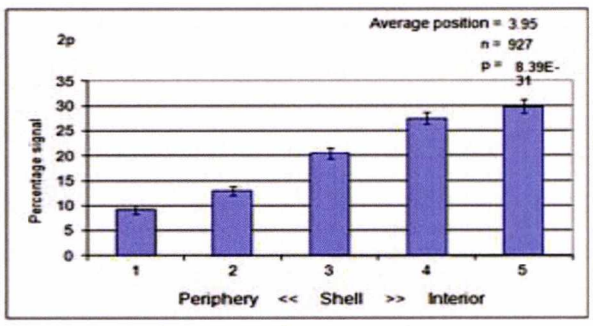


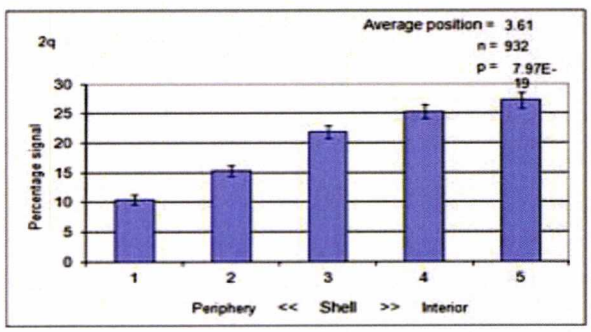


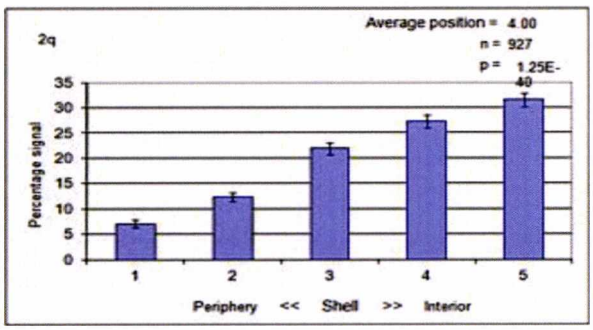


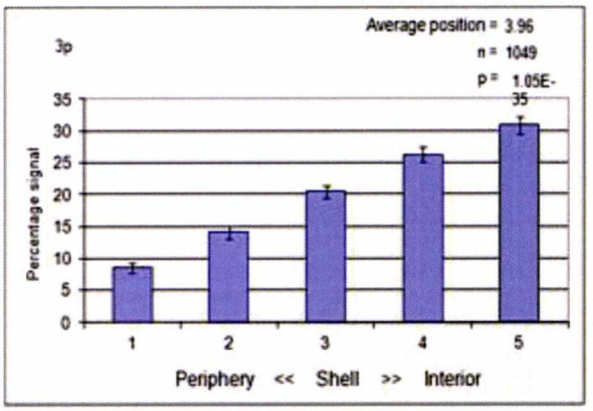








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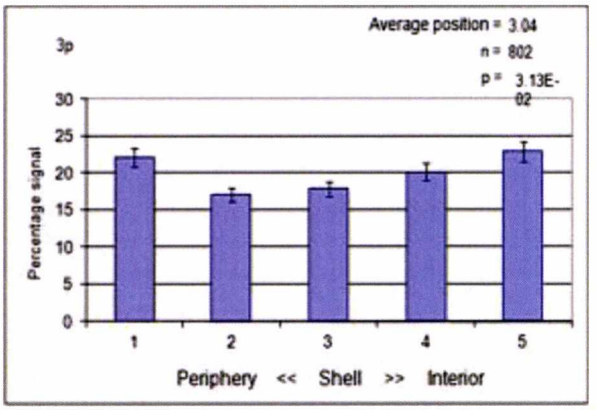
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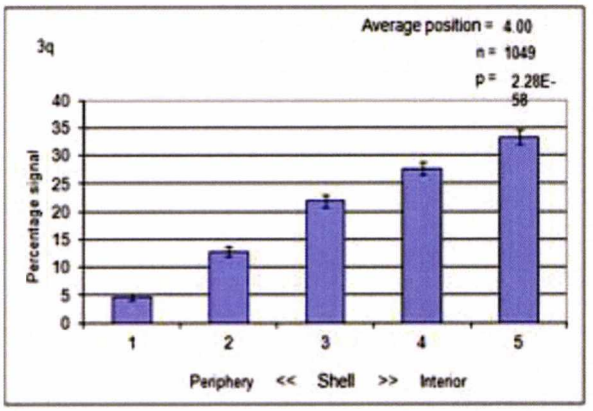
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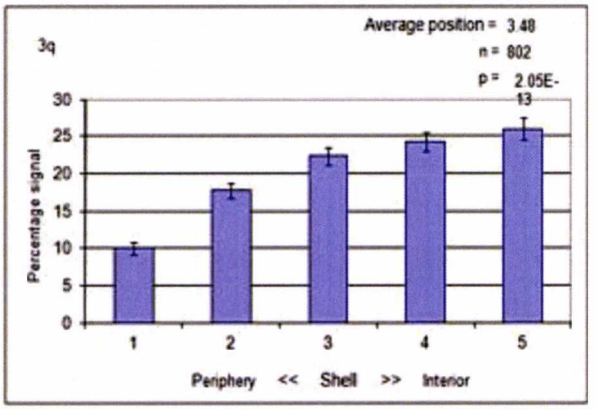
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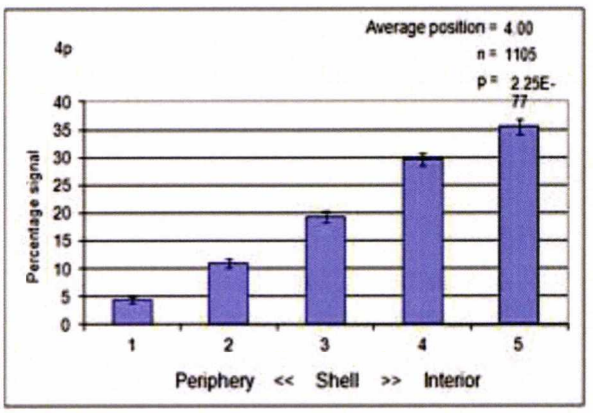
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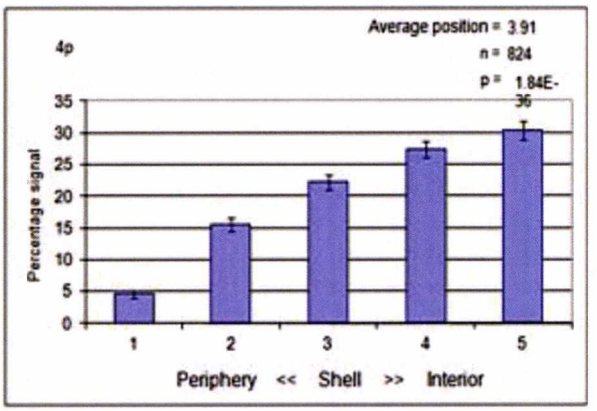
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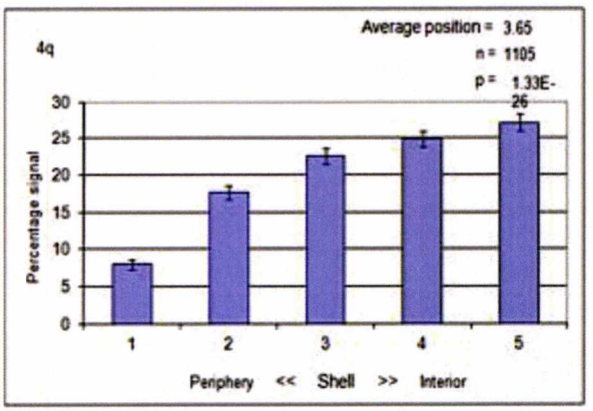
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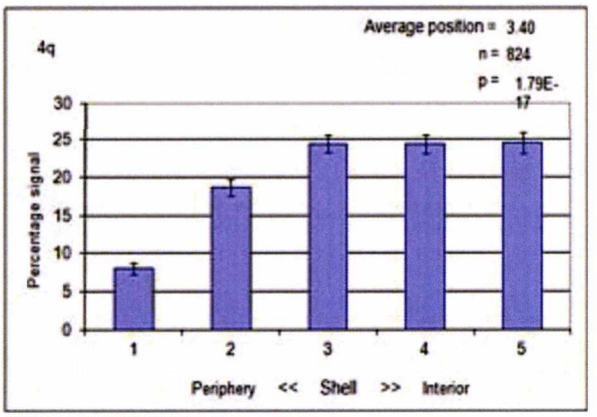
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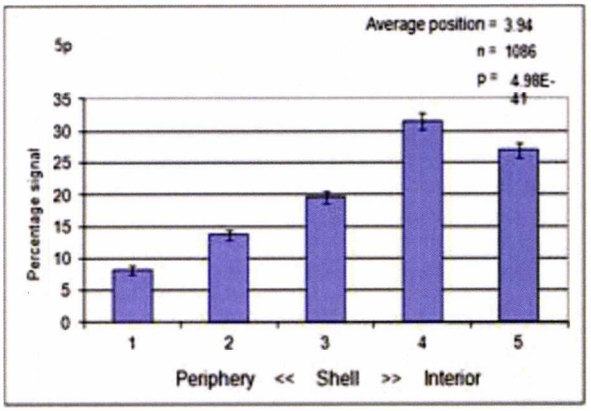
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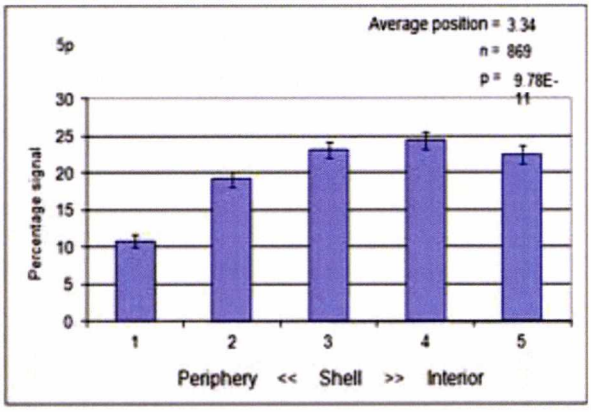
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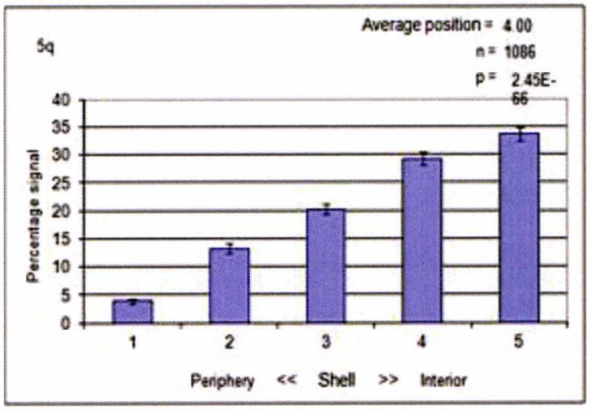
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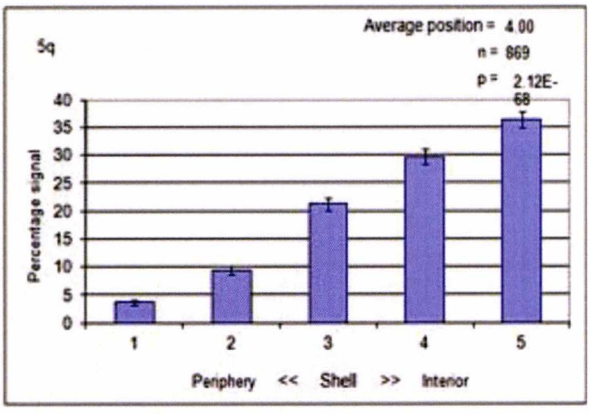
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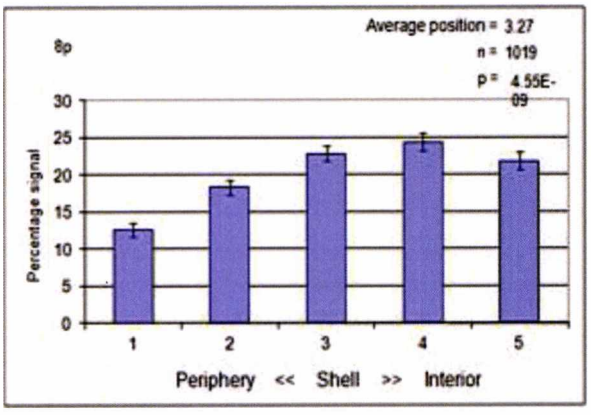
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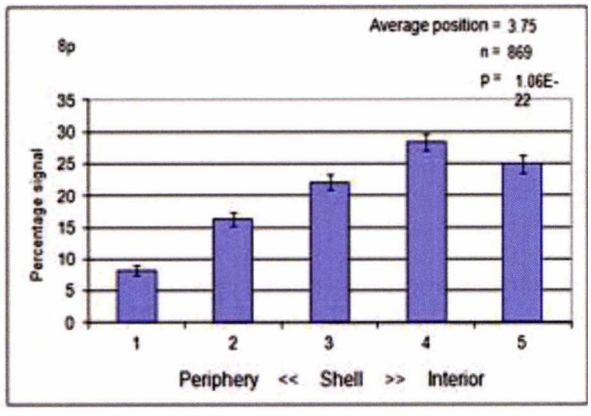
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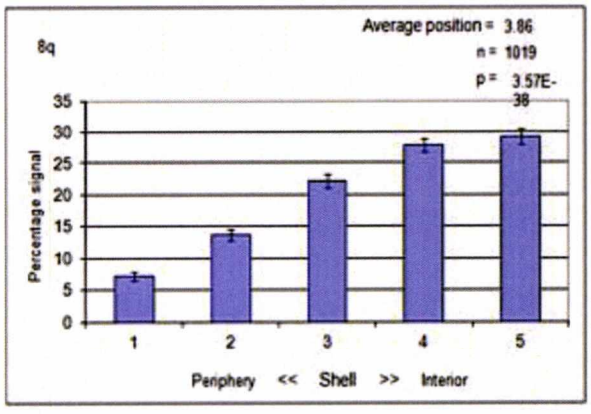
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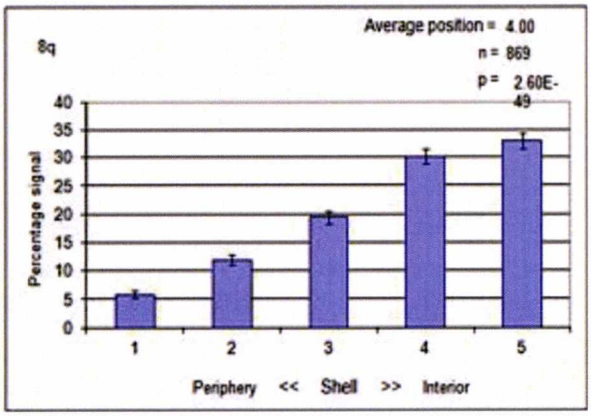
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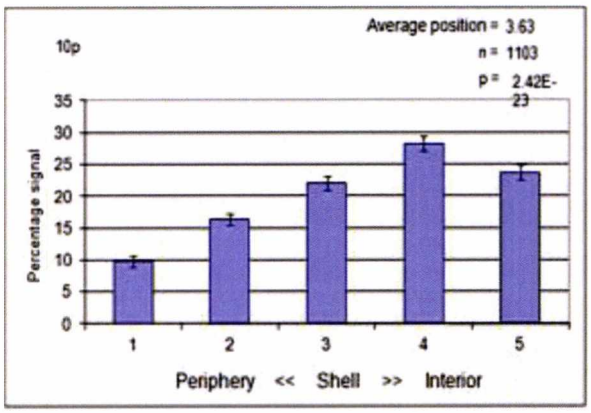
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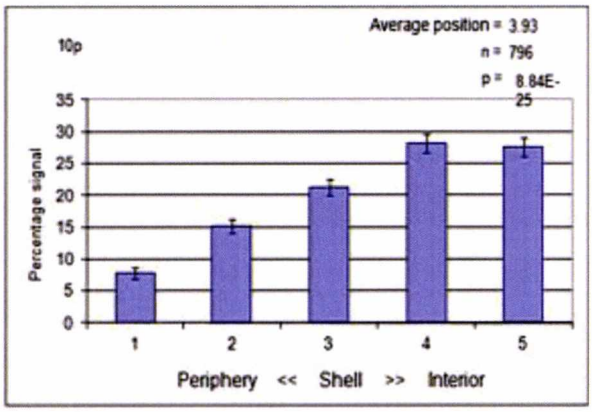
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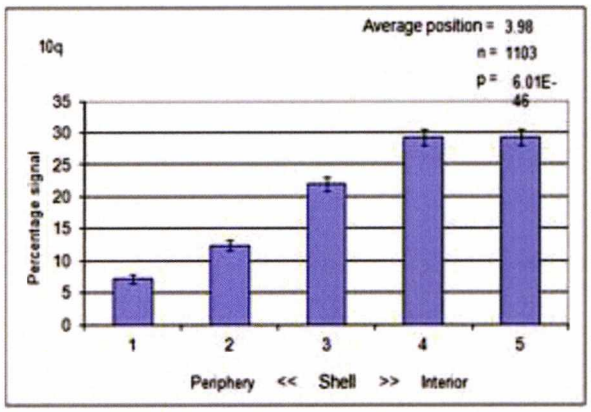
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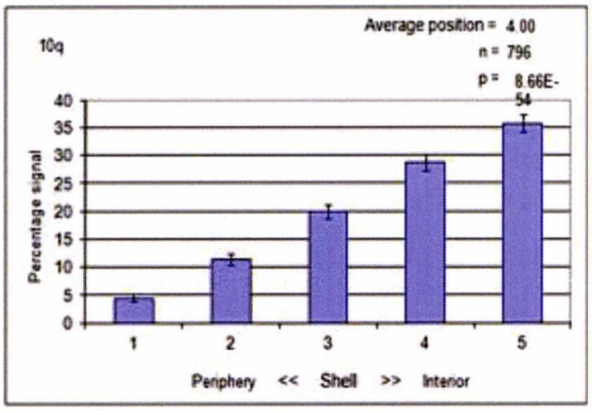
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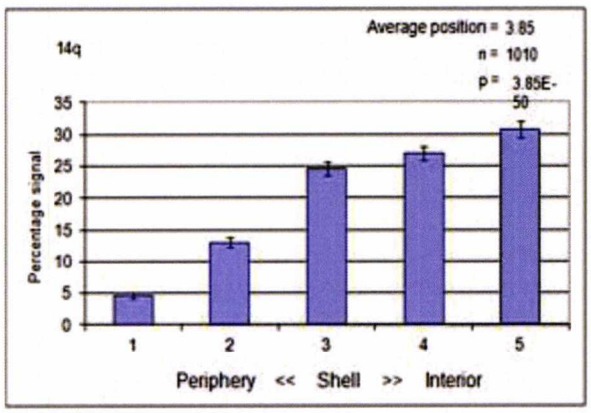
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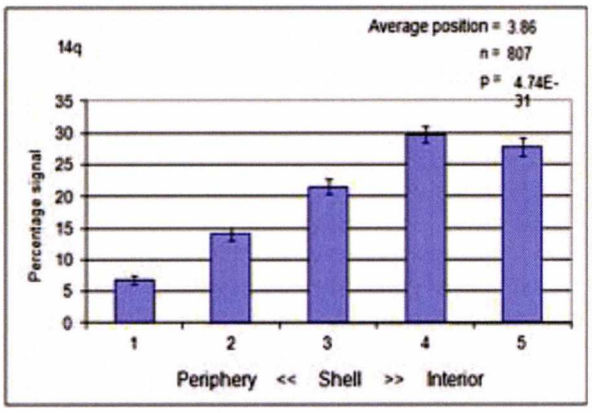
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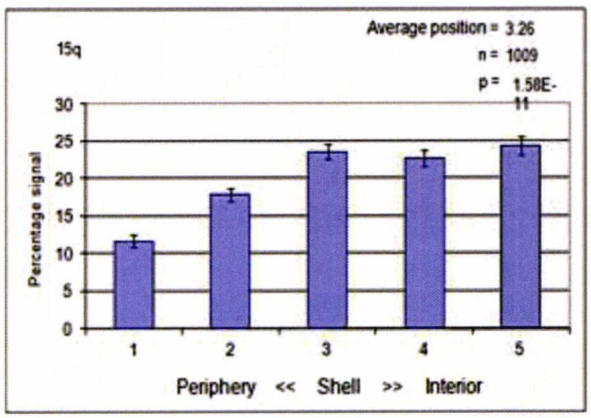
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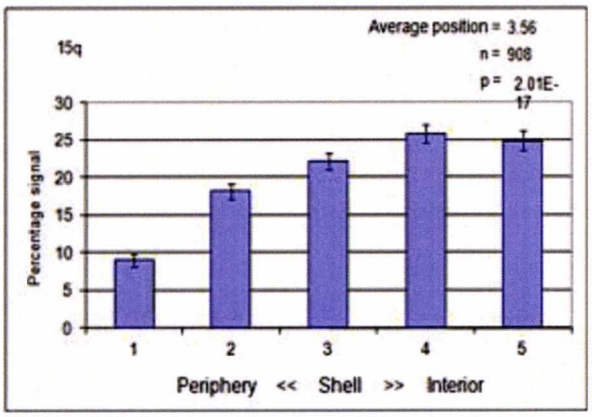
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Percentage signal
Periphery << Shell >> Interior

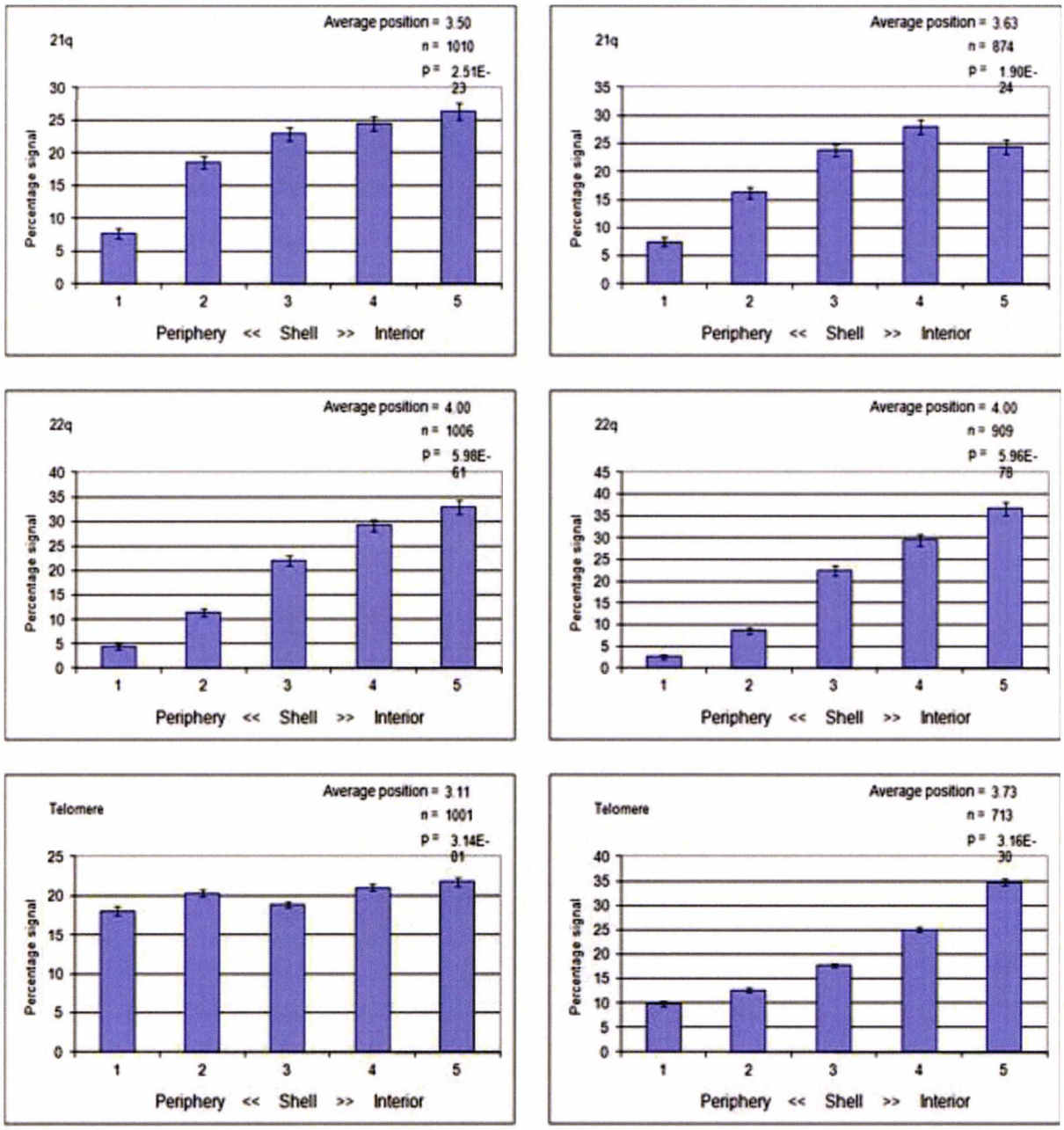
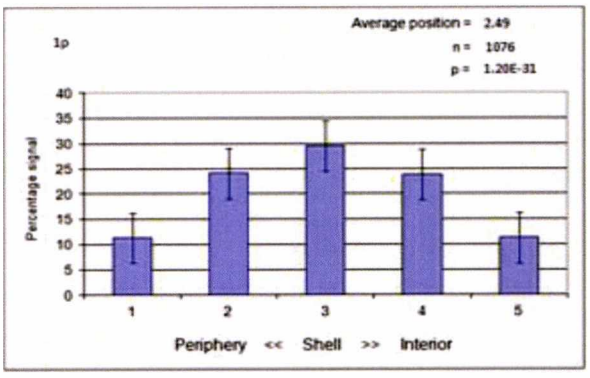
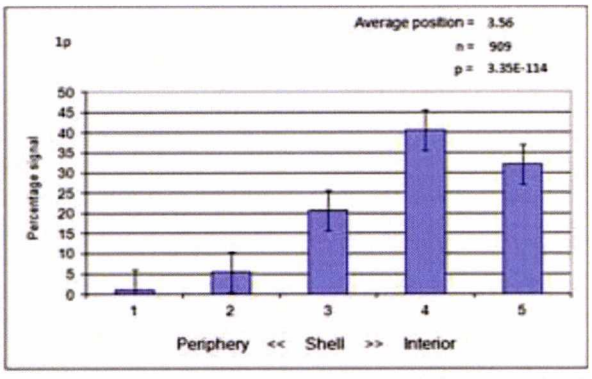
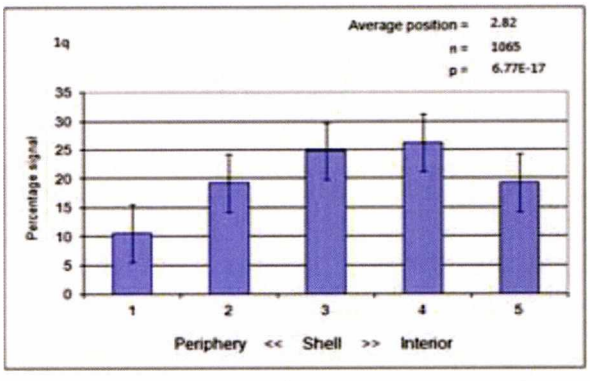


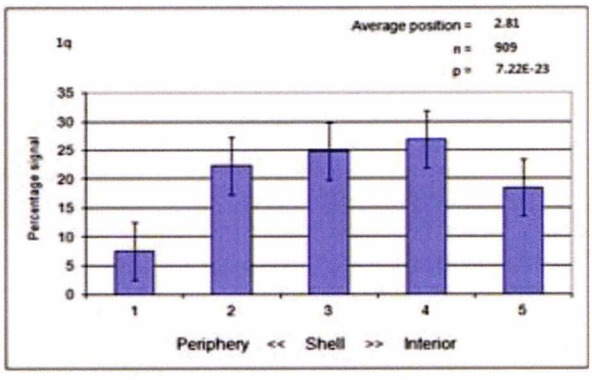
Figure 1: Pooled results for DAPI normalisation template, controls (left) vs. OAT patients (right). Graphs show preferential positions for 1pq, 2 pq, 3 pq, 4 pq, 5 pq, 8 pq, 10 pq, 14q, 15q, 21q, 22q and pan telomere chromosome loci (labelled in top left of each graph). Avarage positions, number of cells analysed and p values from chi squared test is given in the top right in each graph. When p value is less than 0.05 position for the chromosome loci analysed considered to be significant. When p value is higher than 0.05 distribution is considered as random.

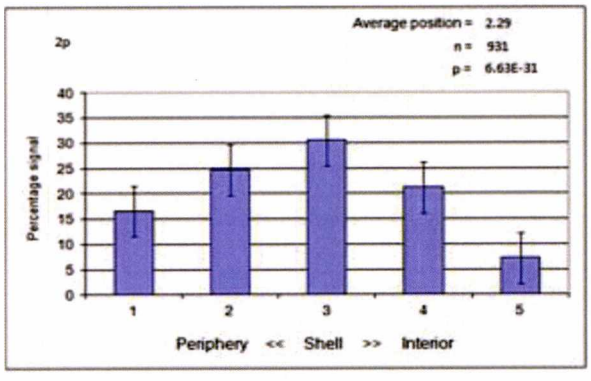
10.1.2.Pooled nuclear organisation of telomeres and sub telomeres (volumetric model) for controls and OAT patients

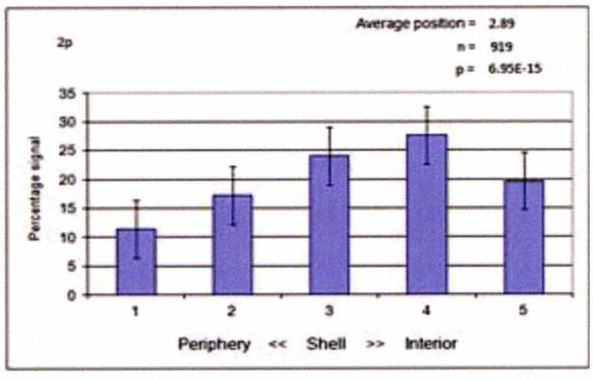


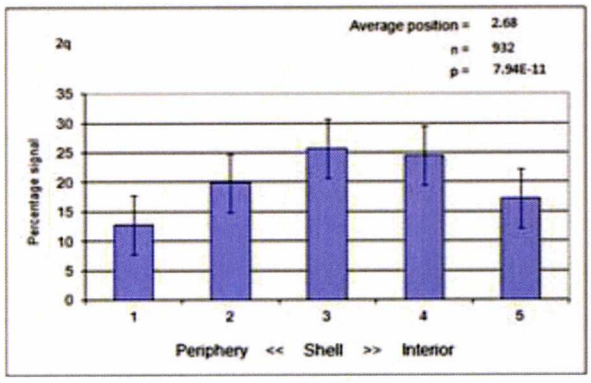


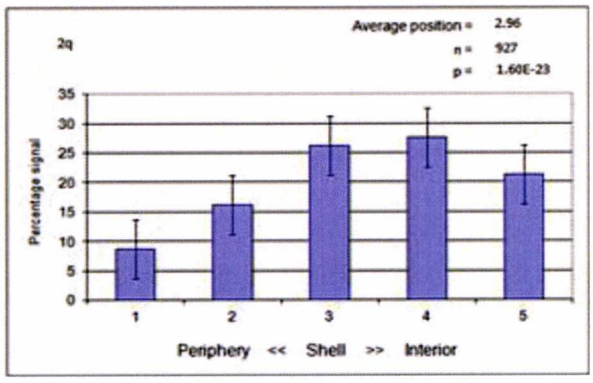


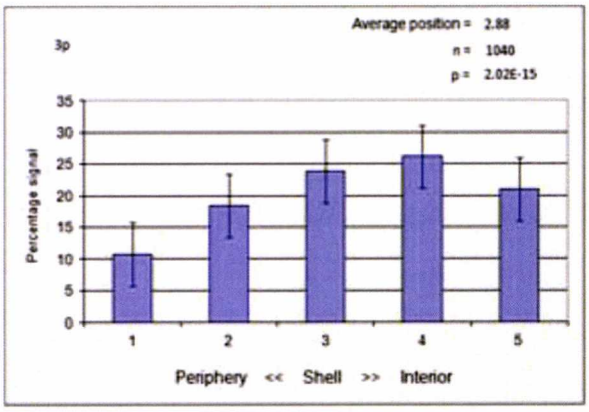








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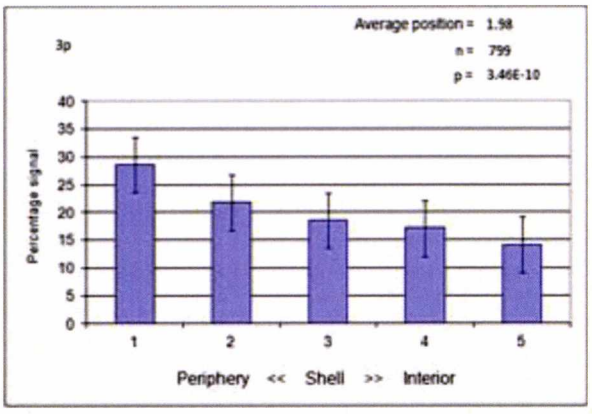
p = 2.02E-15
Percentage signal
Periphery << Shell >> Interior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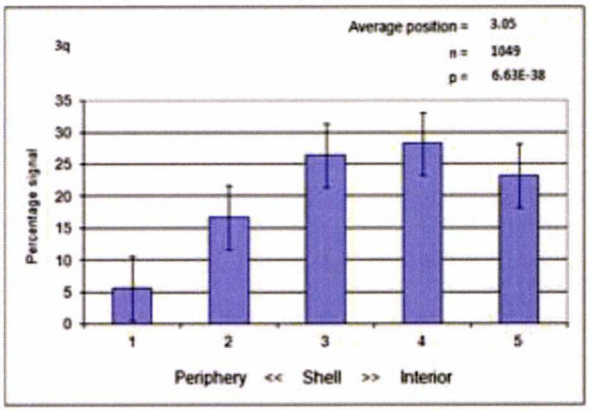
Percentage signal
Periphery << Shell >> Interior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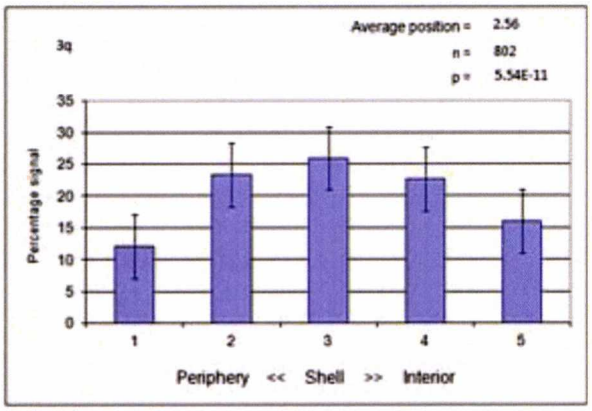
Percentage signal
Periphery << Shell >> Interior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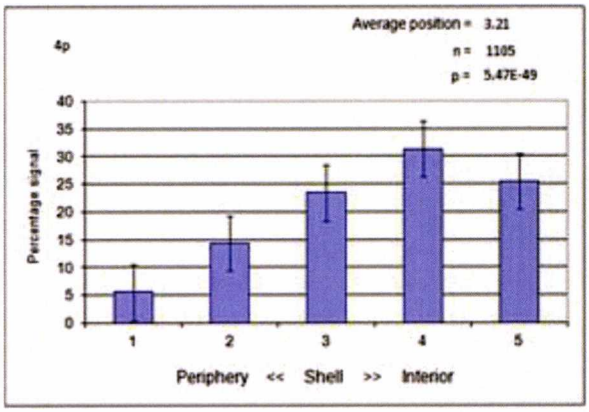
Average position = 2.56
n = 802
p = 5.54E-11
Percentage signal
Periphery << Shell >> Interior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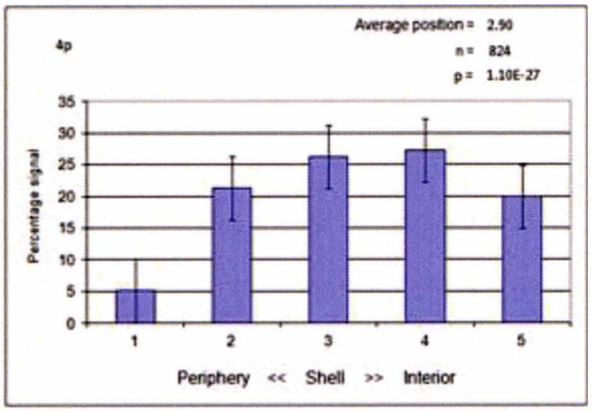
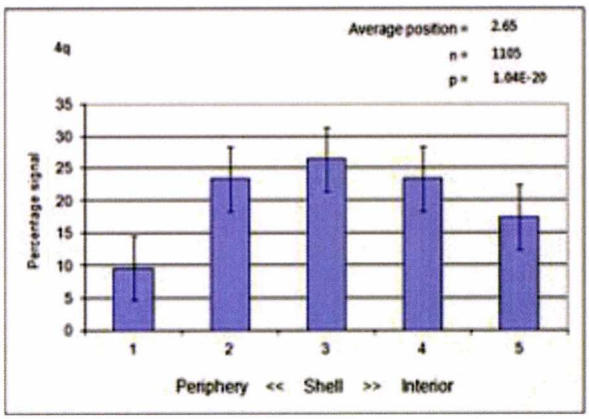
p = 5.47E-49
Percentage signal
Periphery << Shell >> Interior


4p
Average position = 2.90
n = 824
p = 1.10E-27
Percentage signal
Periphery << Shell >> Interior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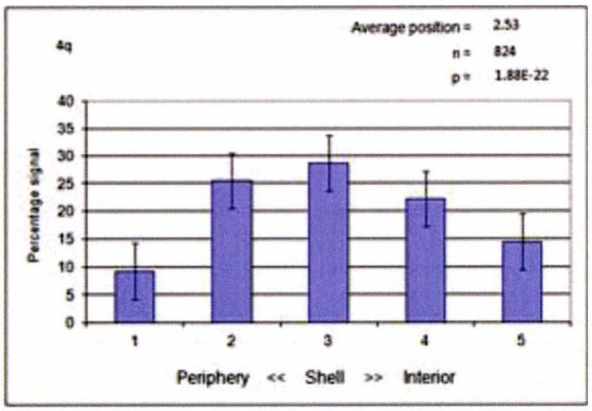
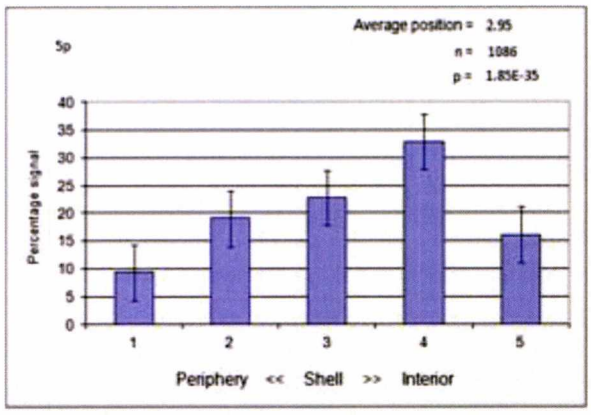
Average position = 2.65
n = 1105
p = 1.64E-20
Percentage signal
Periphery << Shell >> Interior


4q
Average position = 2.53
n = 824
p = 1.88E-22
Percentage signal
Periphery << Shell >> Interior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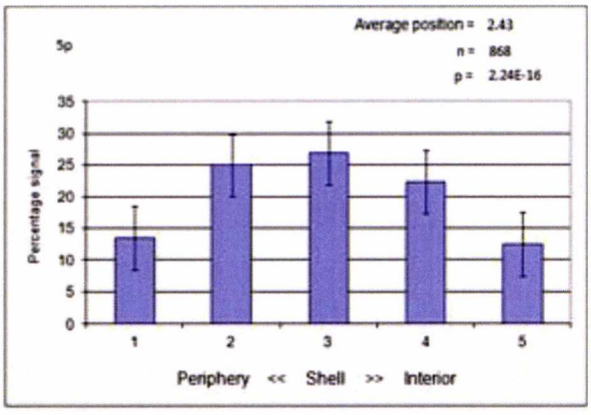
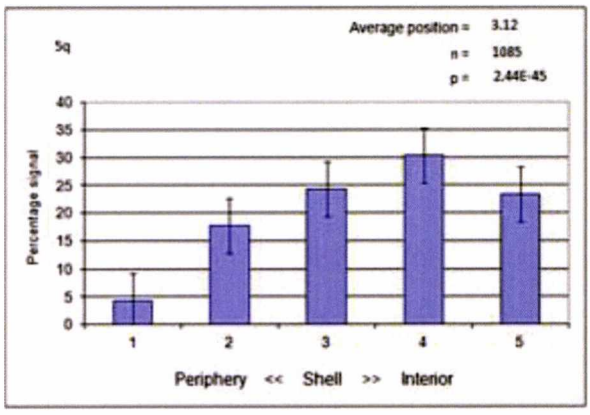
Average position = 2.95
n = 1086
p = 1.85E-35
Percentage signal
Periphery << Shell >> Interior


5p
Average position = 2.43
n = 868
p = 2.24E-16
Percentage signal
Periphery << Shell >> Interior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Average position = 3.12
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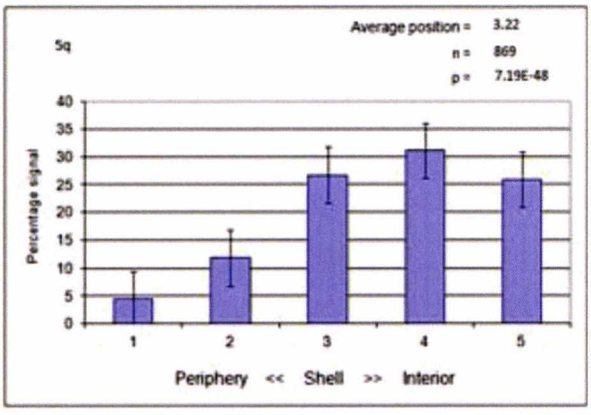
p = 2.44E-45
Percentage signal
Periphery << Shell >> Interior


5q
Average position = 3.22
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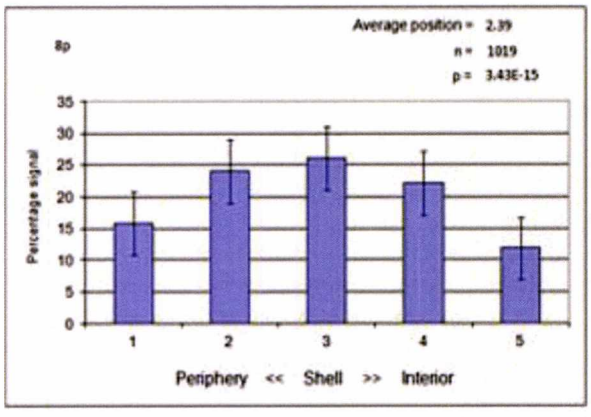
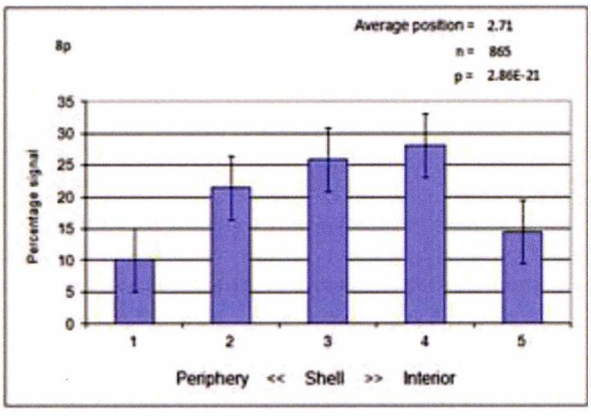
p = 7.19E-48
Percentage signal
Periphery << Shell >> Interior


8p
Average position = 2.39
n = 1019
p = 3.43E-15
Percentage signal
Periphery << Shell >> Interior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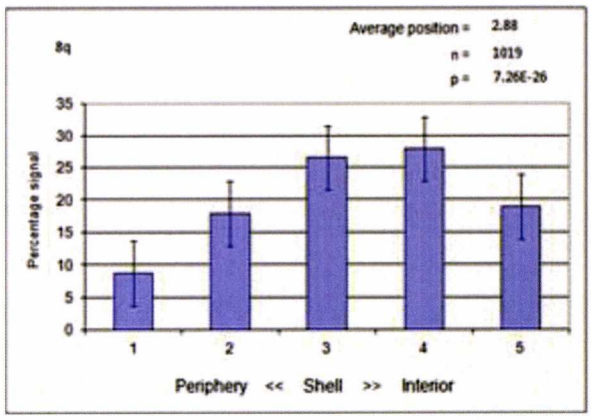
p = 2.86E-21
Percentage signal
Periphery << Shell >> Interior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Average position = 2.88
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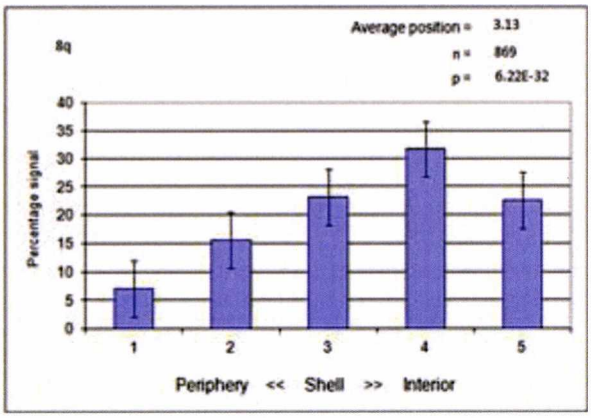
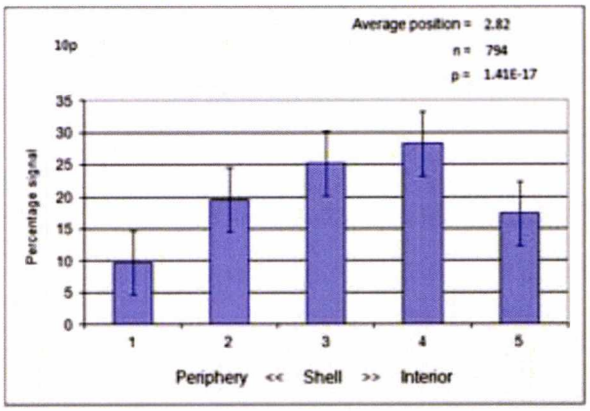
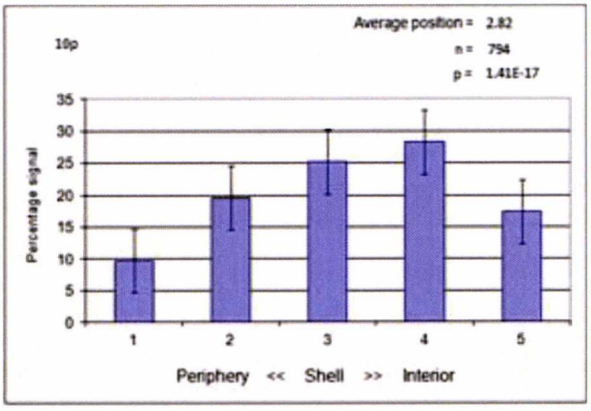
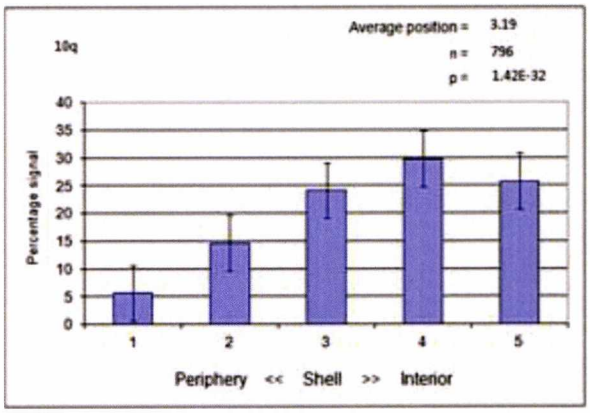
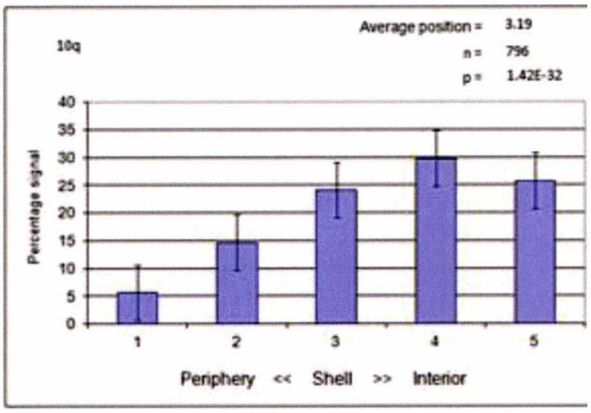
p = 7.26E-26
Percentage signal
Periphery << Shell >> Interior


8q
Average position = 3.13
n = 869
p = 6.22E-32
Percentage signal
Periphery << Shell >> Interior


10p
Average position = 2.82
n = 794
p = 1.41E-17
Percentage signal
Periphery << Shell >> Interior


10p
Average position = 2.82
n = 794
p = 1.41E-17
Percentage signal
Periphery << Shell >> Interior


10q
Average position = 3.19
n = 796
p = 1.42E-32
Percentage signal
Periphery << Shell >> Interior


10q
Average position = 3.19
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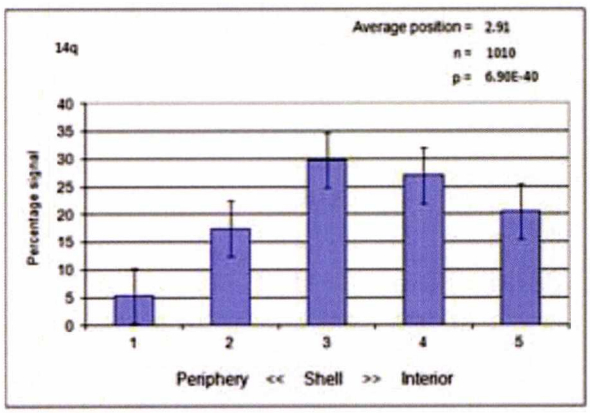
p = 1.42E-32
Percentage signal
Periphery << Shell >> Interior


14q
Average position = 2.91
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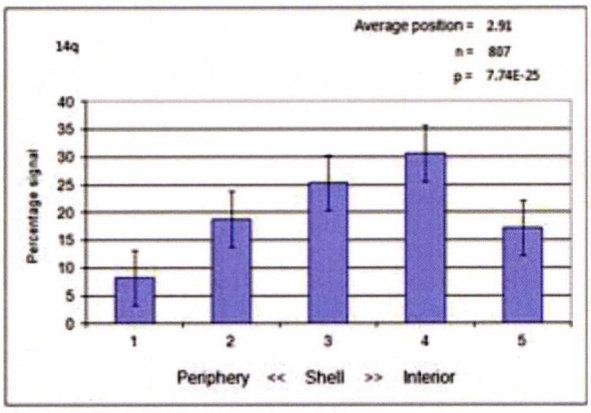
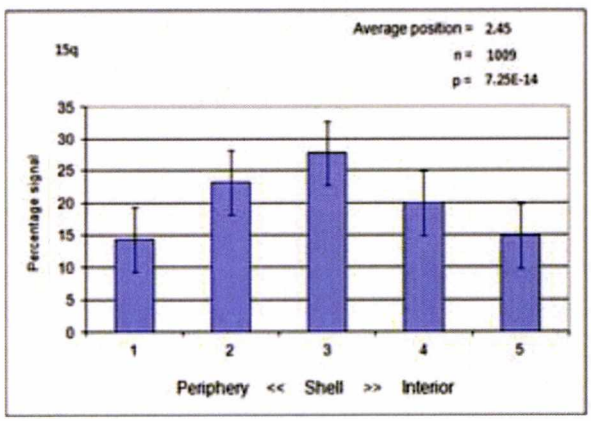
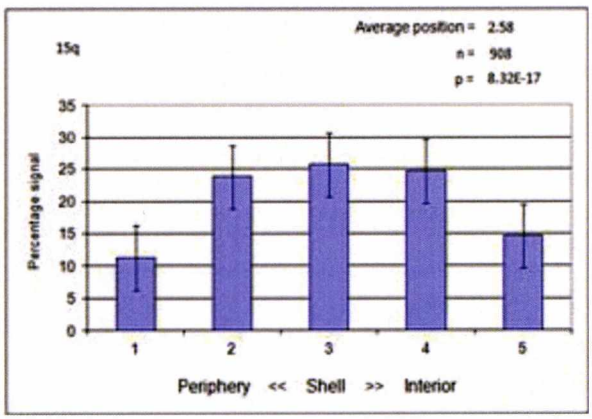
p = 6.90E-40
Percentage signal
Periphery << Shell >> Interior


14q
Average position = 2.91
n = 807
p = 7.74E-25
Percentage signal
Periphery << Shell >> Interior


15q
Average position = 2.45
n = 1009
p = 7.25E-14
Percentage signal
Periphery << Shell >> Interior


15q
Average position = 2.58
n = 908
p = 8.32E-17
Percentage signal
Periphery << Shell >> Interior

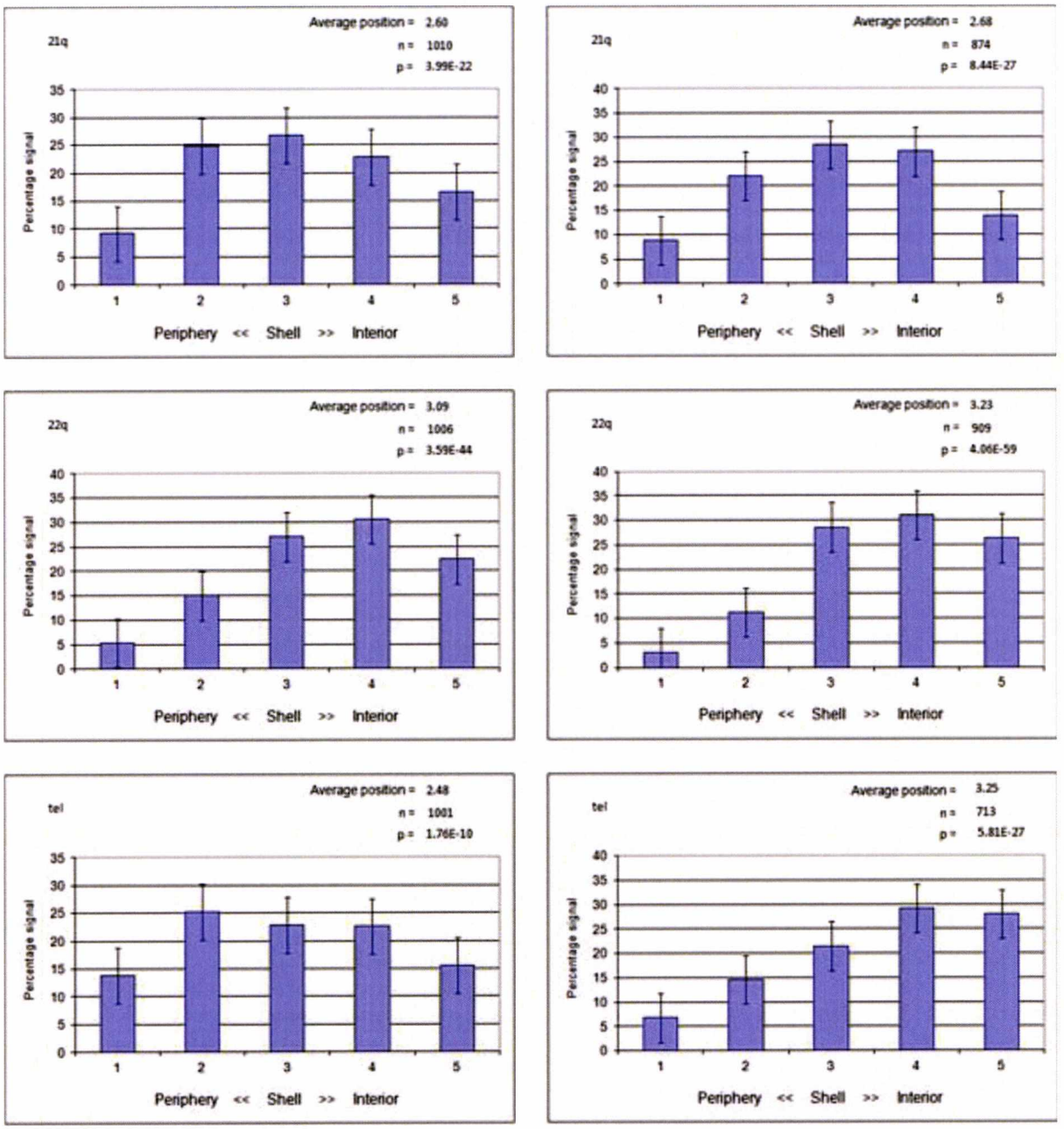


Figure 2: Pooled results for volumetric template, controls (left) vs. OAT patients (right).
Graphs show preferential positions for 1pq, 2 pq, 3 pq, 4 pq, 5 pq, 8 pq, 10 pq, 14q, 15q, 21q, 22q and pan telomere chromosome loci (labelled in top left of each graph). Avarage positions, number of cells analysed and p values from chi squared test is given in the top right in each graph. When p value is less than 0.05 position for the chromosome loci analysed considered to be significant. When p value is higher than 0.05 distribution is considered as random.

10.1. Specific aim 2

10.1.1. Volumetric analysis for autosomes

Table 1: Volumetric analysis for chromosome 1(centromere), 13 & 21(locus specific probes)

	Patient code	0 Month	6 Month	12 Month	18 Month	24 Month
Chromosome 1	1	Central	Central	Central	Central	
	2			Central	Medial	
	3	Central		Central	Central	
	4	Central	Central	Central	Central	
	5	Central	Central	Central		
	6	Central	Central	Central		
	7	Central	Central			
	8	Central		Central		
	9	Central	Central	Central		
	10	Central				
	TC1	Central	Central	Central		
	TC2	Central	Central		Central	Central
	TC3	Central			Central	
	TC4	Central	Central		Central	
	TC5			Central		
	HD1	Central	Central			Central
	HD2	Central	Central	Central		
	HD3	Central	Central	Central	Central	
	HD4	Central				
	HD5	Medial	Medial			
Chromosome 13	1	Central	Medial	Medial	Medial	
	2			Medial	Medial	
	3	Medial	Medial	Medial	Medial	
	4	Medial		Medial	Medial	
	5	Medial	Medial	Medial		
	6	Medial	Central/Medial	Medial		
	7		Medial	Medial		
	8	Medial		Medial		
	9	Central/Medial	Medial	Medial		
	10	Medial				
	TC1	Medial	Medial	Medial		
	TC2	Medial	Medial		Central	Medial
	TC3	Random			Medial	
	TC4	Medial	Medial	Medial		
	TC5			Medial		
	HD1	Medial	Medial			Central/Medial
	HD2	Medial	Medial	Central		
	HD3	Medial	Medial	Medial	Central/Medial	
	HD4	Medial				
	HD5	Central/Medial	Medial			
Chromosome 21	1	Central	Medial	Medial	Central/Medial	
	2			Medial	Medial	
	3	Medial	Medial	Medial	Medial	
	4	Medial		Medial	Medial	
	5	Medial	Central/Medial	Medial		
	6	Medial	Central/Medial	Medial		
	7		35bi	Medial		
	8	Medial		Medial		
	9	Medial	Medial	Medial		
	10	Central				
	TC1	Medial	Peripheral	Medial		
	TC2	Central/Medial	Central/Medial		Medial	Medial
	TC3	Medial			Medial	
	TC4	Central/Medial	Central/Medial	Medial		
	TC5			Peripheral/Medial		
	HD1	Medial	Medial			Medial
	HD2	Medial	Medial	Central		
	HD3	Medial	Medial	Medial	Medial	
	HD4	Medial				
	HD5	3 5 bi	Central/Medial			

Nuclear organisation of controls, Testicular cancer and Hodgkin's lymphoma patients at different time points; 0 months refers to after diagnosis of cancer before any treatments, 6, 12 and 18-24 months refers to number of months after completion of treatments. Controls and patients with different organisation patterns for all of the autosomal chromosome loci analysed; Peripheral – Shell 1 or 1/2, Peripheral/Medial – Shell 2 or 1-3, Medial – Shell 3, 2/3, or 3/4, Central/Medial – Shell 4 or 3-5, Central – Shell 5 or 4/5.

10.1.2.Volumetric analysis for sex chromosomes

Table 4: volumetric analysis for chromosome X &Y (centromeres)

	Patient code	0 Month	6 Month	12 Month	18 Month	24 Month
Chromosome X	1	Central	Central	Central	Central	
	2			Central	Central	
	3	Central		Central	Central	
	4	Central	Central	Central	Central	
	5	Central	Central	Central		
	6	Central	Central	Central		
	7	Central	Central			
	8	Central		Central		
	9	Central	Central	Central		
	10	Central				
	TC1	Central	Central	Central		
	TC2	Central	Central		Central	Central
	TC3	Central			Central	
	TC4	Central	Central		Central	
	TC5			Central		
	HD1	Central	Central			Central
	HD2	Central	Central	Central		
	HD3	Central	Central	Central	Central	
	HD4	Central				
	HD5	Medial	Medial			
Chromosome Y	1	Central	Central	Central	Central	
	2			Central	Central	
	3	Central		Central	Central	
	4	Central	Central	Central	Central	
	5	Central	Central	Central		
	6	Central	Central	Central		
	7	Central	Central			
	8	Central		Central		
	9	Central	Central	Central		
	10	Central				
	TC1	Central	Medial	Central		
	TC2	Central	Central		Central	Central
	TC3	Central			Central	
	TC4	Central	Central		Central	
	TC5			Central		
	HD1	Central	Central			Central
	HD2	Central	Central	Central		
	HD3	Central	Central	Central	Central	
	HD4	Central				
	HD5	Medial	Medial			

Nuclear organisation of controls, Testicular cancer and Hodgkin's lymphoma patients at different time points; 0 months refers to after diagnosis of cancer before any treatments, 6, 12 and 18-24 months refers to number of months after completion of treatments. Controls and patients with different organisation patterns for all of the sex chromosome loci analysed; Peripheral – Shell 1 or 1/2, Peripheral/Medial – Shell 2 or 1-3, Medial – Shell 3, 2/3, or 3/4, Central/Medial – Shell 4 or 3-5, Central – Shell 5 or 4/5.

10.1.1.Pooled nuclear organisation for chromosome 1, 13, 21, X and Y (using DAPI density and volumetric models) for controls, TC and HD patients.

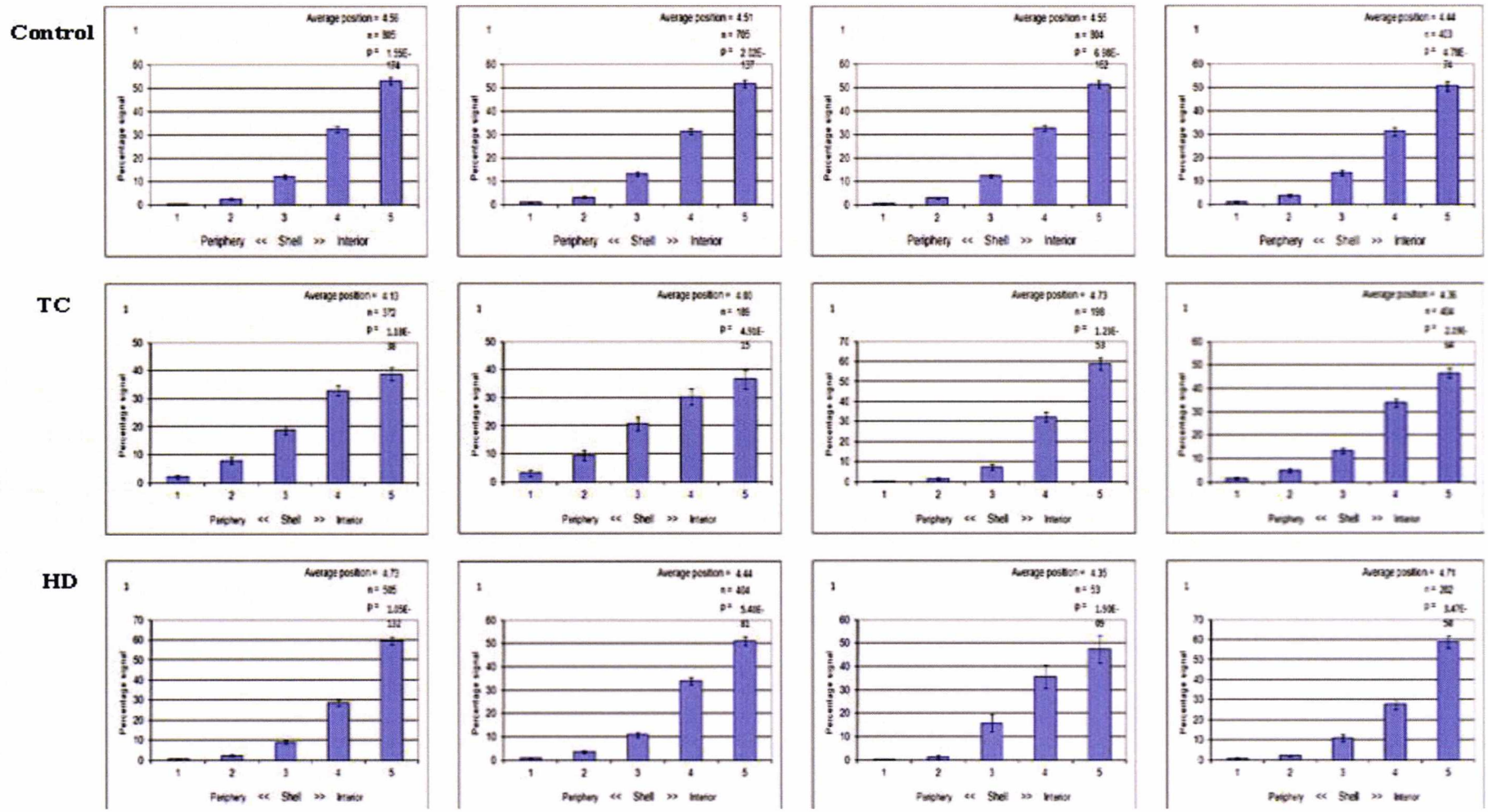


Figure 2: Pooled data analysis for chromosome 1(DAPI density model)
Nuclear organisation for 4 different time points for controls, TC and HD patients. 4 Graphs in each row represent 4 different time points as in 0 months (after cancer before any treatments), 6, 12, and 18-24 months after completion of treatments. Avarage nuclear position for each chromosome, number of cells analysed, and p values from chi squared test are presented in the top right. When $p \leq 0.05$ distribution is considered to be significant.

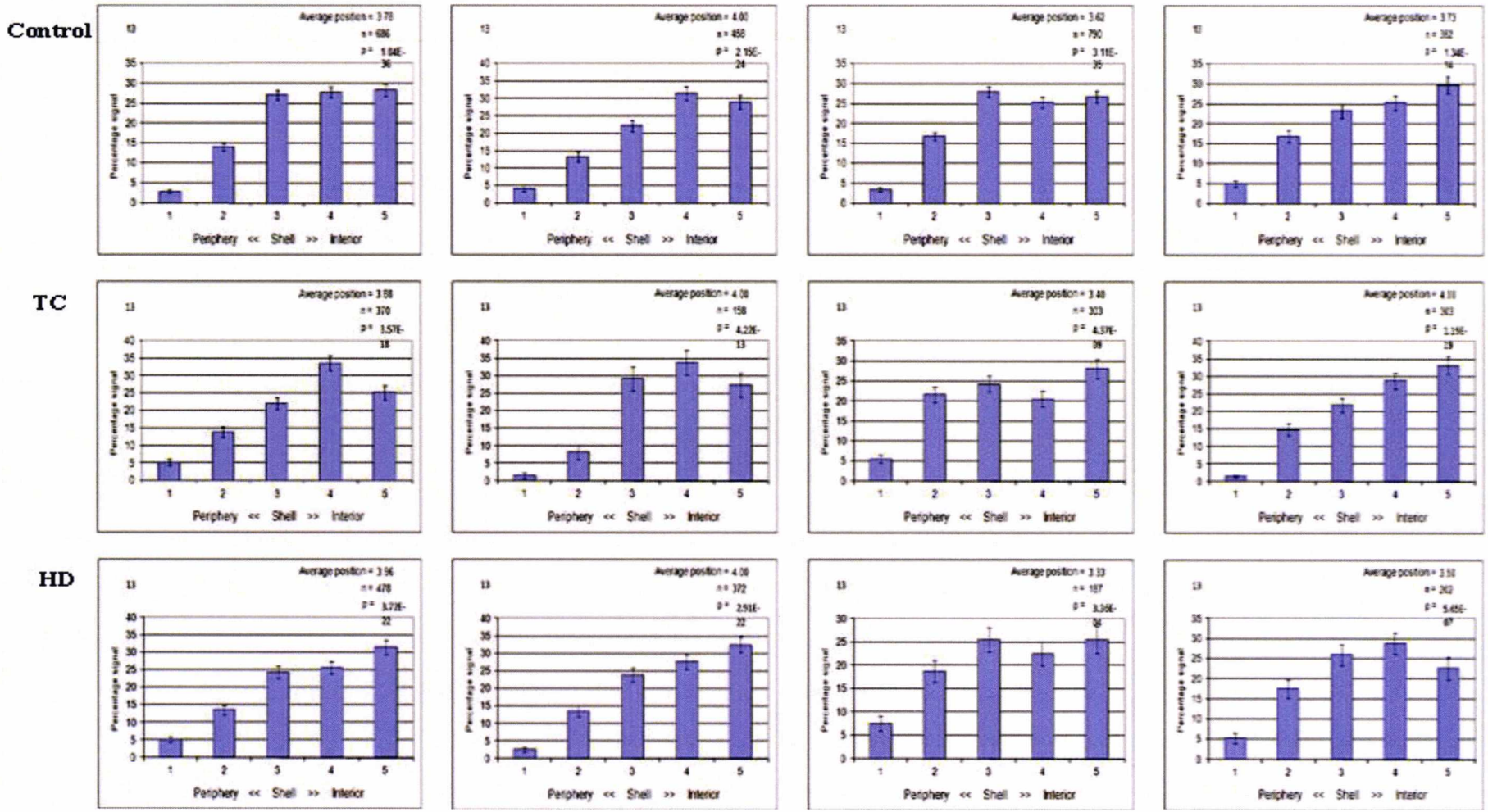


Figure 3: Pooled data analysis for chromosome 13 (DAPI density model)
Nuclear organisation for 4 different time points for controls, TC and HD patients. 4 Graphs in each row represent 4 different time points as in 0 months (after cancer before any treatments), 6, 12, and 18-24 months after completion of treatments. Avarage nuclear position for each chromosome, number of cells analysed, and p values from chi squared test are presented in the top right. When $p \leq 0.05$ distribution is considered to be significant.

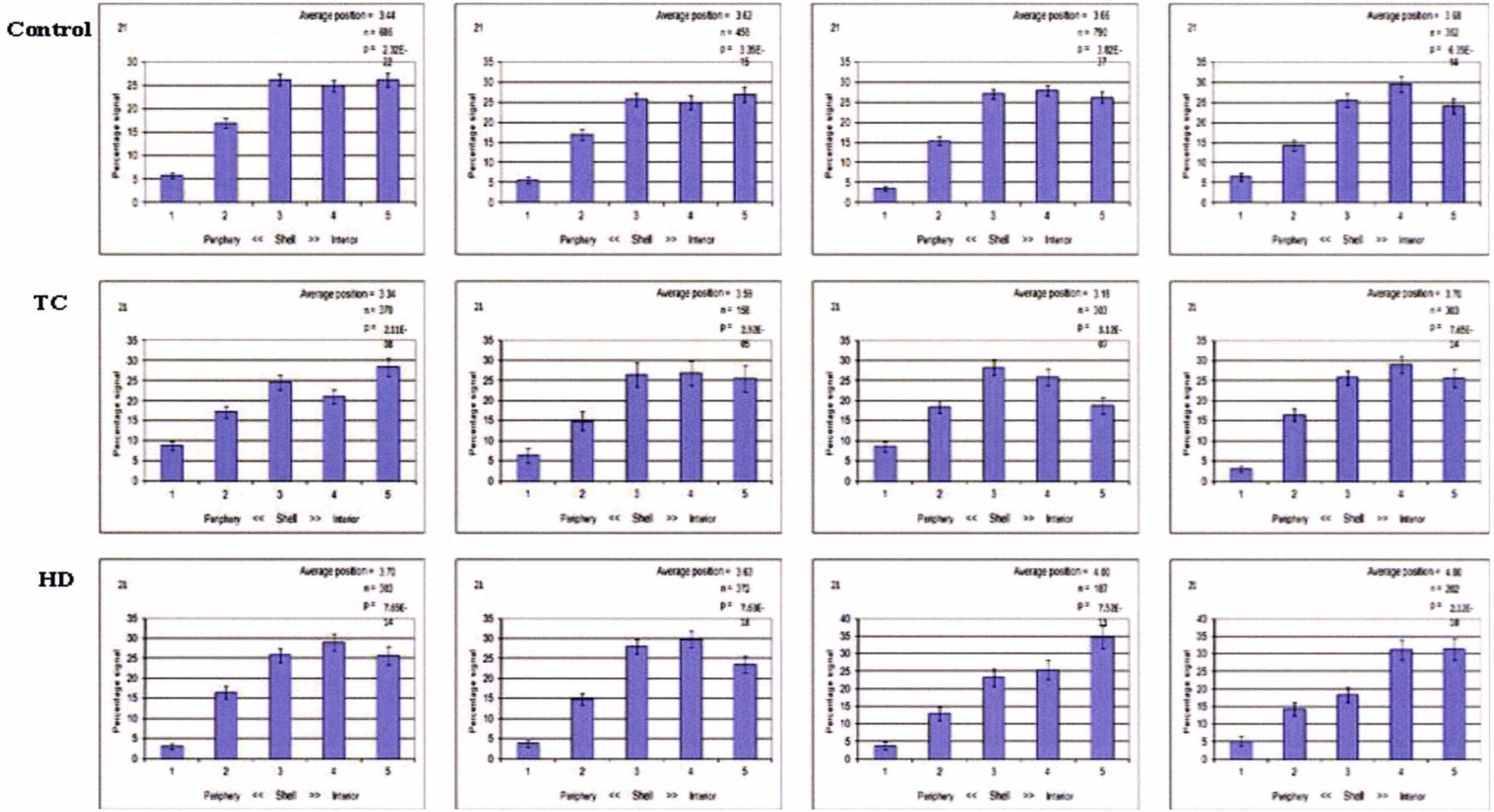


Figure 4: Pooled data analysis for chromosome 21(DAPI density model)
Nuclear organisation for 4 different time points for controls, TC and HD patients. 4 Graphs in each row represent 4 different time points as in 0 months (after cancer before any treatments), 6, 12, and 18-24 months after completion of treatments. Avarage nuclear position for each chromosome, number of cells analysed, and p values from chi squared test are presented in the top right. When $p \leq 0.05$ distribution is considered to be significant.

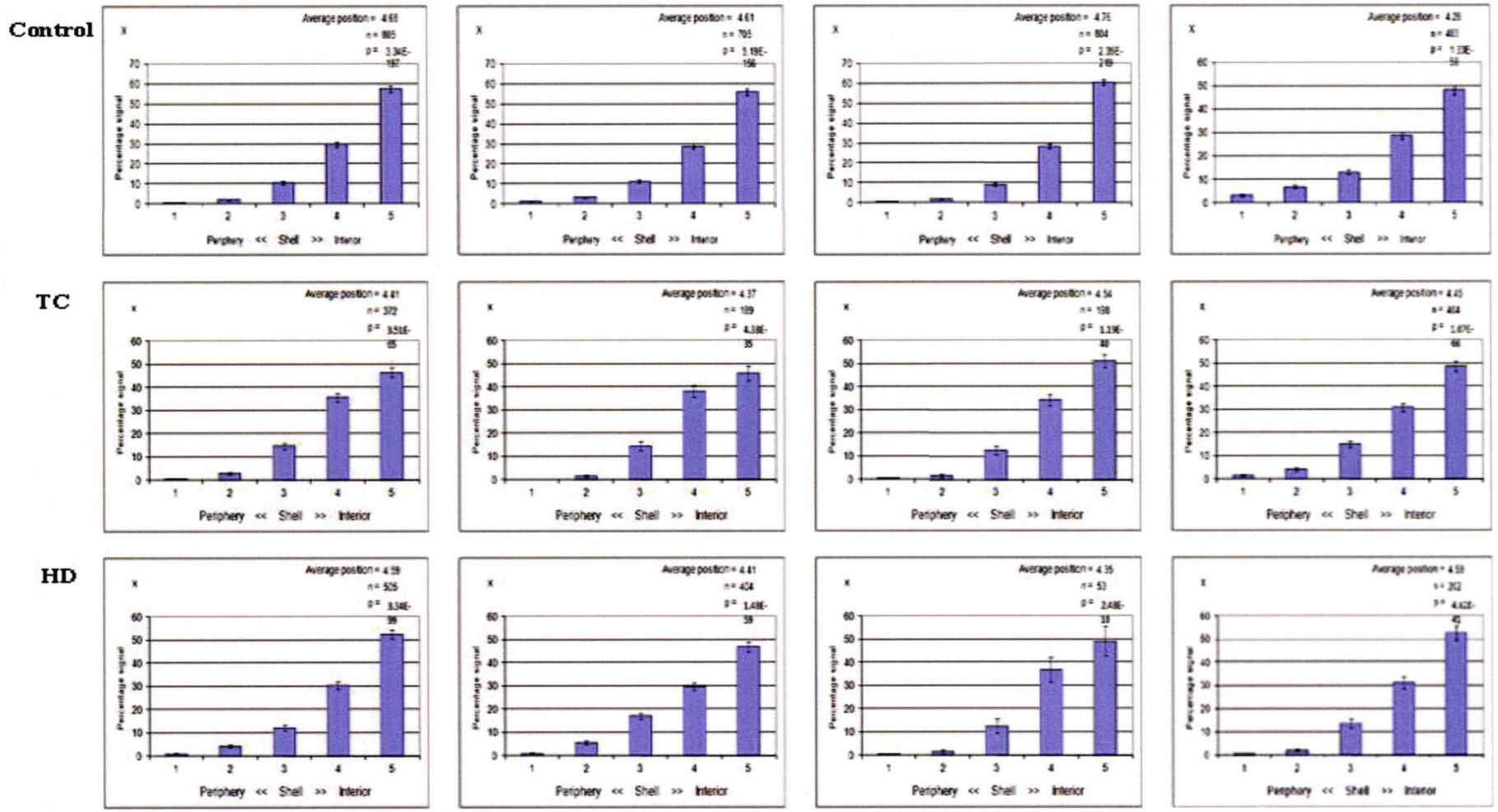


Figure 5: Pooled data analysis for chromosome X (DAPI density model)
Nuclear organisation for 4 different time points for controls, TC and HD patients. 4 Graphs in each row represent 4 different time points as in 0 months (after cancer before any treatments), 6, 12, and 18-24 months after completion of treatments. Avarage nuclear position for each chromosome, number of cells analysed, and p values from chi squared test are presented in the top right. When $p \leq 0.05$ distribution is considered to be significant.

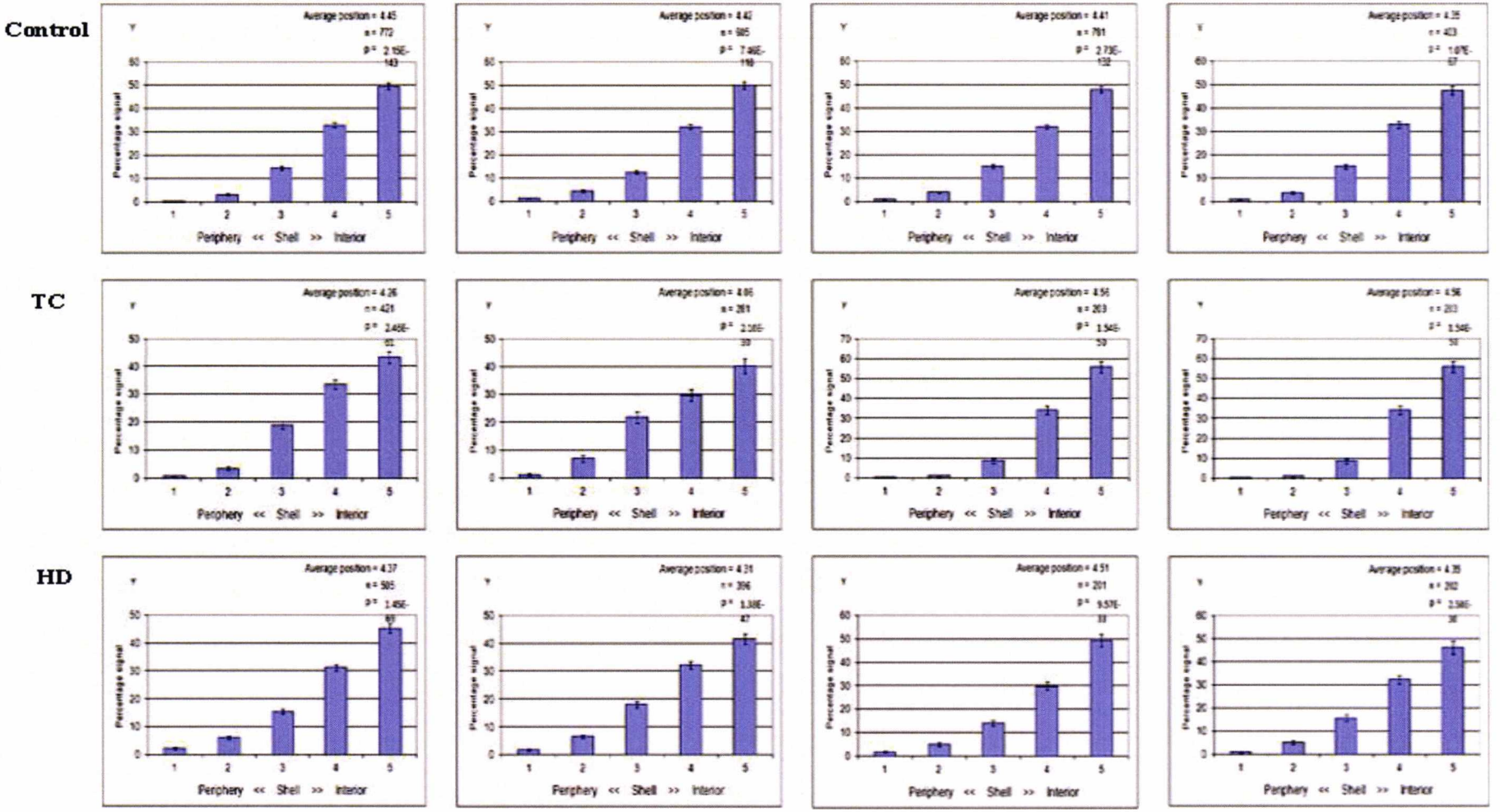


Figure 6:Pooled data analysis for chromosome Y (DAPI density model)
Nuclear organisation for 4 different time points for controls, TC and HD patients. 4 Graphs in each row represent 4 different time points as in 0 months (after cancer before any treatments), 6, 12, and 18-24 months after completion of treatments. Avarage nuclear position for each chromosome, number of cells analysed, and p values from chi squared test are presented in the top right. When $p \leq 0.05$ distribution is considered to be significant.

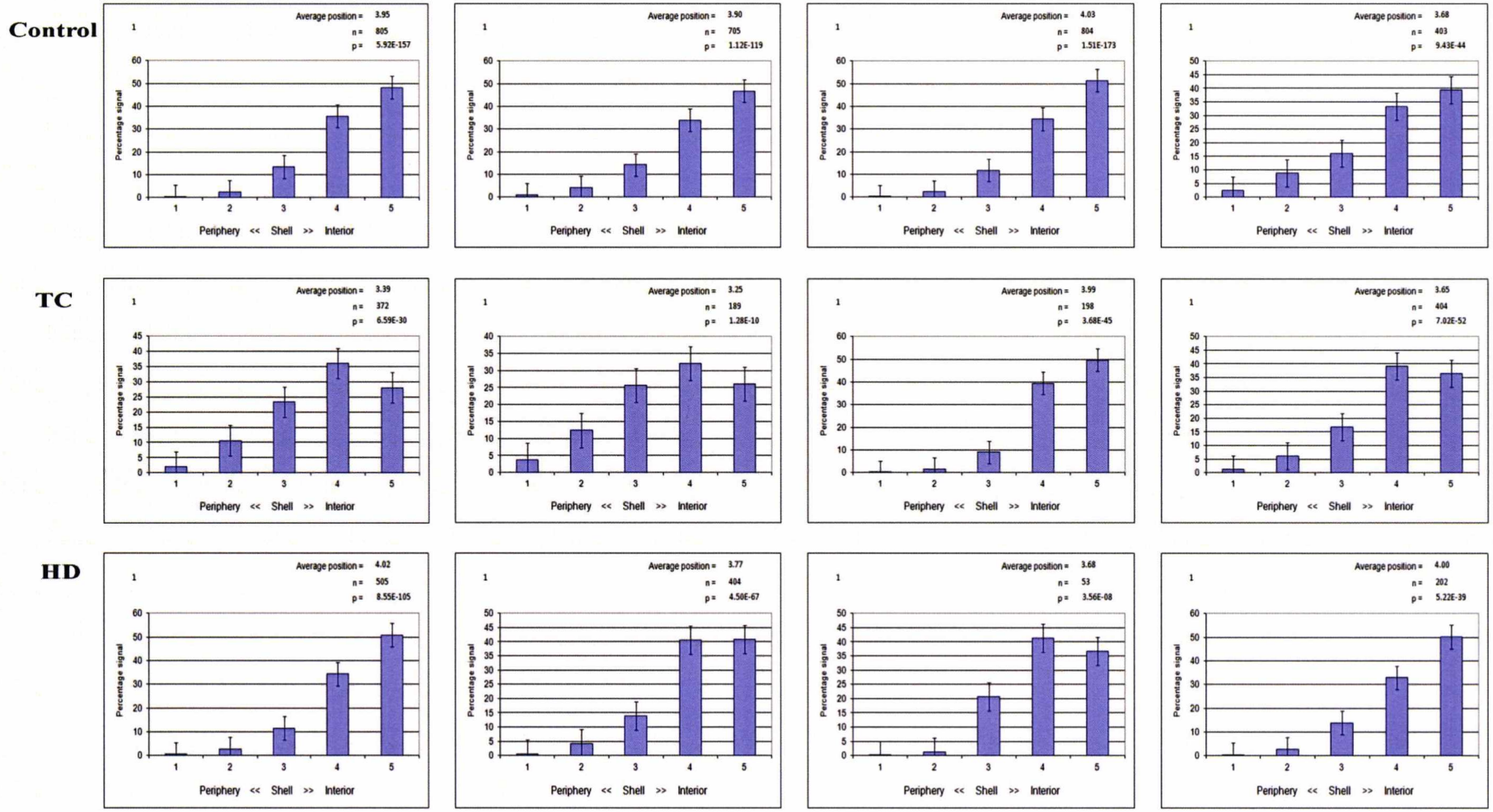


Figure 7: Pooled data analysis for chromosome 1(Volumetric model)
Nuclear organisation for 4 different time points for controls, TC and HD patients. 4 Graphs in each row represent 4 different time points as in 0 months (after cancer before any treatments), 6, 12, and 18-24 months after completion of treatments. Avarage nuclear position for each chromosome, number of cells analysed, and p values from chi squared test are presented in the top right. When $p \leq 0.05$ distribution is considered to be significant.

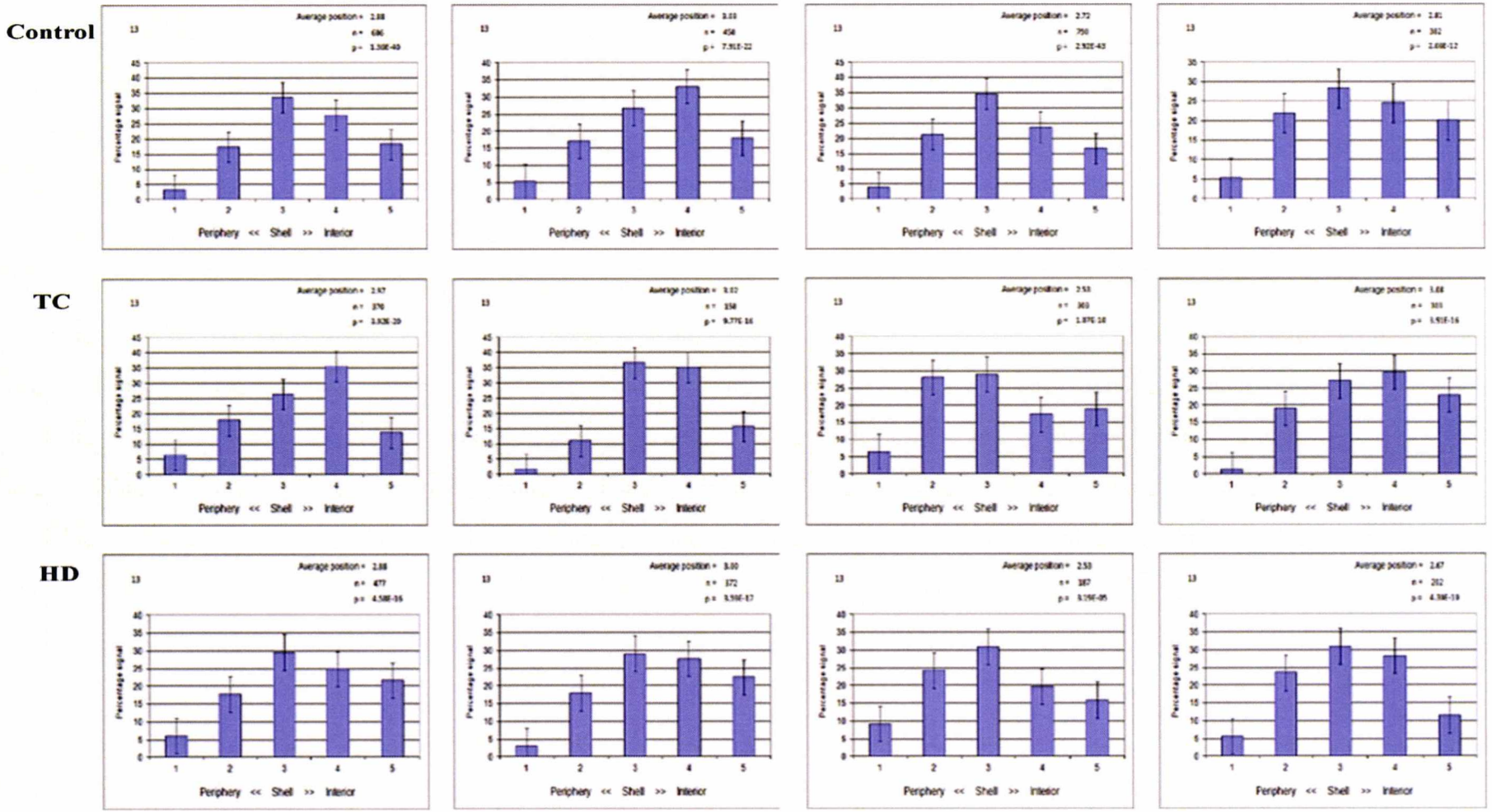


Figure 8: Pooled data analysis for chromosome 13 (Volumetric model)
Nuclear organisation for 4 different time points for controls, TC and HD patients. 4 Graphs in each row represent 4 different time points as in 0 months (after cancer before any treatments), 6, 12, and 18-24 months after completion of treatments. Avarage nuclear position for each chromosome, number of cells analysed, and p values from chi squared test are presented in the top right. When $p \leq 0.05$ distribution is considered to be significant.

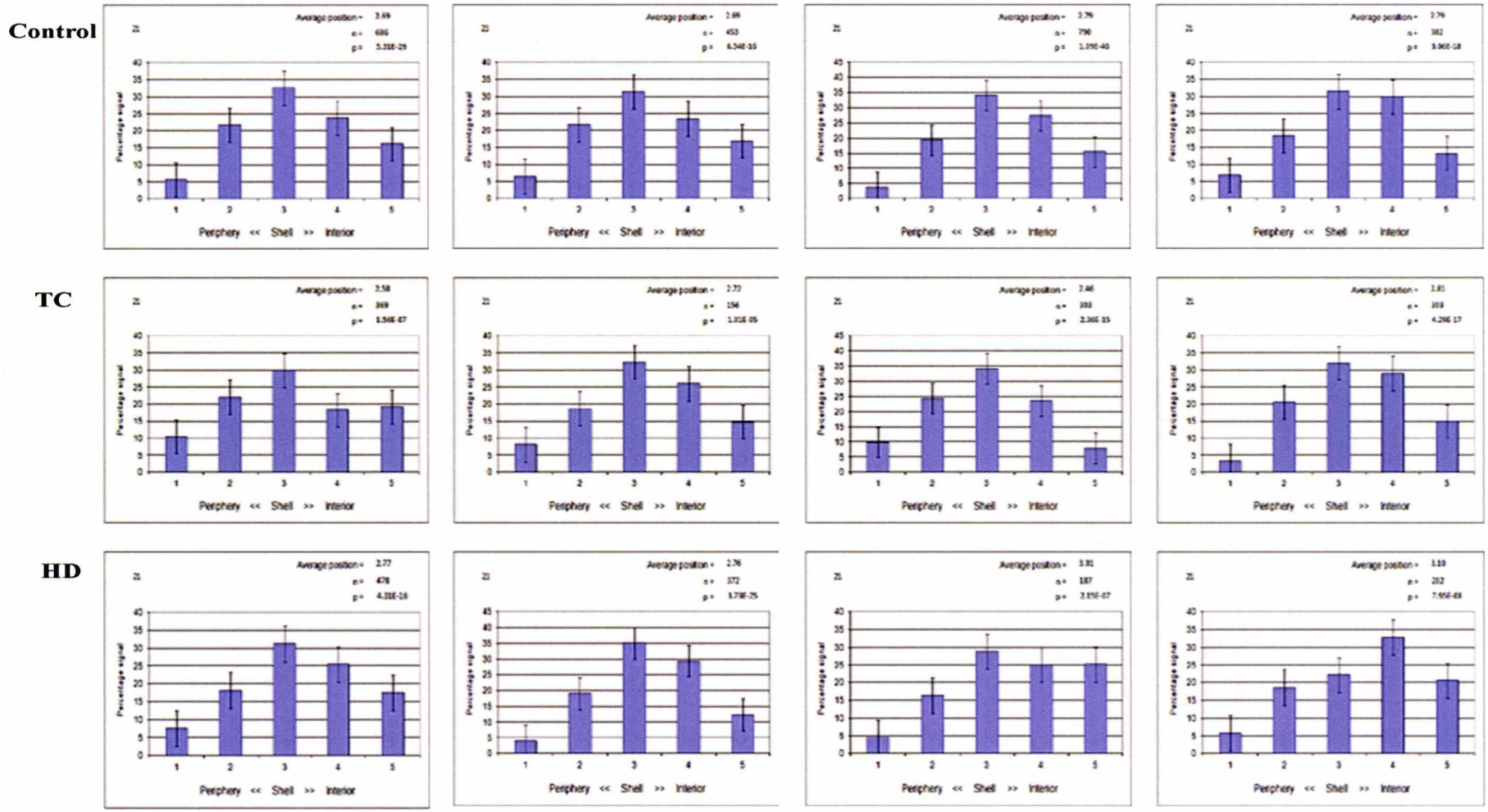


Figure 9: Pooled data analysis for chromosome 21 (Volumetric model)
Nuclear organisation for 4 different time points for controls, TC and HD patients. 4 Graphs in each row represent 4 different time points as in 0 months (after cancer before any treatments), 6, 12, and 18-24 months after completion of treatments. Avarage nuclear position for each chromosome, number of cells analysed, and p values from chi squared test are presented in the top right. When $p \leq 0.05$ distribution is considered to be significant.

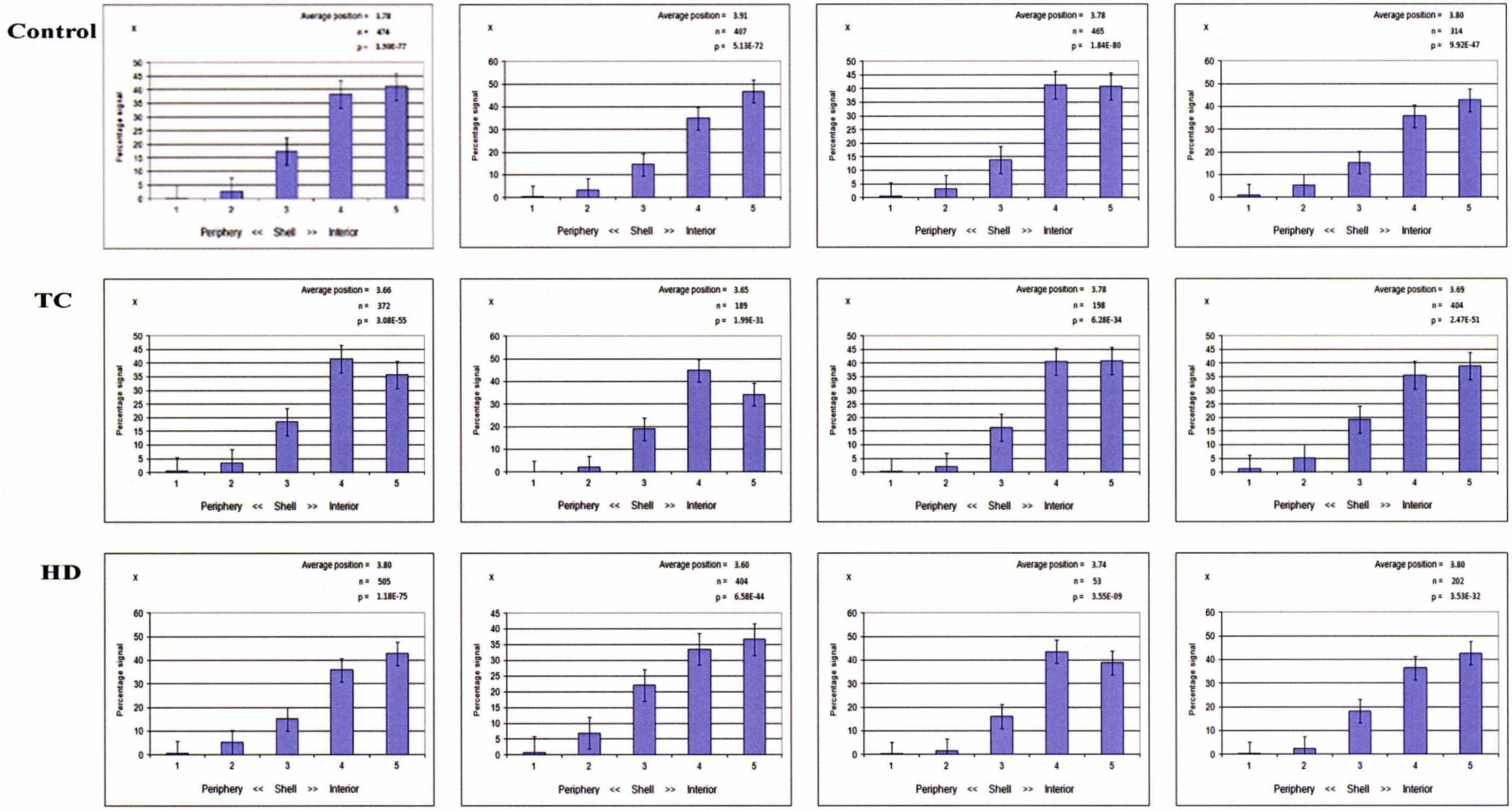


Figure 10: Pooled data analysis for chromosome X (Volumetric model)
Nuclear organisation for 4 different time points for controls, TC and HD patients. 4 Graphs in each row represent 4 different time points as in 0 months (after cancer before any treatments), 6, 12, and 18-24 months after completion of treatments. Avarage nuclear position for each chromosome, number of cells analysed, and p values from chi squared test are presented in the top right. When $p \leq 0.05$ distribution is considered to be significant.

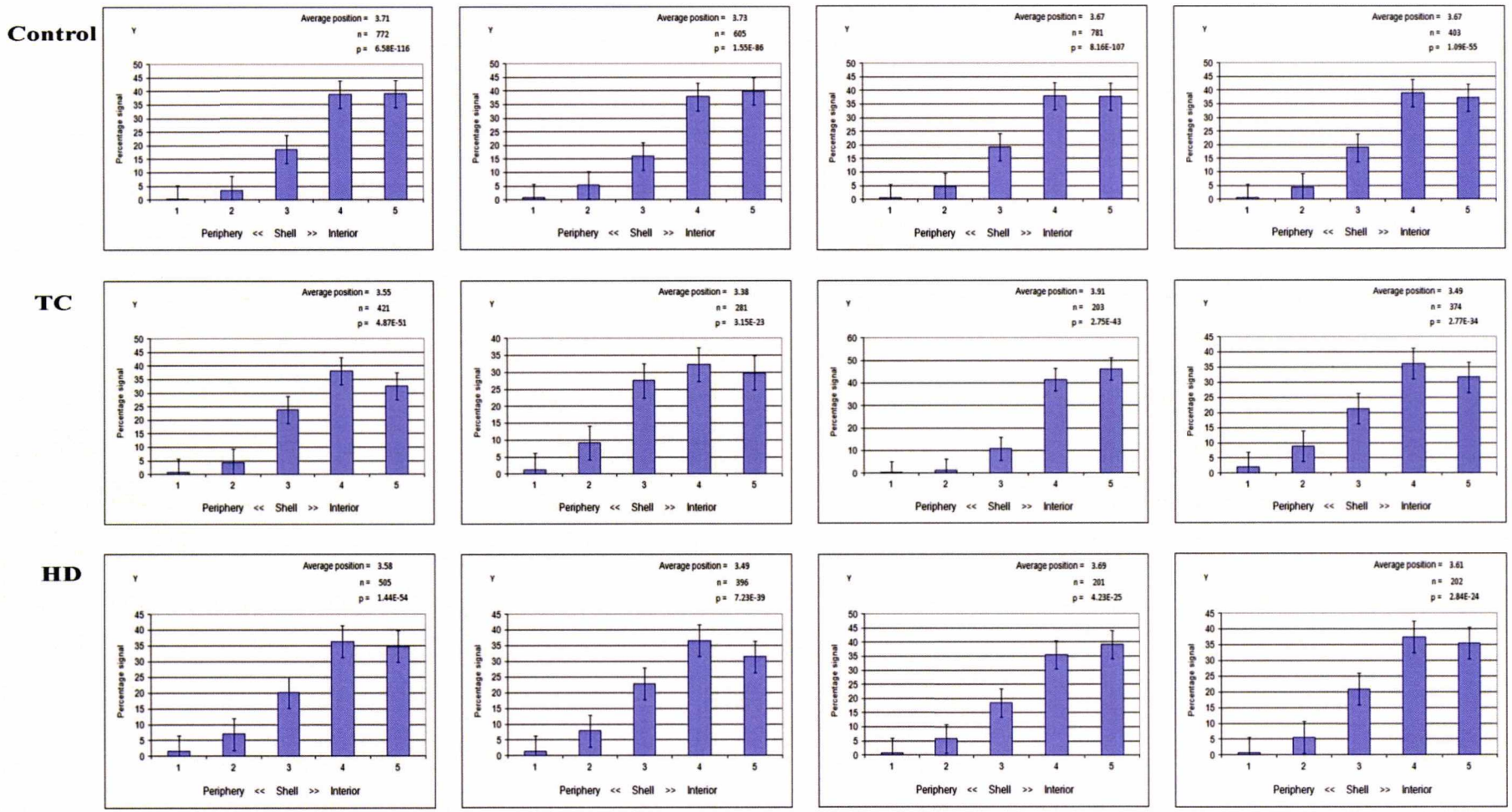


Figure 11: Pooled data analysis for chromosome Y (Volumetric model)
Nuclear organisation for 4 different time points for controls, TC and HD patients. 4 Graphs in each row represent 4 different time points as in 0 months (after cancer before any treatments), 6, 12, and 18-24 months after completion of treatments. Avarage nuclear position for each chromosome, number of cells analysed, and p values from chi squared test are presented in the top right. When $p \leq 0.05$ distribution is considered to be significant.

10.2. Specific aim 4

10.2.1.Summary of cell by cell aneuploidy analysis of embryos

Table 11: Summary of cell by cell aneuploidy analysis of embryos; percentages of nullisomy, monosomy, trisomy, normal and other signals in each embryo

Patient ID	Embryo ID	Cell NO	Chromosomes																						
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
A	1	Nullisomy	31	2	18	41	6	27	29	29	10	2	10	0	0	0	4	6	0	12	0	18	0	0	0	96
A	1	Monosomy	41	6	41	29	8	49	53	39	33	22	20	22	6	6	8	4	16	41	14	57	4	8	0	4
A	1	Trisomy	0	31	4	0	16	4	0	2	8	4	4	4	2	8	6	8	10	0	0	4	4	6	4	0
A	1	Normal	29	61	37	31	67	20	18	31	49	71	65	73	90	84	80	78	73	47	84	20	90	86	94	0
A	1	Others	0	0	0	0	2	0	0	0	0	0	0	0	2	2	2	4	0	0	2	0	2	0	2	0
A	2	Nullisomy	28	0	31	44	0	38	28	9	9	13	3	3	0	0	19	0	6	19	0	34	0	0	0	13
A	2	Monosomy	38	9	25	22	13	38	44	34	53	25	56	44	6	9	3	6	16	28	50	56	3	9	53	81
A	2	Trisomy	0	50	6	0	9	0	3	6	3	6	9	6	0	6	22	28	3	3	3	0	3	0	13	3
A	2	Normal	34	38	34	34	72	25	22	47	31	53	28	44	88	78	53	56	72	47	44	9	88	88	34	3
A	2	Others	0	3	3	0	6	0	3	3	3	3	3	3	6	6	3	9	3	3	3	0	6	3	0	0
A	3	Nullisomy	21	0	14	41	0	26	21	21	2	0	3	0	0	0	14	0	0	5	0	38	0	0	0	0
A	3	Monosomy	52	2	36	41	2	41	41	50	29	19	26	22	2	5	9	5	3	29	10	29	2	3	97	100
A	3	Trisomy	2	52	9	3	0	3	3	2	7	3	5	3	0	0	3	3	9	2	0	2	0	2	0	0
A	3	Normal	26	43	41	14	98	29	34	28	62	78	66	74	98	95	74	91	88	64	90	31	98	95	3	0
A	3	Others	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A	4	Nullisomy	56	0	13	37	8	17	15	17	4	0	2	0	0	0	6	10	13	2	0	4	0	0	0	96
A	4	Monosomy	31	6	38	42	0	38	37	46	19	12	13	13	0	0	2	0	6	21	2	27	0	0	2	4
A	4	Trisomy	0	60	10	2	65	4	4	4	29	46	25	42	62	81	60	67	42	25	56	12	69	77	69	0
A	4	Normal	13	29	38	19	25	40	44	31	46	38	60	40	35	15	31	13	29	52	25	56	29	23	27	0
A	4	Others	0	6	0	0	2	0	0	2	2	4	0	4	4	4	2	10	10	0	17	2	2	0	2	0
A	5	Nullisomy	9	0	36	16	0	16	2	2	0	0	2	0	0	0	14	0	7	0	0	43	0	0	0	2

A	**5**	Monosomy	21	3	36	41	5	50	31	38	17	19	19	16	3	0	14	0	7	48	19	21	2	0	90	86
A	**5**	Trisomy	3	53	2	2	12	2	3	9	29	7	9	9	10	12	14	19	14	3	3	9	12	72	2	0
A	**5**	Normal	66	33	26	41	74	33	60	50	48	67	64	72	83	81	52	76	66	45	72	28	79	16	9	10
A	**5**	Others	2	10	0	0	9	0	3	2	5	7	7	3	3	7	7	5	7	3	5	0	7	12	0	2
A	**6**	Nullisomy	55	0	32	27	36	23	23	5	5	0	14	0	5	0	18	50	36	9	9	45	36	5	0	9
A	**6**	Monosomy	23	14	41	55	5	45	32	55	27	27	32	32	36	32	18	5	32	50	23	36	14	0	82	91
A	**6**	Trisomy	0	27	5	0	5	14	5	0	5	0	5	5	9	0	9	5	0	0	5	5	0	9	0	0
A	**6**	Normal	23	59	23	18	55	18	41	41	64	73	50	64	50	68	55	41	32	41	64	14	50	86	18	0
A	**6**	Others	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
B	**1**	Nullisomy	56	9	67	56	9	22	11	44	9	0	9	0	9	18	0	27	18	9	0	9	18	9	0	100
B	**1**	Monosomy	22	0	22	22	18	33	22	22	27	9	27	18	27	18	27	0	0	18	9	27	55	9	0	0
B	**1**	Trisomy	0	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0	9	9	36	0	0	0	0	0
B	**1**	Normal	22	91	11	22	73	44	67	33	64	82	64	82	64	64	73	73	73	64	55	55	27	82	100	0
B	**1**	Others	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0	0	0	0
B	**2**	Nullisomy	96	9	21	58	12	13	17	15	39	2	49	0	2	16	43	12	21	41	28	45	14	0	4	98
B	**2**	Monosomy	0	19	42	23	10	21	25	35	29	12	12	2	12	12	19	6	19	14	15	31	10	96	2	2
B	**2**	Trisomy	0	11	4	0	6	0	0	6	0	0	0	0	2	2	0	10	21	0	2	0	4	2	2	0
B	**2**	Normal	4	62	33	19	71	67	58	42	33	80	39	94	84	69	38	71	38	45	55	24	71	2	87	0
B	**2**	Others	0	0	0	0	0	0	0	2	0	6	0	4	0	0	0	0	0	0	0	0	0	0	4	0
B	**3**	Nullisomy	35	0	41	24	18	0	0	0	65	0	47	0	12	6	12	29	53	0	18	59	12	0	0	12
B	**3**	Monosomy	29	0	29	0	0	6	0	12	12	6	41	0	0	6	6	0	0	6	0	24	0	0	6	82
B	**3**	Trisomy	6	82	0	0	65	24	29	6	0	76	0	65	76	65	71	47	35	65	76	0	82	88	6	0
B	**3**	Normal	29	12	24	76	18	65	65	76	18	12	6	29	6	18	6	12	12	24	0	12	0	6	82	6
B	**3**	Others	0	6	6	0	0	6	6	6	6	6	6	6	6	6	6	12	0	6	6	6	6	6	6	0
B	**4**	Nullisomy	52	10	31	38		24	0	3	31	3	28	0			17		59	21	48	31			3	100
B	**4**	Monosomy	31	28	28	28		17	28	24	41	14	45	24			24		17	31	17	38			7	0
B	**4**	Trisomy	0	0	3	0		3	7	7	0	7	0	3			0		0	0	3	0			0	0
B	**4**	Normal	17	59	34	24		55	66	66	28	76	28	66			59		24	48	31	31			86	0
B	**4**	Others	0	3	3	10		0	0	0	0	0	0	7			0		0	0	0	0			3	0
B	**5**	Nullisomy	100	0	33	17	30	0	83	0	10	0	0	0	0	0	0	10	20	0	10	10	0	0	10	10
B	**5**	Monosomy	0	10	17	33	20	83	17	50	50	10	60	40	0	10	60	20	20	40	20	50	30	0	70	70
B	**5**	Trisomy	0	0	0	0	20	0	0	17	10	0	0	20	20	0	20	10	20	10	0	0	20	10	0	0

B	**5**	Normal	0	70	50	50	20	17	0	33	30	80	40	40	70	80	10	50	40	50	50	40	30	70	20	20
B	**5**	Others	0	20	0	0	10	0	0	0	0	10	0	0	10	10	10	10	0	0	20	0	20	20	0	0
C	**1**	Nullisomy	30	0	13	13	0	13	26	22	30	0	22	0	0	0	0	0	0	26	0	13	0	0	0	0
C	**1**	Monosomy	43	22	30	61	4	61	57	52	35	17	43	30	0	0	26	9	26	48	26	70	9	13	91	91
C	**1**	Trisomy	0	35	9	0	4	0	0	0	13	0	4	13	13	0	0	0	4	0	0	0	0	0	0	0
C	**1**	Normal	26	43	48	26	91	26	17	26	22	83	30	57	87	100	74	91	70	26	74	17	91	87	9	9
C	**1**	Others	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C	**2**	Nullisomy	8	0	17	8	0	33	0	0	0	0	8	0	0	0	8	0	25	8	0	25	8	0	0	100
C	**2**	Monosomy	50	0	25	25	0	50	67	42	50	33	25	25	0	8	50	25	50	25	33	67	25	8	8	0
C	**2**	Trisomy	0	25	17	0	17	0	0	25	0	8	0	0	25	0	8	17	0	0	0	0	0	33	17	0
C	**2**	Normal	42	75	42	67	83	17	33	33	42	58	67	67	75	92	25	58	17	67	58	8	67	58	75	0
C	**2**	Others	0	0	0	0	0	0	0	0	8	0	0	8	0	0	8	0	8	0	8	0	0	0	0	0
C	**3**	Nullisomy	17	0	4	4	0	9	9	9	4	0	9	4	4	0	4	0	9	4	4	26	0	0	0	78
C	**3**	Monosomy	43	4	43	57	9	52	48	52	13	39	39	22	17	35	9	4	22	48	13	48	48	4	9	9
C	**3**	Trisomy	9	35	0	0	0	4	4	13	9	4	9	9	17	13	13	9	9	9	13	9	22	52	0	4
C	**3**	Normal	30	57	43	39	78	35	39	22	74	57	43	65	48	39	65	78	57	39	65	17	17	30	87	9
C	**3**	Others	0	4	9	0	13	0	0	4	0	0	0	0	13	13	9	9	4	0	4	0	13	13	4	0
C	**4**	Nullisomy	28	0	12	16	0	8	4	44	0	0	0	0	0	0	0	0	0	0	4	12	0	0	0	0
C	**4**	Monosomy	36	20	36	40	0	40	36	40	36	16	12	24	0	0	0	4	100	20	12	24	32	0	8	96
C	**4**	Trisomy	0	0	4	0	8	0	0	0	4	8	12	28	32	12	0	36	0	0	0	8	0	8	38	0
C	**4**	Normal	36	72	48	44	92	52	60	16	60	76	76	48	48	88	100	44	0	80	84	52	68	92	54	4
C	**4**	Others	0	8	0	0	0	0	0	0	0	0	0	0	20	0	0	16	0	0	0	4	0	0	0	0
C	**5**	Nullisomy	21	0	11	32	0	14	14	61	0	0	0	0	4	4	12	38	11	4	11	32	25	13	21	11
C	**5**	Monosomy	43	14	25	36	8	54	32	21	11	7	7	11	4	0	15	21	75	18	14	54	29	21	11	82
C	**5**	Trisomy	7	0	14	11	4	7	7	4	4	0	4	29	21	17	0	0	0	4	0	0	0	0	36	0
C	**5**	Normal	29	79	46	21	75	21	46	14	79	82	81	54	50	67	65	25	14	68	68	11	29	50	21	4
C	**5**	Others	0	7	4	0	13	4	0	0	7	11	7	7	21	13	8	17	0	7	7	4	17	17	11	4
D	**1**	Nullisomy	22	7	26	26	6	17	19	20	13	2	7	2	6	2	35	6	50	13	30	13	4	0	2	37
D	**1**	Monosomy	31	11	48	41	25	41	44	31	33	24	41	43	25	13	35	8	15	33	24	46	15	15	70	61
D	**1**	Trisomy	0	35	4	0	13	2	0	4	4	0	2	2	8	6	4	15	2	2	2	4	2	2	0	0
D	**1**	Normal	46	41	22	33	56	41	37	44	50	74	50	54	60	79	26	71	33	52	44	37	79	83	28	2
D	**1**	Others	0	6	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0

D	**2**	Nullisomy	21	5	56	28	15	28	8	23	3	5	15	0	3	3	33	18	26	15	18	5	18	0	5	97
D	**2**	Monosomy	41	5	23	41	10	28	38	51	31	26	51	26	26	13	49	18	18	41	23	51	15	10	5	3
D	**2**	Trisomy	3	38	0	0	13	5	8	8	8	0	3	0	10	10	0	0	21	0	3	3	8	8	8	0
D	**2**	Normal	36	49	21	31	62	38	46	18	59	69	31	74	62	74	18	56	36	44	56	41	59	82	82	0
D	**2**	Others	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0
D	**3**	Nullisomy	13	0	58	50	6	33	6	8	4	4	4	0	10	0	21	4	33	2	6	4	6	0	0	100
D	**3**	Monosomy	33	6	23	27	6	35	46	50	17	15	23	19	8	12	17	8	12	8	10	29	12	0	0	0
D	**3**	Trisomy	2	58	0	0	6	4	15	8	12	2	2	2	6	2	4	15	17	0	8	2	2	0	4	0
D	**3**	Normal	52	27	19	23	83	29	33	35	67	79	71	79	77	87	58	73	38	90	77	65	81	98	96	0
D	**3**	Others	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
D	**4**	Nullisomy	51	2	41	56	12	29	32	10	36	13	56	0	9	12	13	28	22	27	9	56	7	2	0	100
D	**4**	Monosomy	34	20	37	24	35	37	34	46	24	24	20	24	47	53	47	21	27	49	31	29	33	5	7	0
D	**4**	Trisomy	0	7	12	0	0	5	0	7	4	2	0	2	0	0	2	9	7	0	0	0	5	0	4	0
D	**4**	Normal	15	69	10	20	53	27	34	37	36	60	24	73	44	35	38	42	44	24	60	16	56	93	89	0
D	**4**	Others	0	2	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D	**5**	Nullisomy	21	14	21	29	14	0	7	0	36	0	57	0	29	14	29	43	29	57	29	79	14	0	0	100
D	**5**	Monosomy	43	29	43	36	14	57	57	64	14	29	29	0	29	36	50	29	50	0	21	14	29	7	0	0
D	**5**	Trisomy	0	36	0	7	7	7	7	0	0	0	0	7	0	7	0	7	0	0	7	0	7	0	0	0
D	**5**	Normal	29	21	29	29	64	36	29	29	43	64	14	86	43	43	21	21	21	36	36	7	50	93	93	0
D	**5**	Others	7	0	7	0	0	0	0	7	7	7	0	7	0	0	0	0	0	7	7	0	0	0	7	0
D	**6**	Nullisomy	0	0	0	0	17	0	0	0	0	0	71	0	0	14	43	14	86	0	14	29	0	0	0	100
D	**6**	Monosomy	86	86	86	100	67	100	71	86	100	86	29	86	100	71	57	71	14	86	71	57	86	86	86	0
D	**6**	Trisomy	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
D	**6**	Normal	14	14	14	0	17	0	29	14	0	14	0	14	0	14	0	14	0	14	14	14	14	14	14	0
D	**6**	Others	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E	**1**	Nullisomy	0	6	0	0	44	0	11	0	11	11	50	11	89	22	6	94	6	11	6	22	28	28	6	6
E	**1**	Monosomy	22	0	22	11	39	6	6	6	33	78	22	39	6	56	94	0	17	50	6	28	44	67	50	94
E	**1**	Trisomy	11	28	6	17	0	6	6	17	0	0	0	6	0	0	0	0	0	6	6	0	0	0	0	0
E	**1**	Normal	67	67	72	67	17	83	78	72	56	11	28	44	6	22	0	6	78	33	83	50	28	6	44	0
E	**1**	Others	0	0	0	6	0	6	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E	**2**	Nullisomy	18	0	26	35	1	22	2	8	3	5	15	0	0	1	6	6	5	9	2	15	1	0	0	2

E	**2**	Monosomy	29	9	40	43	16	32	42	42	25	14	26	6	18	9	12	9	23	22	23	18	12	12	88	92
E	**2**	Trisomy	3	39	9	0	9	11	3	9	2	11	9	9	10	3	35	22	15	3	30	9	7	3	2	0
E	**2**	Normal	49	45	23	22	72	35	54	42	66	69	48	80	69	84	42	60	52	65	44	54	76	82	11	6
E	**2**	Others	0	6	2	0	1	0	0	0	5	2	2	5	3	3	5	3	6	2	2	3	3	3	0	0
E	**3**	Nullisomy	51	2	23	44	32	12	2	5	9	0	14	0	14	11	9	41	16	5	9	9	24	3	2	98
E	**3**	Monosomy	42	9	40	40	16	26	56	40	34	11	32	18	27	27	9	11	14	14	26	27	19	95	2	2
E	**3**	Trisomy	0	16	5	0	8	7	2	5	9	2	2	2	5	0	5	19	12	5	2	2	3	0	5	0
E	**3**	Normal	7	72	33	16	43	56	40	51	48	84	52	77	54	59	77	27	49	77	63	59	54	3	91	0
E	**3**	Others	0	0	0	0	0	0	0	0	0	2	0	2	0	3	0	3	9	0	0	2	0	0	0	0
E	**4**	Nullisomy	48	0	5	70	37	68	63	8	28	0	79	10	8	10	25	43	20	54	23	28	13	8	0	90
E	**4**	Monosomy	30	8	33	13	8	10	28	28	18	18	5	38	8	15	18	0	73	21	28	23	10	3	3	5
E	**4**	Trisomy	3	20	8	0	0	0	0	5	21	3	3	3	5	3	0	0	0	0	3	5	0	3	13	0
E	**4**	Normal	20	70	55	18	55	23	10	55	33	79	13	49	80	73	58	58	8	26	48	44	78	88	85	5
E	**4**	Others	0	3	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F	**1**	Nullisomy	64	0	14	20	2	16	14	18	0	0	9	0	2	0	0	7	2	0	2	36	0	0	0	100
F	**1**	Monosomy	27	2	36	43	5	36	41	57	16	9	27	0	10	0	0	5	9	9	7	48	10	2	7	0
F	**1**	Trisomy	0	16	9	0	2	5	2	5	2	2	5	0	2	2	2	34	7	0	7	0	5	0	5	0
F	**1**	Normal	9	82	41	36	90	43	43	20	82	89	59	100	85	98	98	51	80	91	84	16	85	98	89	0
F	**1**	Others	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0
F	**2**	Nullisomy	41	1	11	45	0	5	3	3	2	1	7	0	2	9	4	6	14	3	1	21	1	0	1	4
F	**2**	Monosomy	42	13	46	33	6	22	32	42	26	3	26	7	9	2	11	8	19	11	18	46	4	1	96	88
F	**2**	Trisomy	0	22	0	1	3	3	1	2	2	0	1	2	5	2	18	7	16	1	8	0	1	2	0	0
F	**2**	Normal	16	63	42	21	91	70	64	53	70	96	66	91	84	87	67	78	51	85	73	33	94	97	3	7
F	**2**	Others	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
F	**3**	Nullisomy	5	0	11	0	5	0	0	5	0	11	32	5	11	5	0	11	21	5	0	79	5	0	0	5
F	**3**	Monosomy	37	16	58	58	16	63	16	32	26	21	47	37	26	21	21	0	11	21	16	21	26	11	89	89
F	**3**	Trisomy	0	32	0	5	5	0	5	5	5	5	0	0	0	0	5	26	32	5	16	0	0	0	0	0
F	**3**	Normal	58	53	32	37	74	37	79	53	68	63	21	58	63	74	74	63	37	68	68	0	68	89	5	5
F	**3**	Others	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0
F	**4**	Nullisomy	55	9	9	45	9	15	0	0	36	0	45	3	15	18	45	3	27	6	21	64	0	3	9	100
F	**4**	Monosomy	18	9	18	9	36	9	3	12	30	6	21	3	24	27	0	24	45	24	15	15	15	6	0	0
F	**4**	Trisomy	0	9	18	3	9	3	0	3	3	6	0	0	0	0	3	3	3	3	3	6	3	3	3	0

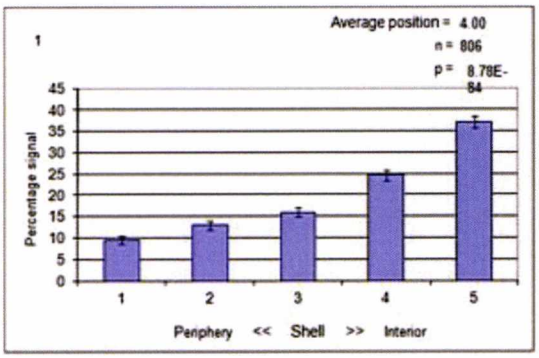
F	**4**	Normal	27	70	55	42	45	73	97	85	30	82	33	91	61	55	52	70	24	67	58	15	82	88	85	0
F	**4**	Others	0	3	0	0	0	0	0	0	0	6	0	3	0	0	0	0	0	0	3	0	0	0	3	0
F	**5**	Nullisomy	60	7	40	13	7	13	0	0	20	0	13	0	7	0	7	0	13	0	0	7	0	0	0	7
F	**5**	Monosomy	7	0	20	47	13	13	40	7	13	0	7	7	7	7	0	0	87	7	13	0	7	7	100	93
F	**5**	Trisomy	0	7	0	0	0	0	0	0	0	7	7	0	7	7	0	20	0	0	0	33	7	7	0	0
F	**5**	Normal	33	87	40	40	80	73	60	93	67	93	73	93	80	87	93	80	0	93	87	60	87	87	0	0
F	**5**	Others	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	**1**	Nullisomy	50	0	0	0	25	0	25	25	0	25	0	0	25	0	25	0	25	25	25	25	25	0	0	100
G	**1**	Monosomy	25	25	50	0	25	0	25	0	50	0	50	0	50	50	0	25	25	0	0	25	0	50	0	0
G	**1**	Trisomy	0	0	25	0	0	0	0	0	0	25	0	0	0	0	0	25	0	0	0	0	25	25	0	0
G	**1**	Normal	25	75	25	100	50	100	50	75	50	50	50	100	25	50	75	50	50	75	75	50	50	25	100	0
G	**1**	Others	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	**2**	Nullisomy	13	0	4	4	4	0	4	0	13	0	22	0	17	4	4	13	9	4	0	13	4	4	0	100
G	**2**	Monosomy	22	9	22	9	13	30	9	26	13	9	30	35	13	9	4	9	13	17	9	30	17	9	9	0
G	**2**	Trisomy	4	9	0	4	4	4	9	0	0	17	0	0	4	9	13	17	9	0	0	4	0	4	4	0
G	**2**	Normal	61	78	74	83	78	65	78	74	74	74	48	65	65	78	78	61	70	74	91	52	74	83	87	0
G	**2**	Others	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0
G	**3**	Nullisomy	57	16	17	52	4	22	26	17	54	0	54	0	4	4	12	8	20	21	20	54	4	4	0	8
G	**3**	Monosomy	22	32	35	13	24	22	22	39	29	13	17	0	56	24	32	20	24	25	24	33	12	8	84	84
G	**3**	Trisomy	0	4	13	4	4	17	4	0	0	17	0	4	4	0	8	0	0	4	0	0	0	8	0	8
G	**3**	Normal	22	48	35	30	68	39	48	43	17	71	29	96	36	72	48	68	56	50	56	13	84	80	16	0
G	**3**	Others	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
G	**4**	Nullisomy	6	2	0	11	9	0	2	0	23	0	19	0	7	9	0	11	2	23	0	30	9	2	0	89
G	**4**	Monosomy	23	19	17	6	9	13	9	13	34	11	34	4	4	2	6	2	19	21	13	32	7	0	4	11
G	**4**	Trisomy	0	6	6	2	7	9	0	2	6	15	2	0	9	7	11	17	6	2	0	2	2	78	2	0
G	**4**	Normal	66	66	72	74	74	72	83	79	32	68	40	87	76	76	79	61	70	49	79	34	76	9	85	0
G	**4**	Others	4	6	4	6	0	6	6	6	4	6	4	9	0	0	4	0	2	4	9	2	0	0	9	0
H	**1**	Nullisomy	90	22	12	61	2	8	4	2	27	4	49	0	10	12	65	4	33	27	37	29	0	0	8	90
H	**1**	Monosomy	0	18	27	14	14	27	18	27	24	12	16	8	14	10	16	8	14	10	24	61	12	4	2	8
H	**1**	Trisomy	0	12	0	0	8	4	2	2	0	0	0	0	8	4	0	20	2	24	4	0	8	4	2	0
H	**1**	Normal	10	47	61	24	76	61	76	69	49	84	35	92	67	73	18	65	51	39	35	10	80	92	88	2
H	**1**	Others	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0

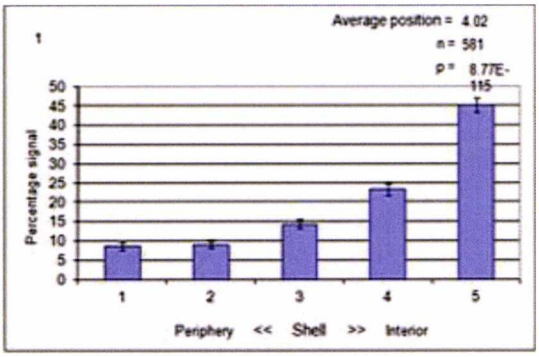
H	**2**	Nullisomy	82	14	80	80	0	29	4	7	14	5	41	0	4	0	84	0	61	13	61	29	0	0	7	98
H	**2**	Monosomy	4	14	7	9	2	31	2	7	29	5	16	4	5	2	7	2	16	32	14	21	4	0	7	2
H	**2**	Trisomy	0	14	0	0	20	2	0	4	2	24	7	7	21	11	2	39	0	4	0	13	21	79	2	0
H	**2**	Normal	15	57	13	11	75	36	93	80	50	60	32	82	68	88	7	52	23	48	25	36	71	14	84	0
H	**2**	Others	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
H	**3**	Nullisomy	73	2	10	35	2	6	0	2	10	2	58	6	0	2	40	4	13	17	9	46	0	0	2	96
H	**3**	Monosomy	27	11	23	27	4	15	88	29	25	2	17	73	15	15	17	4	32	13	11	38	27	4	2	4
H	**3**	Trisomy	0	11	2	8	10	8	0	8	2	67	2	0	19	13	2	44	4	13	21	2	6	10	9	0
H	**3**	Normal	0	74	65	29	79	67	13	52	60	13	23	21	60	63	38	38	49	52	57	13	60	77	83	0
H	**3**	Others	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
H	**4**	Nullisomy	39	5	13	32	0	5	13	29	43	3	13	0	5	3	3	3	5	30	0	48	5	3	0	100
H	**4**	Monosomy	42	0	24	39	3	66	29	55	43	0	53	15	10	5	5	3	5	38	18	43	5	0	3	0
H	**4**	Trisomy	0	0	5	0	0	0	0	0	0	13	0	0	5	0	0	75	3	3	0	0	5	0	0	0
H	**4**	Normal	18	93	58	29	95	29	58	16	15	83	33	83	80	93	90	18	88	30	80	10	85	95	95	0
H	**4**	Others	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0
H	**5**	Nullisomy	8	0	8	0	0	0	0	17	33	0	0	0	0	0	0	8	0	25	0	17	0	0	0	0
H	**5**	Monosomy	42	0	17	0	0	25	25	50	17	0	33	42	25	0	45	92	0	75	9	33	0	0	91	100
H	**5**	Trisomy	0	100	0	0	0	42	0	0	8	42	0	8	0	0	9	0	0	0	0	0	0	0	0	0
H	**5**	Normal	50	0	75	100	100	33	75	33	42	58	67	50	75	100	45	0	100	0	91	50	100	100	9	0
H	**5**	Others	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0
H	**6**	Nullisomy	0	33	11	0	0	0	0	0	44	0	11	0	11	11	22	0	33	0	11	0	0	0	0	11
H	**6**	Monosomy	11	0	33	22	33	22	11	44	0	11	56	11	11	0	0	0	0	11	0	22	0	0	56	56
H	**6**	Trisomy	11	0	11	22	22	22	11	11	11	22	33	22	22	11	33	11	0	22	0	11	11	0	0	0
H	**6**	Normal	67	56	33	44	0	33	56	44	44	44	0	44	0	33	22	33	44	56	56	56	44	56	22	22
H	**6**	Others	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0	0	0

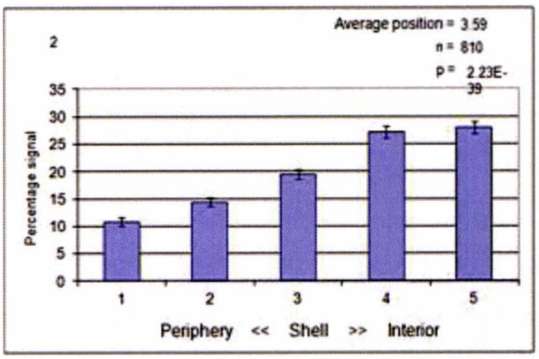
Different combinations of chromosomal normal and abnormal cells in embryos; for each emrbryo, percentage of normal, monosomy, trisomy, nullisomy and other abnormalities percentage is presented for all 24 chromosomes.

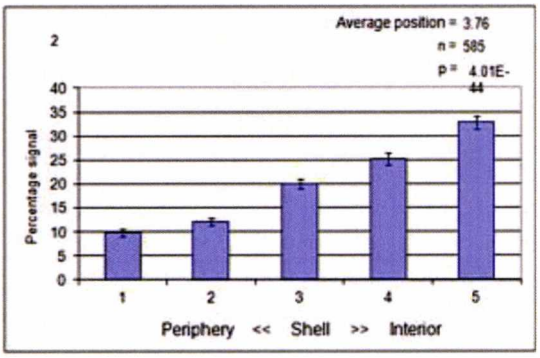
10.3. Specific aim 5

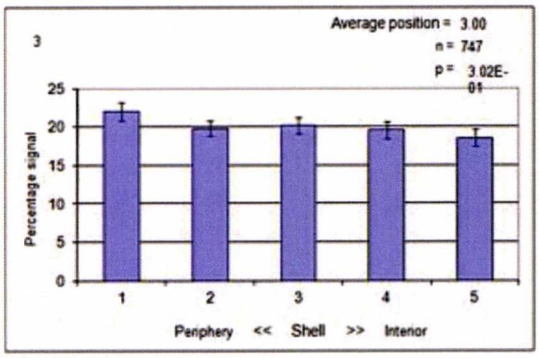
10.3.1.Graphs demonstrate the preferential position of chromosome, when whole embryos (pooled) are compared with one another

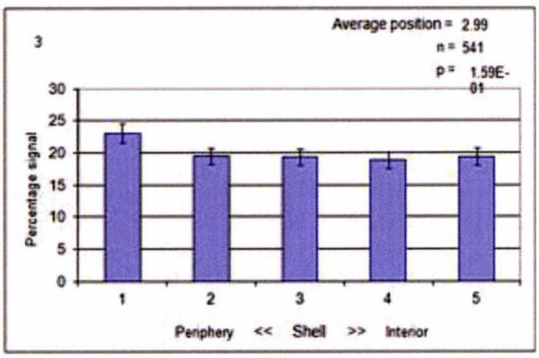


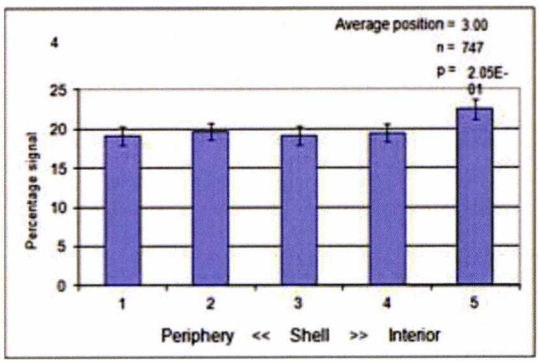


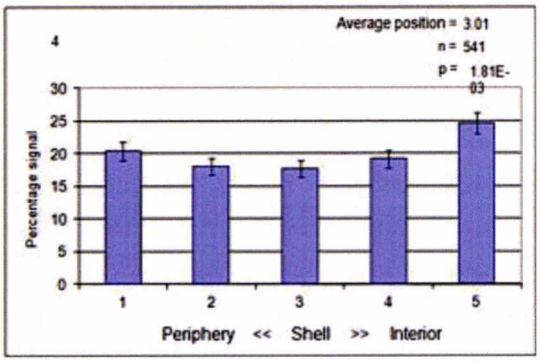


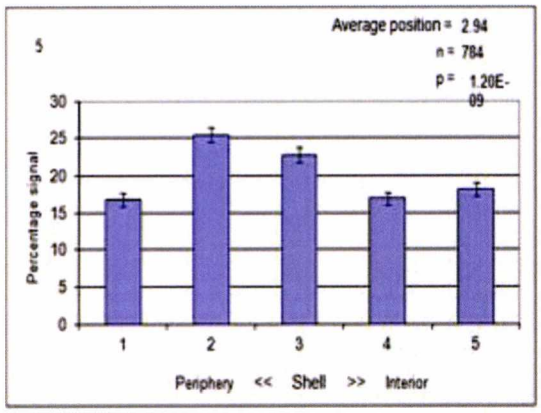








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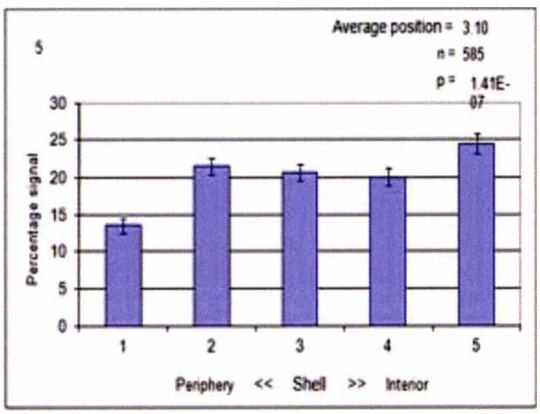
Periphery << Shell >> Interior


5
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p = 1.41E-07
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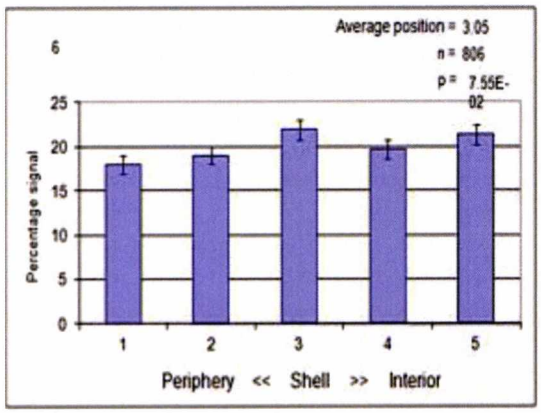
Periphery << Shell >> Interior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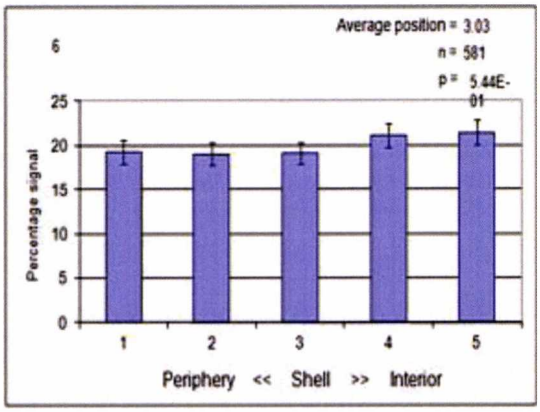
Percentage signal
Periphery << Shell >> Interior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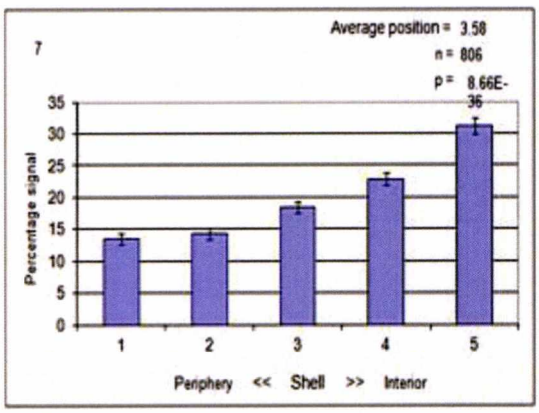
Percentage signal
Periphery << Shell >> Interior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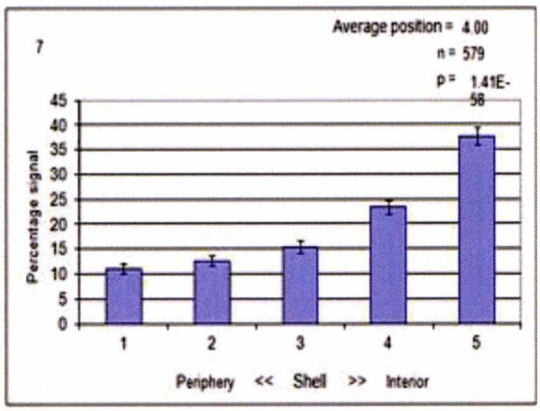
p = 8.66E-36
Percentage signal
Periphery << Shell >> Interior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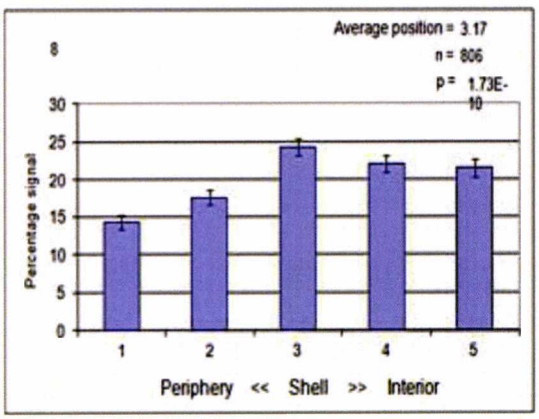
Percentage signal
Periphery << Shell >> Interior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Average position = 3.17
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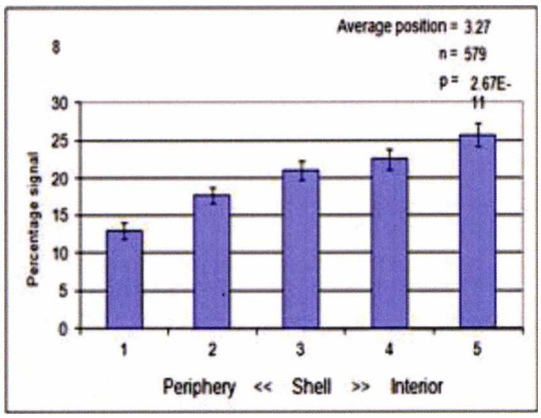
Percentage signal
Periphery << Shell >> Interior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Average position = 3.27
n = 579
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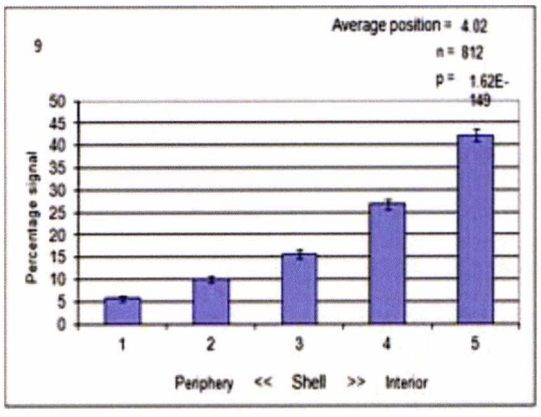
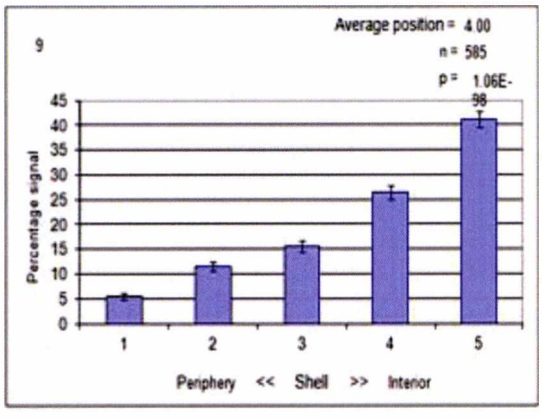
Percentage signal
Periphery << Shell >> Interior


9
Average position = 4.02
n = 812
p = 1.62E-149
Percentage signal
Periphery << Shell >> Interior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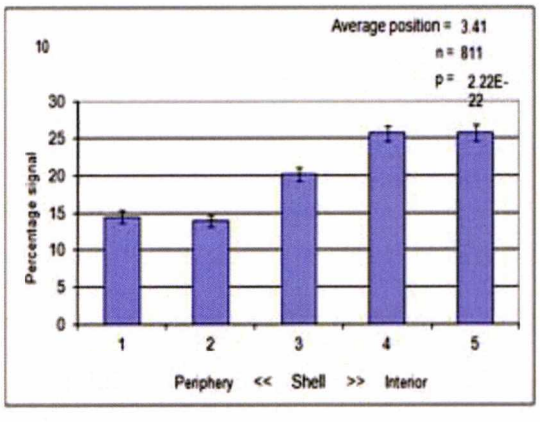
p = 1.06E-98
Percentage signal
Periphery << Shell >> Interior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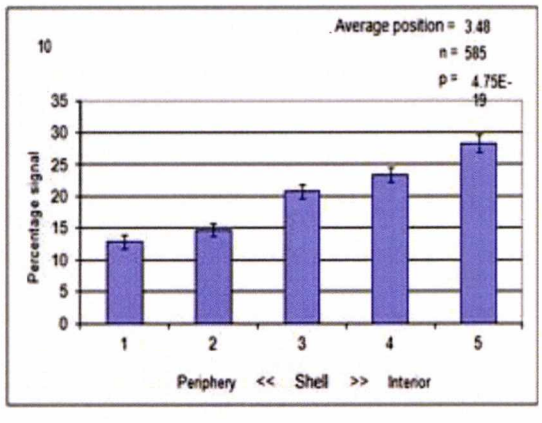
Average position = 3.41
n = 811
p = 2.22E-22
Percentage signal
Periphery << Shell >> Interior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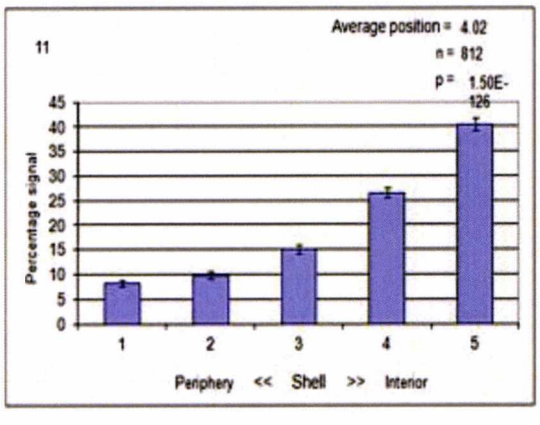
Periphery << Shell >> Interior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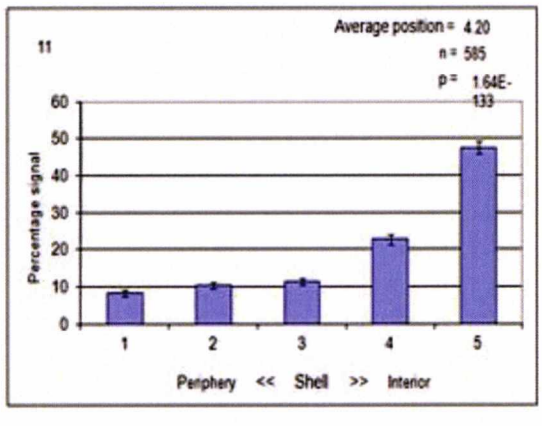
p = 1.50E-126
Percentage signal
Periphery << Shell >> Interior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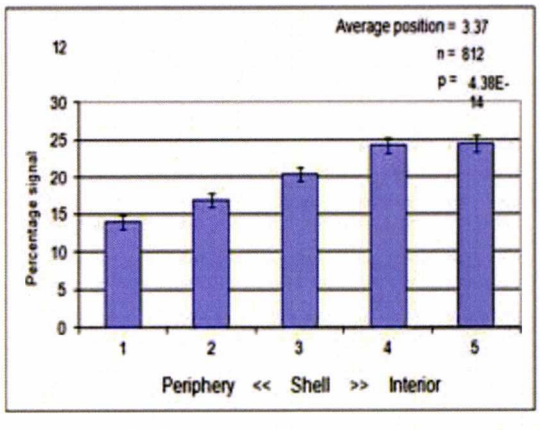
Average position = 4.20
n = 585
p = 1.64E-133
Percentage signal
Periphery << Shell >> Interior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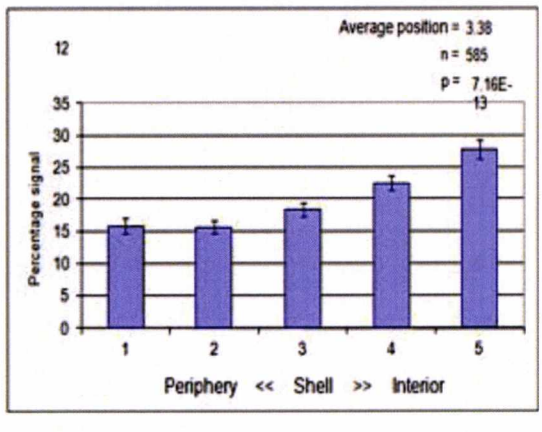
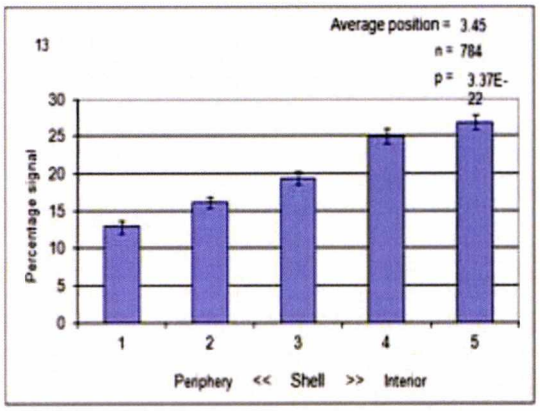
Average position = 3.37
n = 812
p = 4.38E-14
Percentage signal
Periphery << Shell >> Interior


12
Average position = 3.38
n = 585
p = 7.16E-13
Percentage signal
Periphery << Shell >> Interior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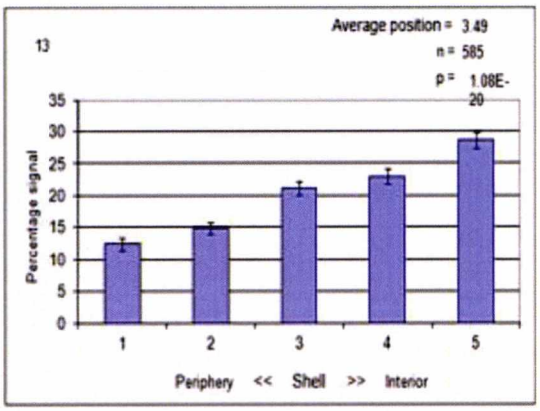
Average position = 3.45
n = 784
p = 3.37E-22
Percentage signal
Periphery << Shell >> Interior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Average position = 3.49
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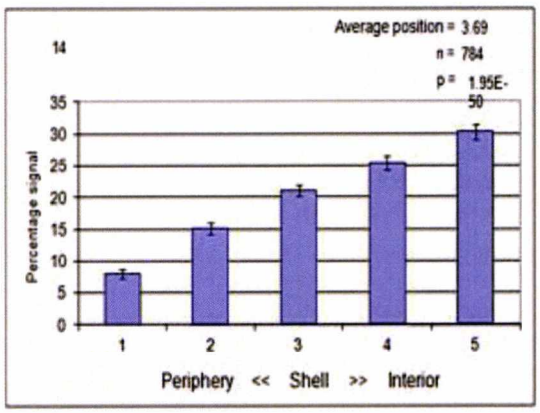
p = 1.08E-20
Percentage signal
Periphery << Shell >> Interior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Average position = 3.69
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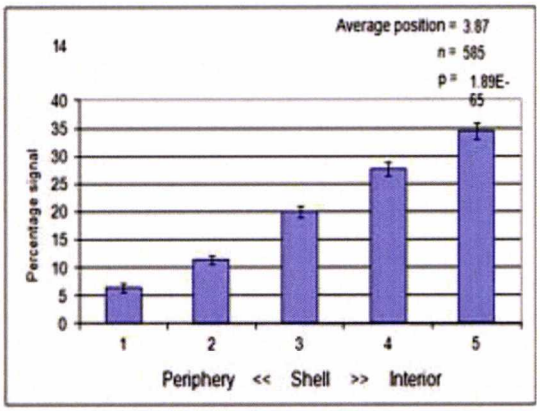
p = 1.95E-50
Percentage signal
Periphery << Shell >> Interior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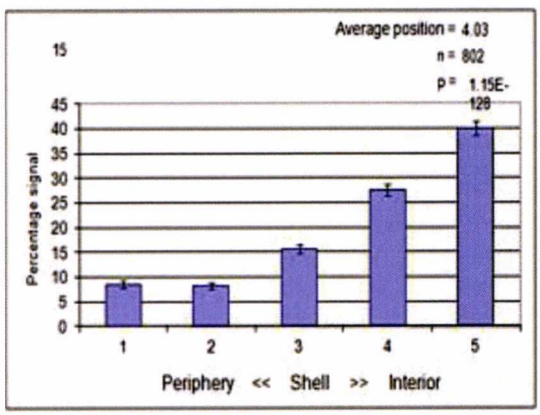
Average position = 3.87
n = 585
p = 1.89E-65
Percentage signal
Periphery << Shell >> Interior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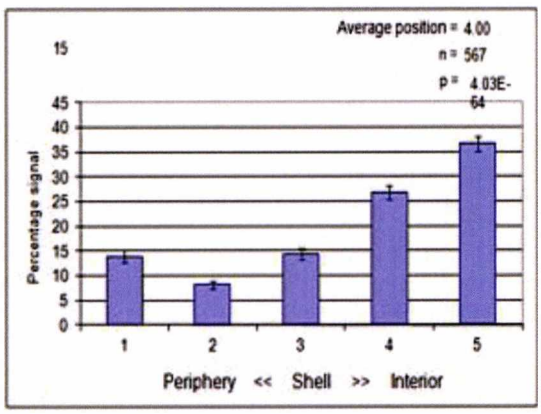
Average position = 4.03
n = 802
p = 1.15E-128
Percentage signal
Periphery << Shell >> Interior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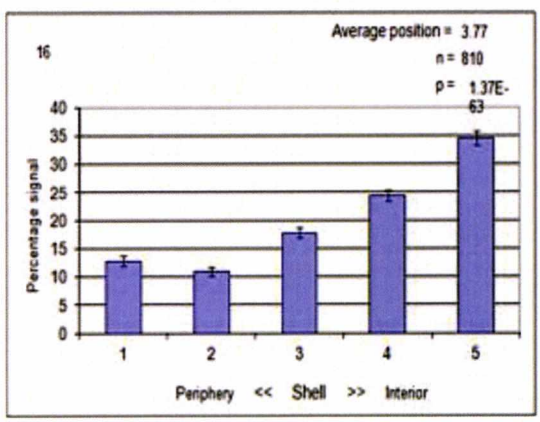
Average position = 4.00
n = 567
p = 4.03E-64
Percentage signal
Periphery << Shell >> Interior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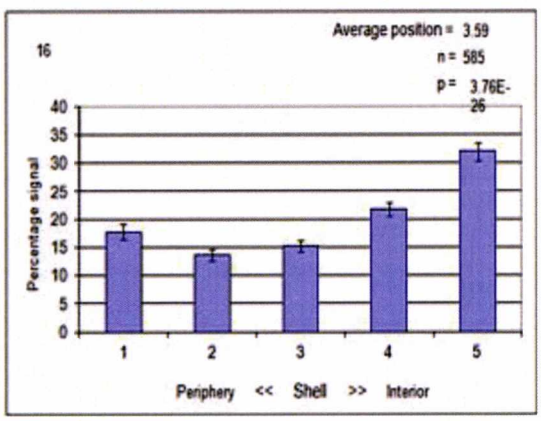
Average position = 3.77
n = 810
p = 1.37E-63
Percentage signal
Periphery << Shell >> Interior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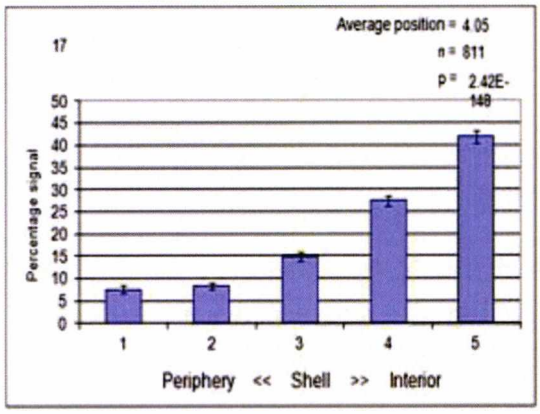
Average position = 3.59
n = 585
p = 3.76E-26
Percentage signal
Periphery << Shell >> Interior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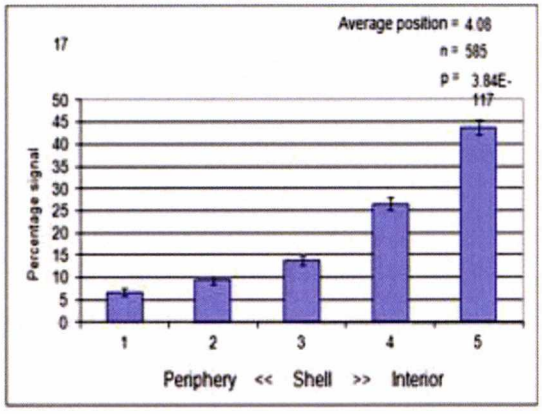
Average position = 4.05
n = 811
p = 2.42E-148
Percentage signal
Periphery << Shell >> Interior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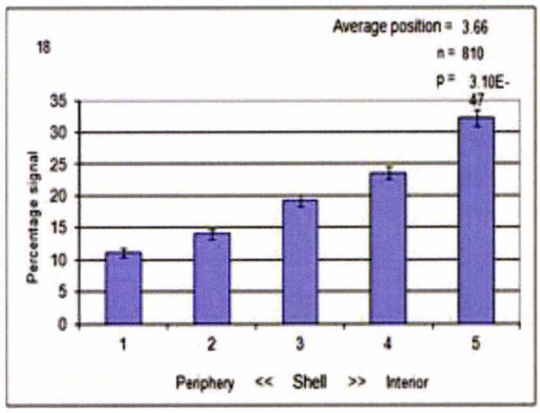
Average position = 4.08
n = 585
p = 3.84E-117
Percentage signal
Periphery << Shell >> Interior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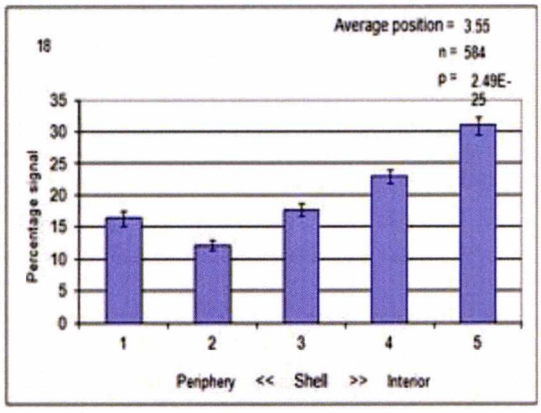
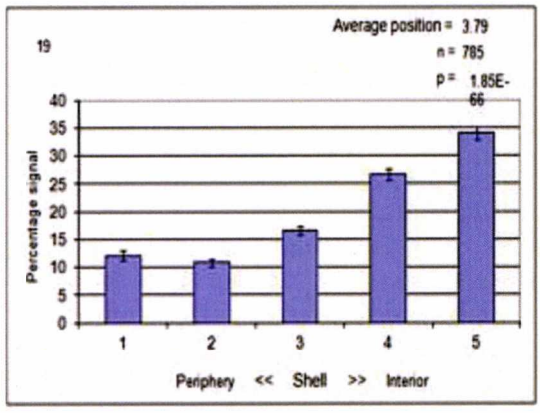
Average position = 3.66
n = 810
p = 3.10E-47
Percentage signal
Periphery << Shell >> Interior


18
Average position = 3.55
n = 584
p = 2.49E-25
Percentage signal
Periphery << Shell >> Interior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Average position = 3.79
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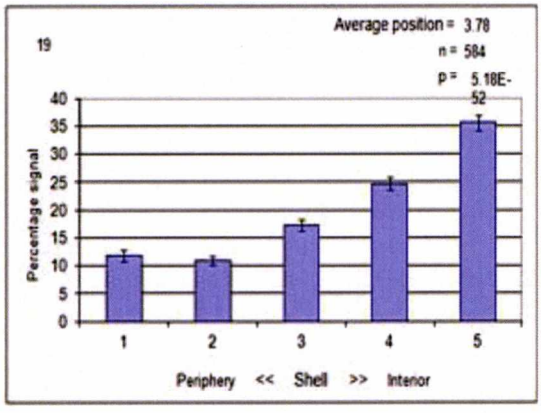
p = 1.85E-66
Percentage signal
Periphery << Shell >> Interior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Average position = 3.78
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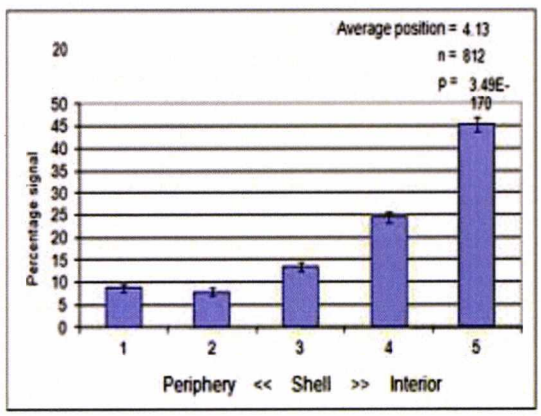
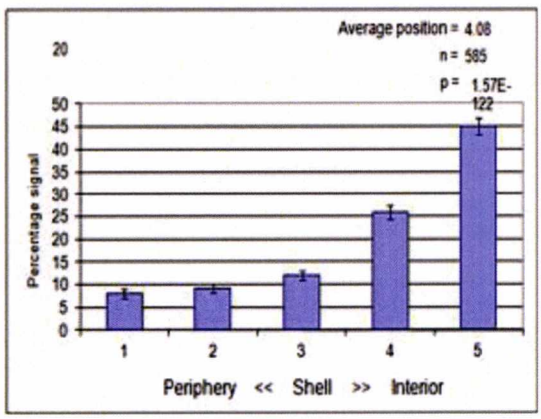
p = 5.18E-52
Percentage signal
Periphery << Shell >> Interior


20
Average position = 4.13
n = 812
p = 3.49E-170
Percentage signal
Periphery << Shell >> Interior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Average position = 4.08
n = 585
p = 1.57E-122
Percentage signal
Periphery << Shell >> Interior

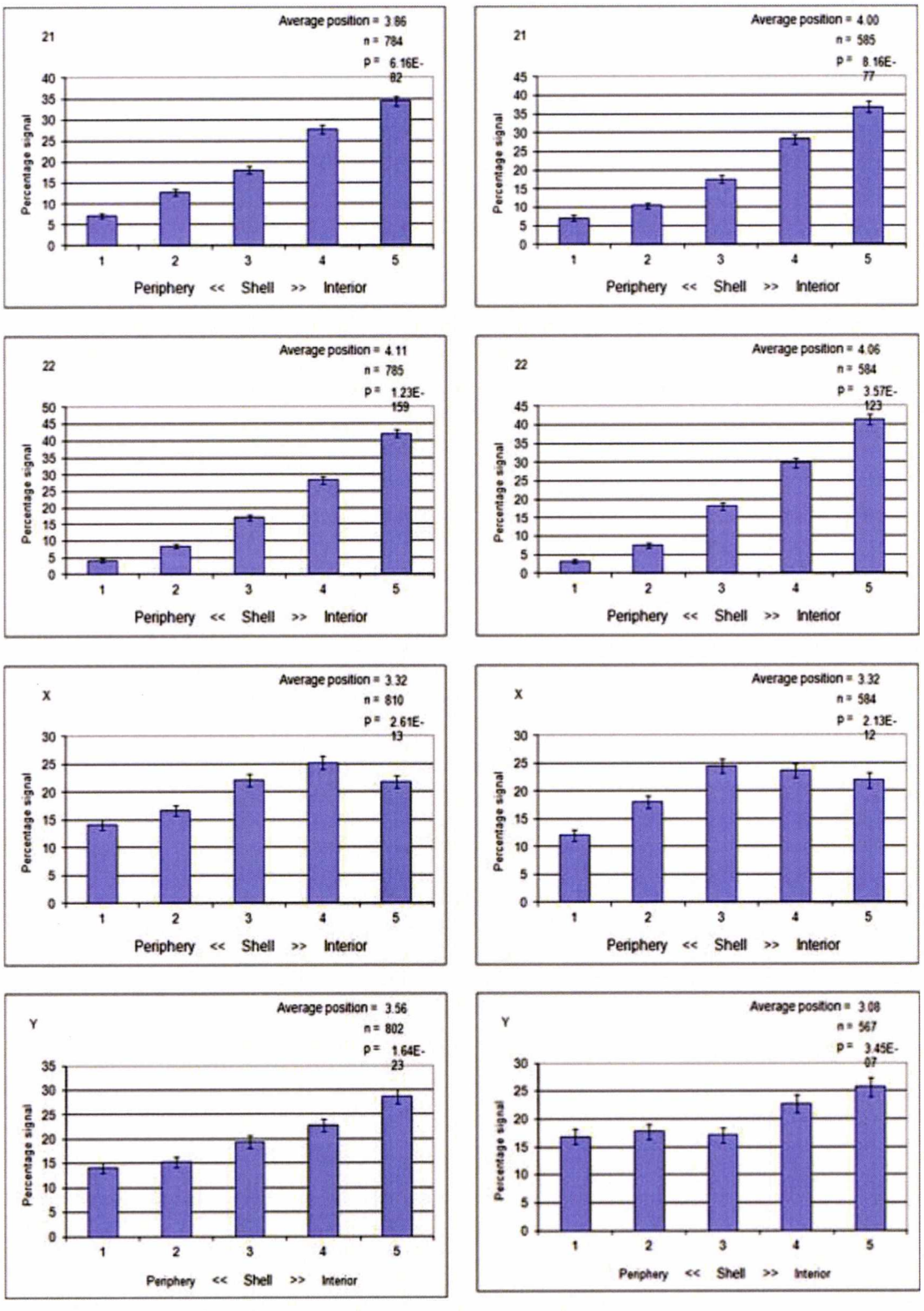
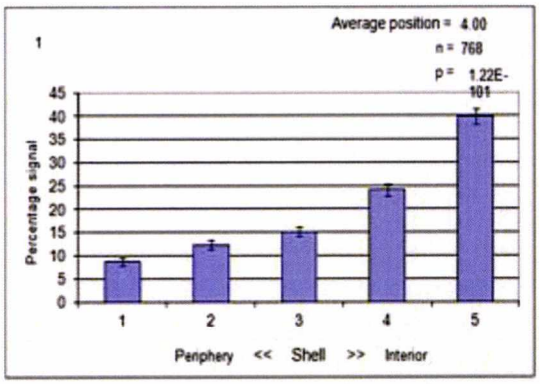
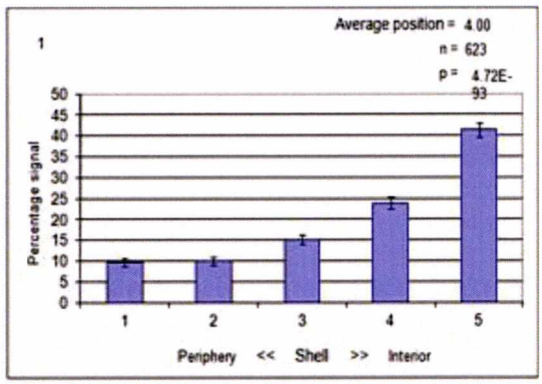
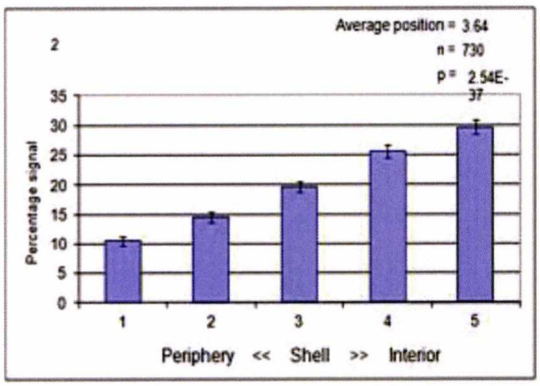


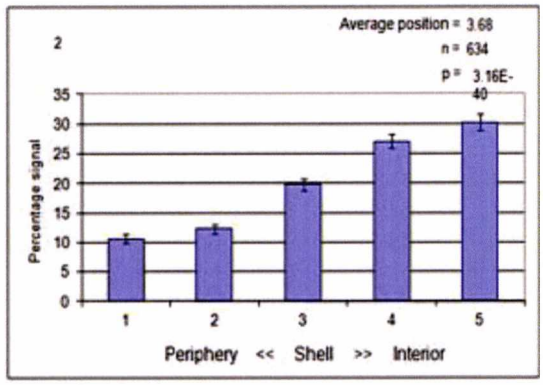
Figure 1: Graphs for pooled chromosome positions in normal (left) and abnormal (right) embryos. Graphs show preferential positions for loci form all 24 chromosomes (labelled in top left of each graph). Avarage positions, number of cells analysed and p values from chi squared test is given in the top right in each graph. When p value is less than 0.05 position for the chromosome loci analysed considered to be significant. When p value is higher than 0.05 distribution is considered as random.

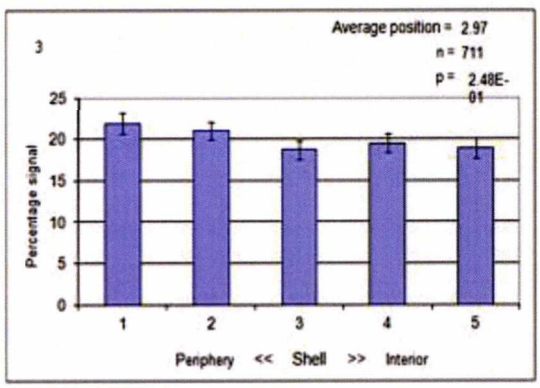
10.3.2.Graphs demonstrate the preferential position of chromosome, when nuclei are compared blastomere by blastomere

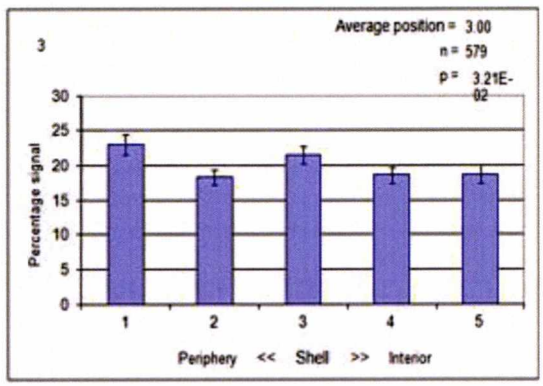


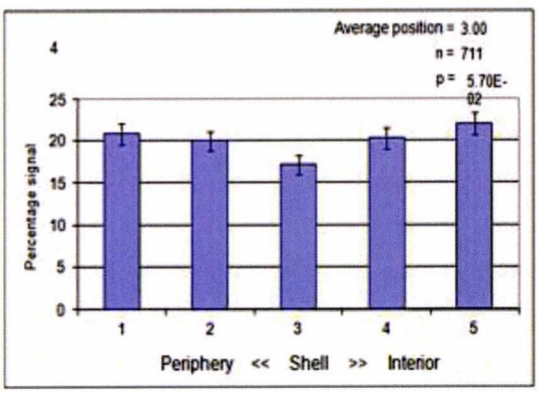


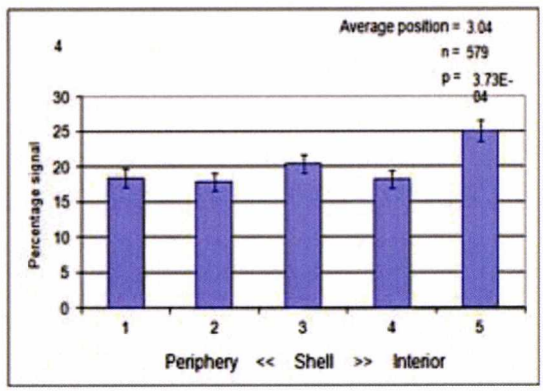


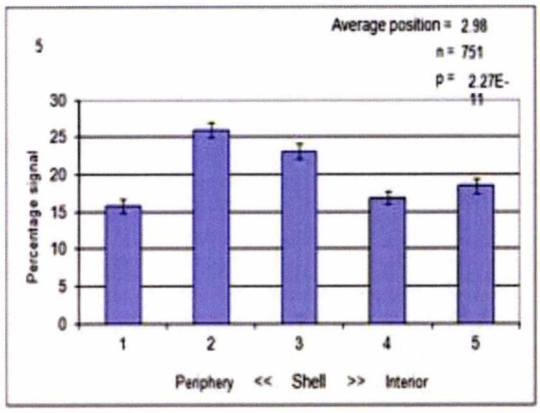








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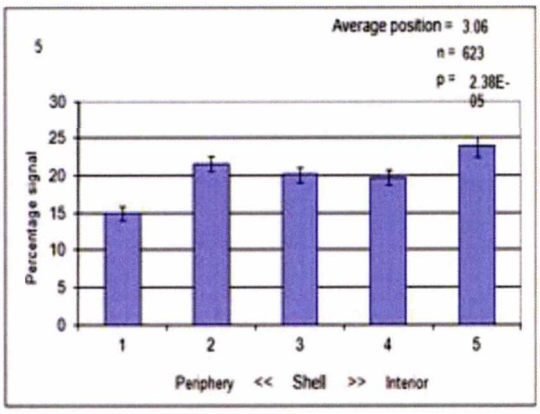
n = 751
p = 2.27E-11
Percentage signal
Periphery << Shell >> Interior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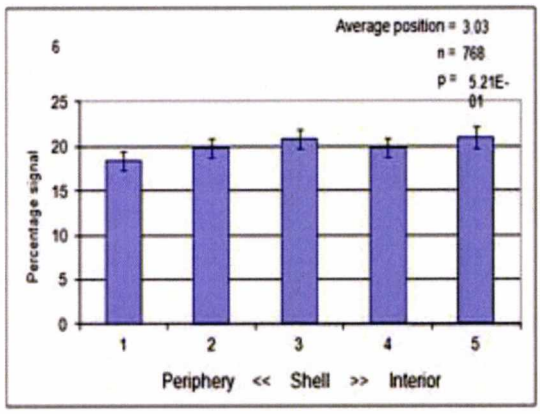
p = 2.38E-05
Percentage signal
Periphery << Shell >> Interior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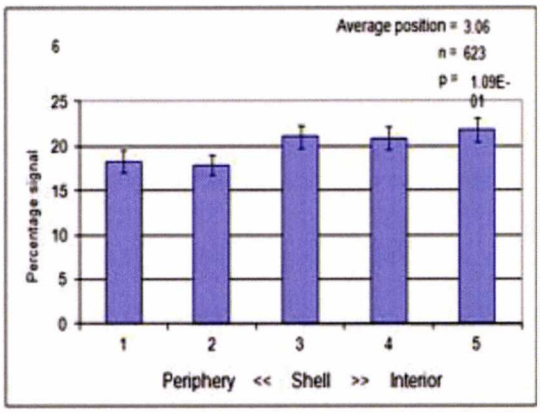
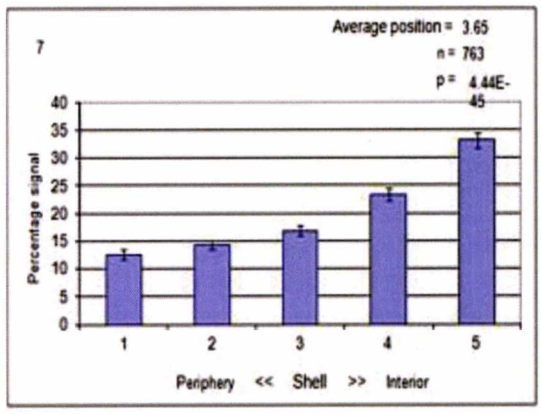
Percentage signal
Periphery << Shell >> Interior


6
Average position = 3.06
n = 623
p = 1.09E-01
Percentage signal
Periphery << Shell >> Interior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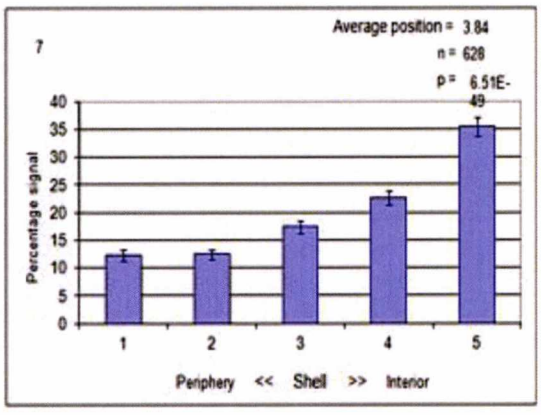
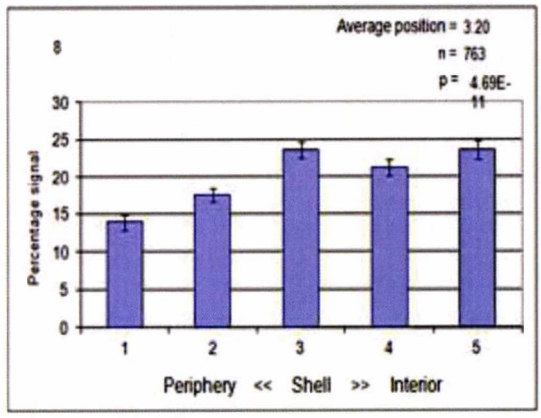
Average position = 3.65
n = 763
p = 4.44E-45
Percentage signal
Periphery << Shell >> Interior


7
Average position = 3.84
n = 628
p = 6.51E-49
Percentage signal
Periphery << Shell >> Interior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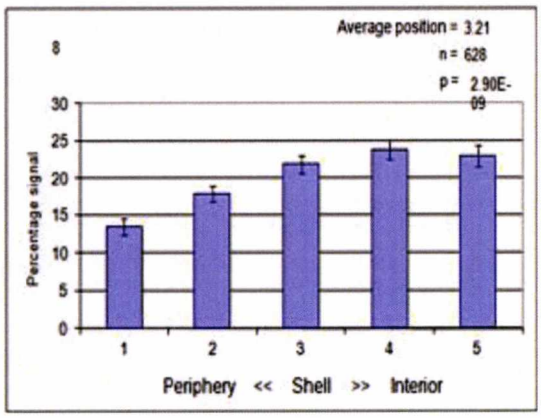
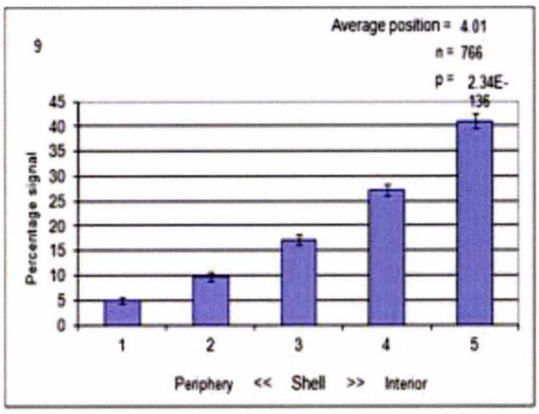
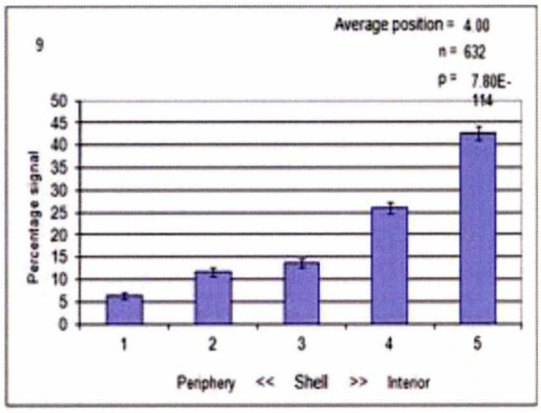
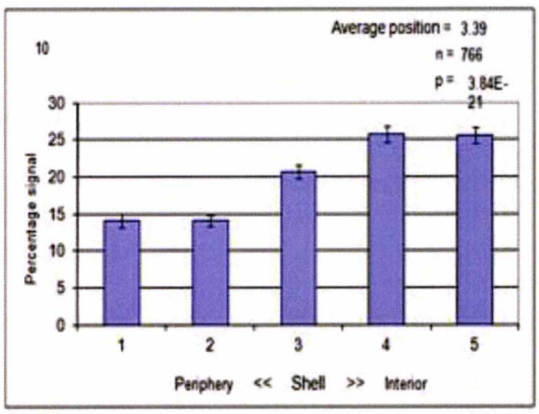
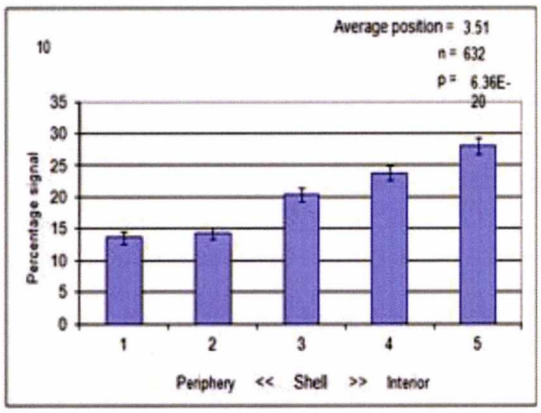
Average position = 3.20
n = 763
p = 4.69E-11
Percentage signal
Periphery << Shell >> Interior

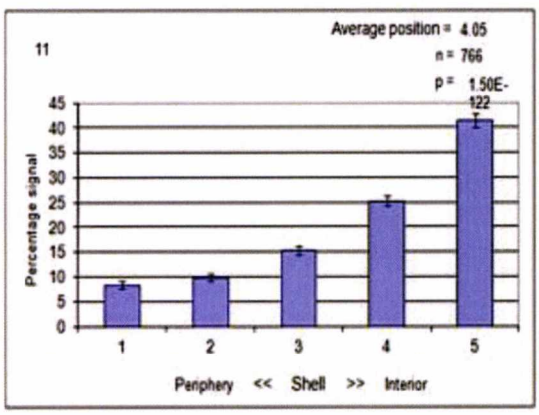

8
Average position = 3.21
n = 628
p = 2.90E-09
Percentage signal
Periphery << Shell >> Interior

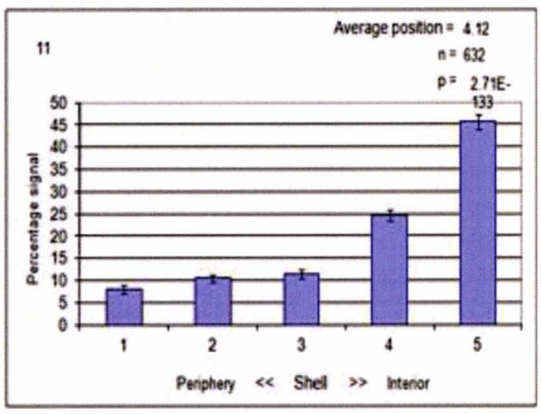

9
Average position = 4.01
n = 766
p = 2.34E-136
Percentage signal
Periphery << Shell >> Interior

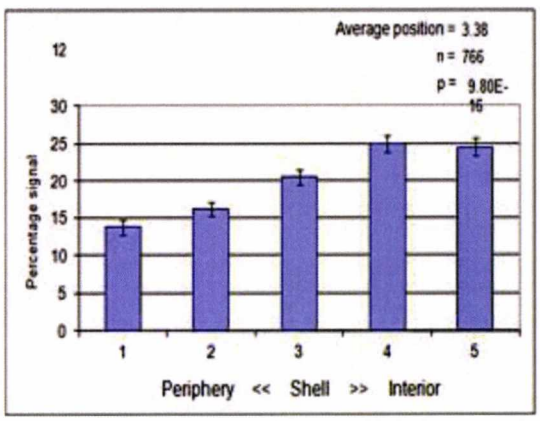

9
Average position = 4.00
n = 632
p = 7.80E-114
Percentage signal
Periphery << Shell >> Interior

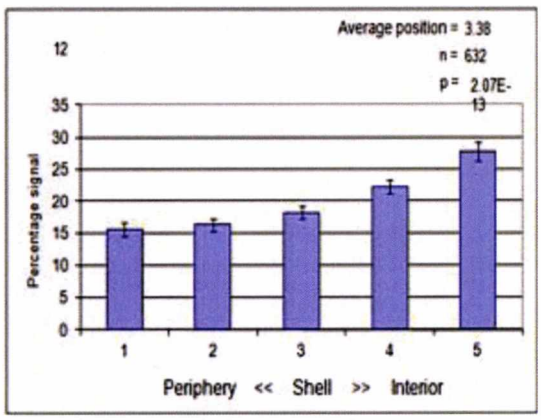

10
Average position = 3.39
n = 766
p = 3.84E-21
Percentage signal
Periphery << Shell >> Interior

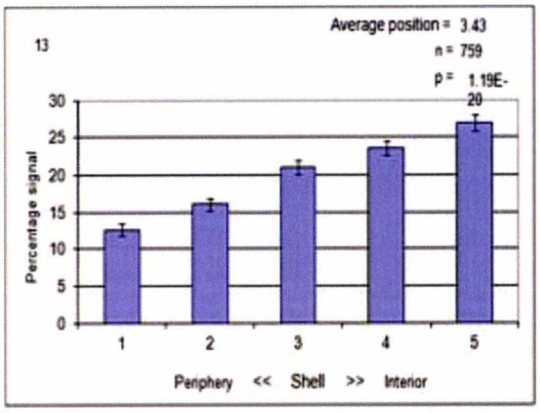

10
Average position = 3.51
n = 632
p = 6.36E-20
Percentage signal
Periphery << Shell >> Interior

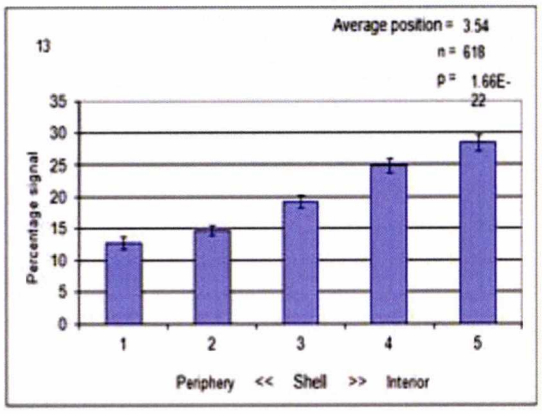

11
Average position = 4.05
n = 766
p = 1.50E-122
Percentage signal
Periphery << Shell >> Interior

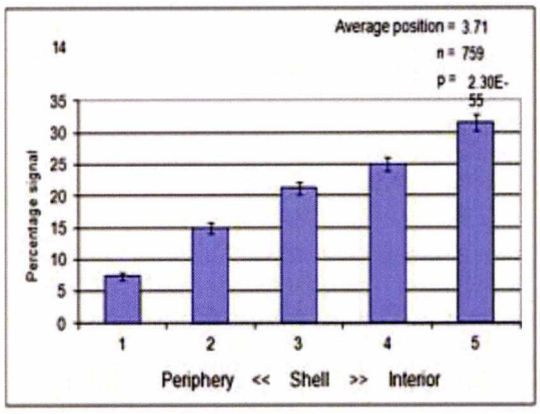

11
Average position = 4.12
n = 632
p = 2.71E-133
Percentage signal
Periphery << Shell >> Interior

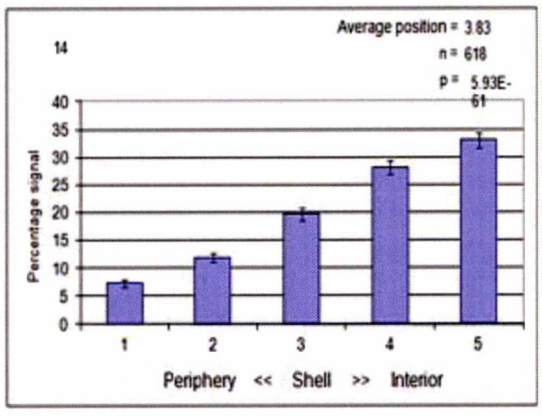

12
Average position = 3.38
n = 766
p = 9.80E-16
Percentage signal
Periphery << Shell >> Interior

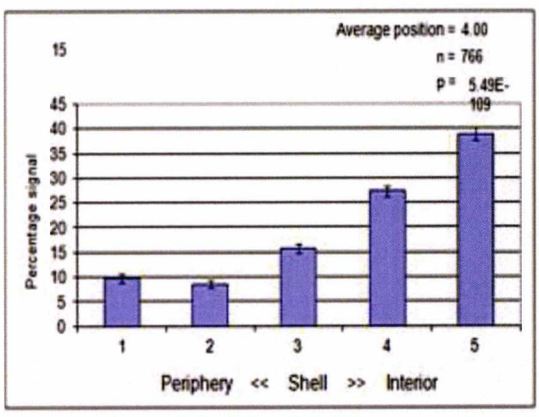

12
Average position = 3.38
n = 632
p = 2.07E-13
Percentage signal
Periphery << Shell >> Interior

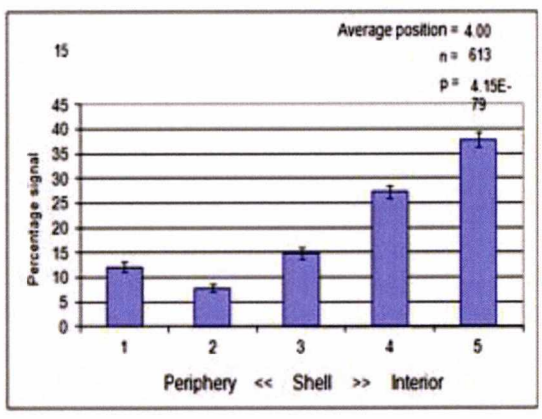

13
Average position = 3.43
n = 759
p = 1.19E-20
Percentage signal
Periphery << Shell >> Interior

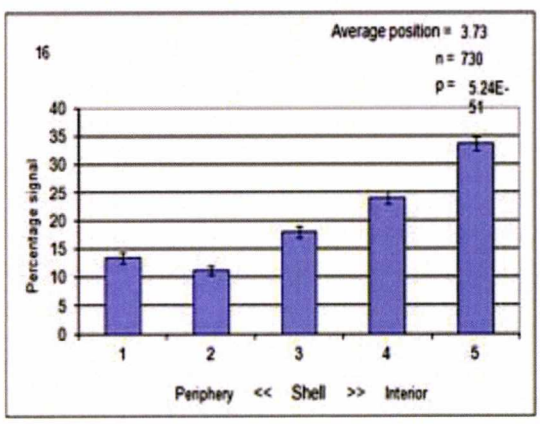

13
Average position = 3.54
n = 618
p = 1.66E-22
Percentage signal
Periphery << Shell >> Interior

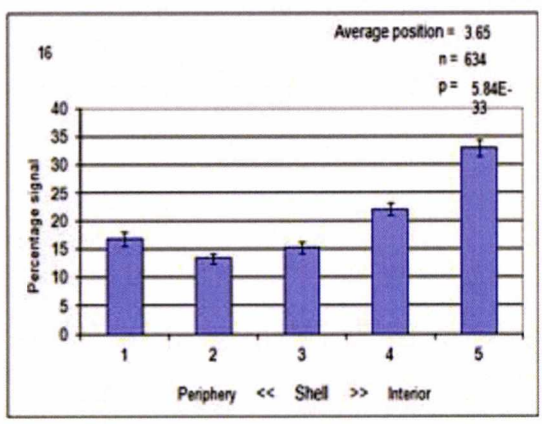

14
Average position = 3.71
n = 759
p = 2.30E-55
Percentage signal
Periphery << Shell >> Interior

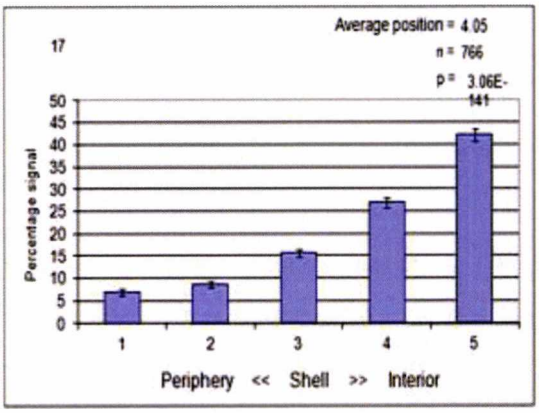

14
Average position = 3.83
n = 618
p = 5.93E-61
Percentage signal
Periphery << Shell >> Interior


15
Average position = 4.00
n = 766
p = 5.49E-109
Percentage signal
Periphery << Shell >> Interior


15
Average position = 4.00
n = 613
p = 4.15E-79
Percentage signal
Periphery << Shell >> Interior


16
Average position = 3.73
n = 730
p = 5.24E-51
Percentage signal
Periphery << Shell >> Interior


16
Average position = 3.65
n = 634
p = 5.84E-33
Percentage signal
Periphery << Shell >> Interior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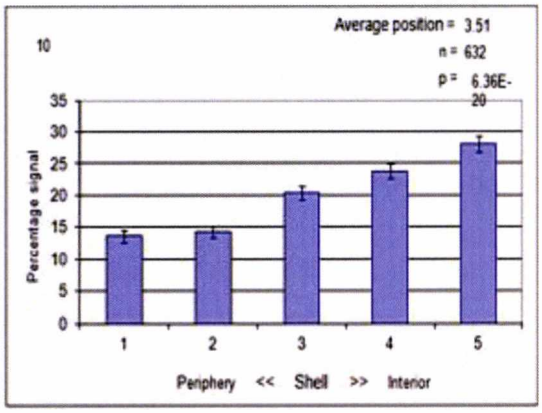
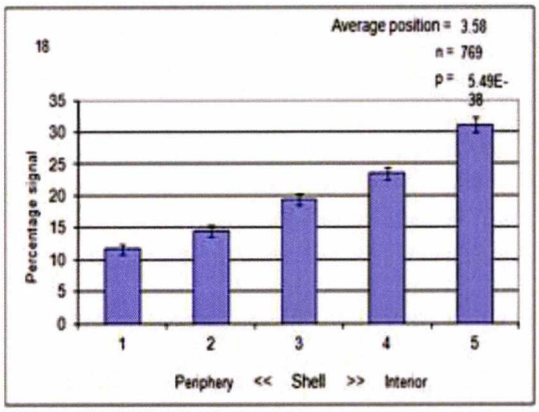
n = 766
p = 3.06E-141
17
Percentage signal
Periphery << Shell >> Interior


Average position = 3.51
n = 632
p = 6.36E-20
10
Percentage signal
Periphery << Shell >> Interior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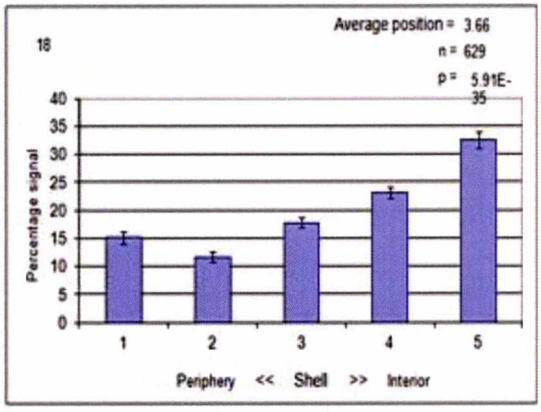
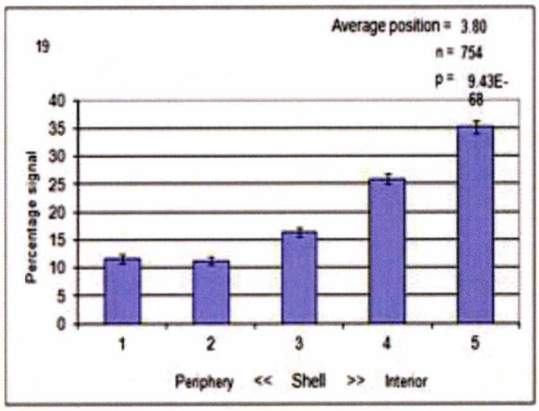
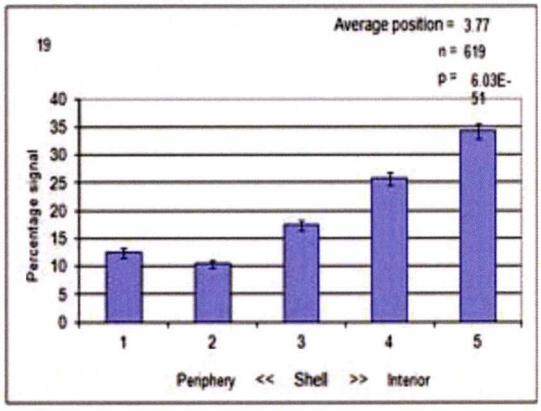
n = 769
p = 5.49E-38
18
Percentage signal
Periphery << Shell >> Interior


Average position = 3.66
n = 629
p = 5.91E-35
18
Percentage signal
Periphery << Shell >> Interior


Average position = 3.80
n = 754
p = 9.43E-68
19
Percentage signal
Periphery << Shell >> Interior


Average position = 3.77
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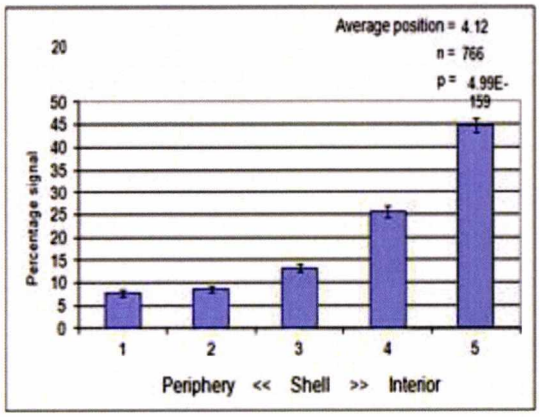
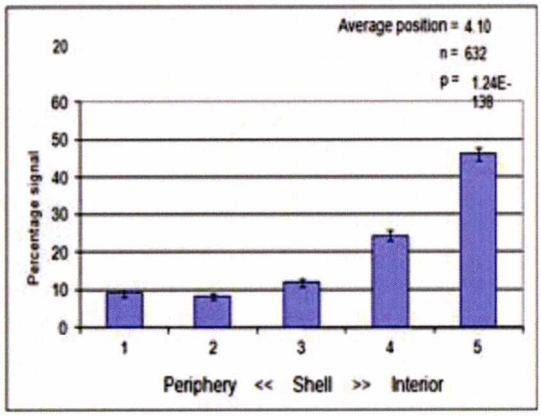
p = 6.03E-51
19
Percentage signal
Periphery << Shell >> Interior


Average position = 4.12
n = 766
p = 4.99E-159
20
Percentage signal
Periphery << Shell >> Interior


Average position = 4.10
n = 632
p = 1.24E-138
20
Percentage signal
Periphery << Shell >> Interior

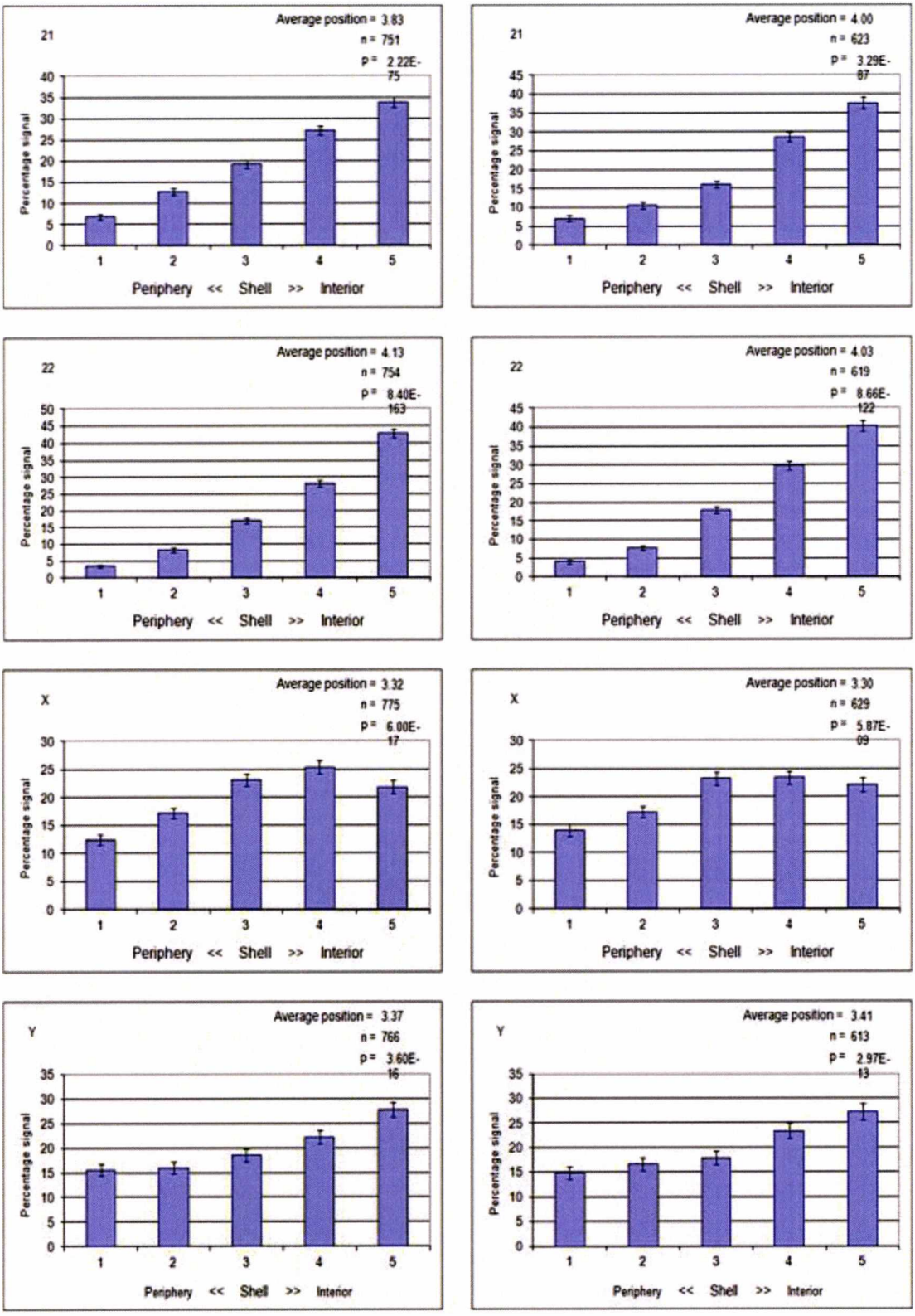


Figure 2: graphs for pooled chromosome positions in normal (left) and abnormal (right) embryo cells from cell by cell analysis. . Graphs show preferential positions for loci form all 24 chromosomes (labelled in top left of each graph). Avarage positions, number of cells analysed and p values from chi squared test is given in the top right in each graph. When p value is less than 0.05 position for the chromosome loci analysed considered to be significant. When p value is higher than 0.05 distribution is considered as random.

10.3.3.To assess whether nuclear organisation is affected by the spreading tequniques by comparing current and previous data.

Table 1: median positions of normal and abnormal cells analysed by embryo by embryo and cell by cell basis using above 2 spreading techniques and p values from T test.

	Cell by cell analysis				Embryo by embryo analysis			
	Combine method		HCL tween		Combine method		HCL tween	
	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
1	4	4	3.91	3.95	4	4.02	3.84	4
2	3.64	3.68	3.18	3.62	3.59	3.76	3.48	3.58
3	2.97	3	3.14	3.57	3	2.99	3.54	3.34
4	3	3.04	3.08	3.48	3	3.01	3.36	3.53
5	2.98	3.06	3.32	2.96	2.94	3.1	3.02	3.07
6	3.03	3.06	3.16	3.47	3.05	3.06	3.27	3.76
7	3.65	3.84	3.58	3.74	3.58	4	3.71	3.65
8	3.2	3.21	3.29	3.49	3.17	3.27	3.51	3.38
9	4.01	4	3.5	3.75	4.02	4	3.77	3.64
10	3.39	3.52	3.85	3.66	3.41	3.48	3.71	3.58
11	4.05	4.12	3.9	3.71	4.02	4.2	3.9	3.5
12	3.33	3.37	3.76	3.72	3.37	3.38	3.72	3.75
13	3.43	3.54	3.57	3.08	3.45	3.49	3.45	3.96
14	3.71	3.38	3.88	4	3.69	3.87	3.88	4
15	4	4	4.15	4	4.03	4	4.01	4
16	3.73	3.65	4.04	3.81	3.77	3.59	4	3.81
17	4.05	4.05	4.26	4	4.05	4.08	4	4
18	3.57	3.66	3.88	3.65	3.66	3.55	3.83	3.37
19	3.8	3.77	-	-	3.79	3.78	3.25	3.35
20	4.12	4.1	4.18	4	4.13	4.08	4	4
21	3.83	3.3	4.23	4	3.86	4	4.23	4
22	4.13	4.03	4.58	4.13	4.11	4.05	4.34	4
X	3.32	3.3	4	3.35	3.32	3.32	3.81	3.29
Y	3.37	3.41	4	3.98	3.56	3.08	4	4

	Compare 2 methods by Cell by cell analysis		Compare 2 methods by Embryo by embryo analysis	
T test P values	Normal –Normal	Abnormal- Abnormal	Normal –Normal	Abnormal- Abnormal
	0.18	0.26	0.22	0.57

Table above compares HCl/Tween method and methanol:acitic acid/HCl tween combination method interms of nuclear organisation results; firstly using cell by cell analysis for normal and abnormal embryo cells and then using embryo by embryo analysis for normal and abnormal embryos. For each category median position of all 14 chromosomes were compared using studnt T test. When $p<0.05$ resutls are significant at 95% confidence level.