Cassandra Francelina Sousa Santos

Licenciada em Bioquímica

Non-invasive Profile of Embryo Culture Media as a Preimplantation Genetic Diagnosis for Aneuploidy

Dissertação para obtenção do Grau de Mestre em

Bioquímica para a Saúde

Orientador:

Doutor Luís Gafeira Gonçalves, PhD, Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa

Coorientador:

Doutora Sofia Gouveia Nunes, PhD, Instituto Valenciano de Infertilidade em Reprodução Medicamente Assistida, Lisboa

24 de Março de 2022

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24 de Março de 2022

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Agradecimentos

Neste último ano e meio, para além do árduo trabalho e dedicação completa que o ano de dissertação exige, tivemos que lidar com todos os entraves, fragilidades, medos e angústias que a pandemia impôs. Foi um acontecimento ímpar, que ainda hoje nos afeta e que no futuro, mais que superar este problema, teremos de aprender a viver com ela e ter em conta todas as lições que ela nos deu. Num mestrado dedicado à ciência, como é o caso de Bioquímica para a Saúde, compreendemos o quão complexa é uma investigação científica e como foi sobre-humano o trabalho dos cientistas envolvidos em todas as fases de desenvolvimento de tratamento/ prevenção da COVID-19. Contudo, esta pandemia demonstrou as fragilidades de comunicar ciência de forma simples e direta. A ciência é uma interpretação objetiva da realidade; e ela não quer saber dos nossos quereres, nem desejos, ideologias, crenças ou opiniões. Quero aproveitar e começar por agradecer a todos os cientistas, profissionais de saúde e políticos que aguentaram esta luta em duas frentes, contra um vírus e contra a desinformação.

A elaboração desta dissertação de mestrado contou com a contribuição de vários apoios fulcrais, sem os quais esta etapa académica e pessoal não poderia ter sido concluída. Assim sendo, e porque "*It takes a village*", quero expressar os meus sinceros agradecimentos a todas as pessoas que, tanto direta como indiretamente, contribuíram.

Em primeiro lugar, quero agradecer à Doutora Sofia Nunes por ter acreditado e lutado por este projeto desde o dia 1. Agradeço-lhe pela oportunidade, pela confiança que depositou em mim, por toda a paciência, apoio, resiliência e motivação. Agradeço também ao Doutor Luís Gonçalves por ter aceitado embarcar nesta aventura connosco e por ter feito tudo para levarmos este projeto a bom porto, pela sua orientação, pelas suas dicas e por toda a sua disponibilidade.

O meu profundo agradecimento ao Instituto Valenciano de Infertilidade de Reprodução Medicamente Assistida (IVI RMA) por ter financiado este projeto. Agradeço também às instituições da Universidade Nova de Lisboa que me receberam durante estes últimos 27 meses: FCT, ITQB e NMS; e aos coordenadores deste mestrado, Professora Doutora Teresa Catarino, Professor Doutor Pedro Matias e Professor Doutor Sebastião Rodrigues, pelo apoio, ânimo e esforços devotados a este projeto.

A toda a equipa do IVI RMA Lisboa, agradeço por me receberem e guiarem tão bem. Um agradecimento especial ao David Torrado, por me ter acompanhado e apoiado no trabalho clínico; bem como à Doutora Carla Jorge e ao Doutor Samuel Ribeiro, que acompanharam o desenrolar do projeto e fizeram com que tudo isto se tornasse realidade. A todos os meus colegas do grupo laboratorial Proteómica de Organismos Não-Modelo, agradeço o companheirismo e apoio. À Professora Doutora Ana Varela Coelho agradeço por me ter acolhido no seu grupo de investigação, por ter estimulado o meu pensamento crítico e por toda a atenção e cuidado que dedicou ao meu projeto.

Agradeço à minha incondestável FAM de sempre, Beatriz Santos, Manuela Eusébio e Sónia Portugal, por estarem presentes em todas as etapas (importantes ou não) da minha vida, pelo vosso apoio, por me manterem humilde (eu bem sei o que custa não se rirem das minhas piadas incríveis), pela vossa boa disposição, leveza, motivação e pelos diabetes que provavelmente me irão causar no futuro.

Disseram-me que os amigos que se fazem na faculdade são para a vida, espero que assim seja, porque eu tive o privilégio de conhecer pessoas incríveis! À Isabel Lemos, agradeço de forma abesbílica por nunca ter dado chance aos erros de logística e por ter aprendido com ela que há verdades sobrevalorizadas na vida. À Carolina Buga, Rita Marrazes, Isabel Barata, Joana Bernardino, Madalena Parrado e Sara Salgado, agradeço pela vossa amizade e empatia, que nem a distância se interpôs, pelo apoio e por todos os desafios que travamos juntas. Os melhores momentos que levo desta etapa são com vocês. Espero que a vida vos traga muitos sucessos!

Finalmente, agradeço a toda a minha família! Este último ano ensinou-me o quão imprescindível (e frágil) é a vossa presença na minha vida. Aos meus avôs, obrigada pelo vosso carinho, por todas as chamadas e preocupação (e por verem a CM*tv* e me avisarem das greves com antecedência), mas principalmente pela vossa presença na minha vida! Aos meus patrocinadores pessoais, pai e mãe, agradeço-vos com tudo o que sou tudo aquilo que fizeram por mim, pelos vossos sacrifícios, por tudo aquilo que abdicaram em prole do meu futuro e do João e por nunca terem deixado de acreditar nos meus sonhos (mesmo quando eu o fiz). Obrigada por terem criado um lar onde sempre me senti segura e respeitada, repleto de amor (às vezes demais), por me incentivarem a voar e serem a minha rede de segurança. (Se fazer 684km em agosto sem ar condicionado não é sinónimo de amor incondicional, então não sei o que será.) Não existe nada neste mundo que retribua tudo o que fizeram por mim. Ao meu irmão, por partilhar o meu sentido de humor e alinhar sempre nas minhas brincadeiras. Ver-te a lutar pelo teu futuro e pela pessoa que queres ser, inspira-me a fazer o mesmo.

"Justice weights her scales, sets you this trial:
no wisdom without pain. And all you dream,
your fragile futureness—best let it go,
best wait until you see, not hope unseen.
Clarity comes with time, and it dawns meanwhile."

Agamemnon, by Aeschylus

Resumo

A aneuploidia é o principal fator de risco associado com a falha na implantação e desenvolvimento do embrião. Atualmente, os métodos de teste e diagnóstico precoce de aneuploidias embrionárias debatem-se com várias limitações: invasividade, consomem bastante tempo, são dispendiosos, requerem técnicos altamente especializados e vários passos de manuseamento embrionário até se obter o diagnóstico. Assim, o desenvolvimento de métodos de deteção de biomarcadores específicos para a aneuploidia no meio de cultura embrionária (MCE) é bastante importante. O objetivo desta dissertação é aplicar a metabolómica utilizando ^{1}H-NMR na deteção precoce da ploidia embrionária, assim como obter mais informações acerca das alterações metabólicas entre embriões euplóides e aneuplóides. Neste estudo, os metabolitos presentes no MCE foram quantificados utilizando espetros de ^{1}H-NMR em dois períodos sequenciais de incubação embrionária e a concentração dos metabolitos foi utilizada para a construção de modelos de classificação PLS-DA. As maiores diferenças foram observadas no dia 5, período no qual os embriões euplóides apresentaram uma atividade metabólica mais elevada, com especial ênfase na metabolização de aminoácidos essenciais. Este estudo indicou que a ploidia tem um impacto direto na atividade metabólica do embrião. Os resultam mostram que o modelo de classificação conseguiu discriminar entre os embriões euplóides e aneuplóides, com 92% e 83% de exatidão na sua capacidade de previsão no dia 3 e 5, respetivamente. Apartir deste estudo preliminar foi possível observar que o perfil metabólico do MCE oriundo da incubação de embriões aneuplóides está alterado face ao dos embriões euplóides. Ainda, o modelo de previsão construído teve uma alta exactidão. Seria interessante aplicar este conhecimento adquirido na prática clínica, de forma a diminuir o tempo até se atingir uma gravidez, os custos associados e os danos inflingidos no embrião quando se utilizam técnicas de diagnóstico invasivas.

Palavras-chave: Aneuploidia, Meio de Cultura Embrionário, Técnicas de Procriação Medicamente Assistida, ^{1}H-NMR, Biomarcadores, Metabolómica

Abstract

Aneuploidy is the leading risk factor associated with implantation and embryo's development failure. Nowadays, the screening methods for early diagnosis of embryo aneuploidy struggle with several limitations such as: invasiveness, be very time-consuming and costly, requirement of highly specialized technicians and several manipulation steps of embryo until diagnosis. For that, the development of a method for detect specific aneuploidy biomarkers in the spent embryo culture media (SECM) is of high interest. The objective of this dissertation was to apply ^{1}H-NMR based metabolomics for the detection of embryo's ploidy, and, as such, gain more insights in the metabolic alterations among euploid and aneuploid embryos. In this study, the metabolites present in the SECM were quantified using ^{1}H-NMR spectra at two embryo sequential incubation times, and the metabolites concentrations were used to build a PLS-DA classification model. The major differences were observed in day 5, in which euploid embryos tend to have a higher metabolic activity, with an especial uptake of essential amino acids. This study indicates that ploidy has a direct impact on embryo's metabolic activity. Results show that a classification model could discriminate between aneuploid and euploid embryos, with a 92% and 83% of prediction accuracy in day 3 and day 5 model, respectively. From this preliminary study it was possible observe that SECM profile of aneuploid embryos is altered compared to euploid embryos, and it was obtained an accurate predictive model. This workflow could be interesting to apply in clinical practice, to potentially decrease time-to-pregnancy, costs and harms inflicted to the embryo during invasive aneuploidy diagnosis approaches.

Keywords: Aneuploidy, Spent Embryo Culture Media, Assisted Reproductive Techniques, ^{1}H-NMR, Biomarkers, Metabolomics

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Abbreviation list

1D: one-dimensional

^{1}H: proton

2D: two-dimensional

AI: artificial intelligence

ASEBIR: *Asociación para el Estudio de la Biología de la Reproducción*

AFC: antral follicle count

AMH: anti-Müllerian hormone

ART: assisted reproductive technologies

ATP: adenosine triphosphate

AUC: area under curve

BMI: body mass index

CNEAA: Conditionally non-essential amino acids

ChA: chromosomal abnormalities

CNPMA: *Conselho Nacional de Procriação Medicamente Assistida*

CO_2: carbon dioxide

CoA: coenzyme A

D_2O: deuterium oxide

DFA: discriminant function analysis

DNA: deoxynucleotide acid

EAA: essential amino acids

ECM: embryo culture media

ESHRE: European Society of Human Reproduction and Embryology

ETs: embryo transfers

$FADH_2$: flavinadenine dinucleotide

FDR: false discovery rate

FID: free induction decay

FISH: fluorescence *in situ* hybridization

FSH: follicle stimulating hormone

GCP: good clinical practices

GnRH: gonadotrophin-releasing hormone

HCG: human chorionic gonadotrophin

HMDB: human metabolome database

HPLC: high-pressure liquid chromotography

HSQC: heteronuclear single quantum coherence

ICSI: intracytoplasmic sperm injection

IR: infra-red

IRB/IEC: Institutional Review Board/ Independent Ethics Committee

IU: international units

IVF: *in vitro* fertilization

IVI: Instituto Valenciano de Infertilidad

LH: luteinizing hormone

MCE: meio de cultura embrionária

mRNA: messenger ribonucleic acid

MS: mass spectrometry

NaH_2PO_4: monosodium phosphate

Na_2HPO_4: disodium hydrogen phosphate

NADH: nicotinamide adenine dinucleotide

NEAA: non-essential amino acids

NGS: next generation sequencing

NMR: nuclear magnetic resonance

NPB: nucleolar precursor bodies

O_2: oxygen

orthoPLS-DA: orthogonal partial least squares discriminant analysis

OS: ovarian stimulation

PCA: principal component analysis

PCR: polymerase chain reaction

PGT: preimplantation genetic testing

PGT-A: preimplantation genetic testing for aneuploidy

PLS: partial least squares

PLS-DA: partial least squares discriminant analysis

ppm: parts per million

PPP: pentose phosphate pathway

PVP: polyvinylpyrrolidone

RIF: recurrent implantation failure

ROC: receiver operating characteristics

ROS: reactive species of oxygen

SAC: spindle assembly checkpoint

SECM: spent embryo culture media

SET: single embryo transfer

SNP: single nucleotide polymorphism

TCA: tricarboxylic acid

TOCSY: total correlation spectroscopy

TSP: 3-(trimethyl-silyl)propionic-2,2,3,3-d_4 acid sodium salt

VIP: Variable Importance in Projection

WHO: World Health Organization

1. Introduction

1.1. ART, Aneuploidy, and Infertility: The Good, the Bad, and the Ugly

Fertility is the capacity of generate descendants in result of same specie individuals crossing, and it has been regarded as a fundamental requirement for the survival and perpetuation of species. Recent data indicates that the rate of the capacity to conceive and produce a live birth in women of peak reproductive age who has a regular menstrual cycle and engages in regular unprotected sex is only of about 22% (1). Nowadays, epidemiological studies have shown an increase in the prevalence of human infertility worldwide. According to World Health Organization (WHO), infertility can be defined as the inability to achieve a pregnancy after regular and unprotected sexual intercourse for 12 months (2). Infertility affects about 15% of the couples worldwide in fertile age, amounting up to 186 million couples, and it is estimated to affect 300 thousand of Portuguese couples (3, 4). About 30% of cases are due to female infertility, other 30% are due to male infertility, the combination of both male and female infertility amount about 30%, and the last 10% of the couple infertility are idiopathic (5, 6). In developing countries the percentages are even more disturbing, since it is predicted to affect 50% of all women (7). Associated with this low human fertility, about 1 in every 8 clinical pregnancies results in fetal loss (8).

Research data suggest that lifestyle factors, as fat-rich diets, smoking, alcohol/ drug misuse, anxiety/ depression, physical activities, and radiation exposition, play an important role in the fertility of the individual (7). The most common cause of male infertility is a health condition called varicocele, which leads to one or both of the testicular veins draining from the testes to became swollen, resulting in poor circulation and spermatozoa's malnutrition (9). The semen analysis has been used as the primary biomarker to evaluate male fertility and the overall health in the preceding 2 months. The main indicators of male infertility diagnosis are a low sperm count, low total and progressive motility and aberrant sperm morphology (10). Although men are able to retain their fertile potential into old age, men with fifty years older have a decreased fertility and their offspring have higher incidence on the development of congenital anomalies (8). However, despite some factors have been correlated with male infertility, up to 80% of male infertility remains idiopathic. Recent studies indicate the need of new male fertility biomarkers (11). In a recent study with a large cohort (n=140 men presenting with recurrent pregnancy loss), 40% of male partners resorting to assisted reproductive technologies (ART) with normal semen parameters and with a couple history of recurrent pregnancy loss, had significantly high levels of sperm aneuploidy (11). In the last years, evidence have grown to suggest aneuploidy as an important factor contributing for male infertility (12). In women, the main causes for infertility are anovulation, premature ovarian failure, pelvic inflammatory disease, polycystic ovarian syndrome, and endometriosis. Since women born with all their gametes formed that remain dormant until menarche, these prolonged dormancy largely contributes to the higher incidence of chromosomal abnormalities (ChA) in their offspring (13).

Nowadays, couples struggling with fertility have a large variety of medical options to help them conceive a healthy baby. Due to the low rates of viable embryos, around 10% of them suffer from recurrent implantation failure (RIF), even after multiple cycles of in vitro fertilization (IVF) (14). The success behind ART procedures, as embryo transfer (ET) and implantation, are mainly determined by embryo quality and number of embryos per transfer (15). There are many key steps involved in achieving a healthy euploid embryo. It starts with the formation of the gametes itself, their union and subsequent cellular divisions. These are meticulous and sensitive steps and any error can result in an aneuploid embryo, which is associated with low implantation and development rates (1). Aneuploidy has been considered the "bad guy" in ART, since it is the main cause for the high unsuccessful rates (16). It was proposed that it is more likely occurred RIF due to embryonic origins than involve endometrial receptivity, as it was previously proposed (17). A recent study with a larger cohort of sequential ET indicates that RIF is rare when it is use euploid embryos (18).

1.2. How to Build a Human? – a series doomed to unfortunate events

The main aim of the human reproductive system is to produce differentiated and mature male and female gametes in order to, after their union, give rise to a new self-organism. Unlike any other anatomical structure, the reproductive system is not functional at birth. Only around puberty by the influence of sex hormones, it matures and becomes completely active and operational for its purpose. Both female and male reproductive systems are required to produce two types of gametes that complement each other in their common purpose of producing offspring (6, 9).

The human genome has approximately 23,000 genes comprised on 22 pairs of homologous chromosomes, and 1 pair of sex chromosomes, in an euploid individual (figure 1.2.1.) (19). The gametes transport only half of the maternal and paternal chromosomes, thus after their union the human ploidy is restored. Since the genome influences the future traits of the new formed organism, is essential to prevent any possible errors during their development. Although the existence of mechanisms that ensure the complete genome inheritance during cell division, the process is not entirely error-proof. Major variations in DNA and their functional repercussion, alongside the environmental component, contribute to disease processes, some of them not compatible with life. This can lead to cells with ChA, as numerical abnormalities (aneuploidy) or structural abnormalities. Other genetic anomalies identifiable in humans non-related to the mitotic division are related to single gene disorders (e.g., cystic fibrosis, phenylketonuria and hemophilia), epigenetics and complex disorders (20). The main cause of death during infancy is due to genetical and developmental anomalies, in which aneuploidies account for 10% of major birth defects, and gene mutations account for an additional 8% (21). Although scientific advances have led to understand the genetic regulation of morphogenesis, and how cells are committed

to rearrange the different parts of the embryo and develop into a mature human being, there are limited information available regarding prevention and correction of congenital anomalies (22).

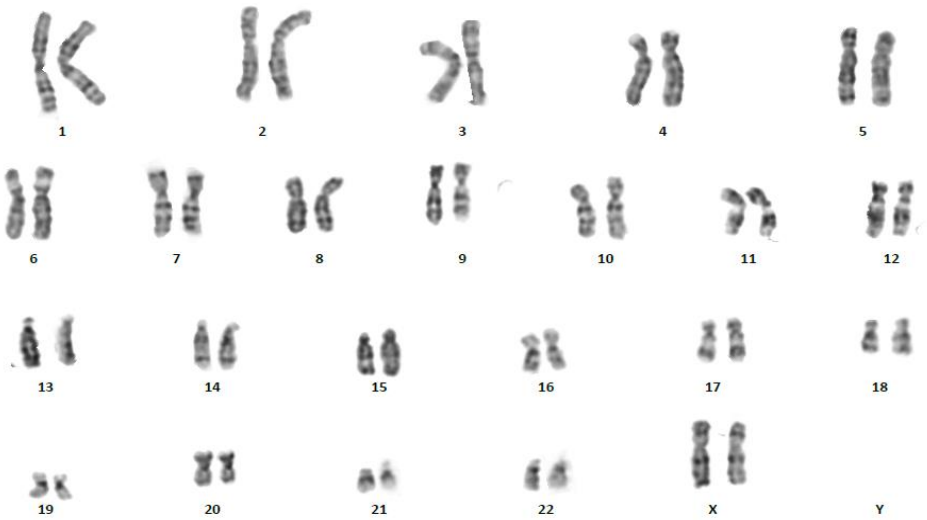

Figure 1.2.1. Euploid Human Karyotype. Display of 22 pairs of homologous chromosomes, and 1 pair of sex chromosomes, characteristic of an euploid cell. In a haploid cell karyotype, would be displayed only 1 of each pair of chromosomes. If any error occurs during gametogenesis or after fertilization, the karyotype may exhibit more/ less paired chromosomes, characteristic of an aneuploid cell. Image ceded by the cytogenetic laboratory of Institute of Biomedical Sciences Abel Salazar.

Gametogenesis is the specialized process of cell division and maturation of primordial germ cells within the functional organ of the reproductive system, that implies some rearrangements and distribution of chromosomes among the gametes. Gametogenesis comprises of an initial phase, in which the stem cells multiply and populate the gonads by mitosis; whereas in the second phase occurs the cell differentiation associated with the reduction to half of the cells' ploidy by meiosis. However, aiming the same purpose, gametogenesis in females and males (oogenesis and spermatogenesis, respectively) occur differently (1, 6, 9).

Aneuploidies can be a direct cause of errors occurred both in meiosis or in mitosis on an early embryo stage, due to malfunctions of recombination, improper spindle formation, microtubule-kinetochore interactions, or defects in the spindle assembly checkpoint (SAC) (23, 24). The errors in meiotic chromosome segregation arise at anaphase I and II. The majority of aneuploidies are a direct consequence of the homologous chromosomes non-disjunction, during anaphase I (16). Recent experimental studies in mice have observed that cells with nondisjunction chromosomes at the end of meiosis I are eliminated in males, but tolerated in females, highly contributing to aneuploidy in the offspring (25). Further investigation is required to ensure if a comparable mechanism occurs in humans.

The oogenesis diverges from spermatogenesis in several aspects: time of occurrence, number of gametes formed, multiplication and development steps, until the nuclear and cytoplasmic cell differentiation and maturation. A single man is able to produce roughly 200 to 300 million spermatozoa per day throughout his life, but it requires around 2 months for each spermatogonia to become four fully mature spermatozoa and only about 100 million become a viable mature gamete (9, 26). Differently,

newborn females possess fewer than 2 million primary oocytes. This number largely degenerates by atresia to about 40,000 during childhood until menarche, and of these only 400 are able to develop and to be ovulated (22). In contrast of what happens in spermatogenesis, one primary oocyte is only able to form one mature oocyte. When spermatozoa are ejaculated their process of meiosis and differentiation is fully finished. This is not the case in women. When they born their oocytes are in a resting stage trapped in prophase I. Only after menarche, a small fraction of their oocyte reserve is activated to continue meiosis and develop, but arrests it in metaphase II before ovulation. Meiosis II finishes, only after fertilization. The meiotic process of oogenesis is associated with an asymmetrical division of the cytoplasm, leading to the formation of one oocyte and two polar bodies. During the oocyte maturation, the formation of the first polar body indicates the finalization of meiosis I, and the formation of the second polar body is an indicative of the oocyte's fertilization (6, 21, 22, 27).

For that is easily comprehensible how the female factor is the most limiting of human fertility, womens only have a defined and reduced number of viable gametes in a strict time-window. However, oogenesis is associated with higher occurrences of errors. For instance, most of the aneuploidies in human embryos are originated from errors in maternal meiosis I, in a matter of fact it was reported that 3 to 5% of sperm cells and 20 to 25% of oocytes are aneuploid, in fertile and healthy people (12, 16). When comparing the timeline of oogenesis and spermatogenesis, the possible causality of error-prone stages of maternal meiosis becomes clearer. In the maternal cases of meiotic nondisjunction the errors in meiosis I are three times higher than in meiosis II, when in men they are equally error-prone (28). This is not entirely surprising given the long meiotic arrest in prophase I, which can last among 10 to 50 years, until their ovulation time (29). Recent studies have identified a direct correlation between parent age and the incidence of aneuploidies and mutations. Several hypothesis have been proposed to explain the maternal age effect: accumulation of toxic insults inflicted by the environment during the oocyte arrested stage; dysfunction of meiotic machinery (e.g. spindle function, sister chromatid adhesive proteins, microtubule motor proteins) over time, resulting in suboptimal resumption of oocyte meiotic stages; suboptimal hormonal signaling changes the ovarian functioning; and degradation of the female reproductive environment (16, 28-31). There is some evidence to suggest that only 36% of the embryos from women younger than 38 years were considered genetically normal (32), and the risk of trisomy increases from 1.9% in 25 to 29 years old women to over 19% in women with, at least, 39 years (20). Embryos from females with increased maternal age tend to have decreased implantation and pregnancy rates, fact that has been correlated to aneuploidy (33, 34). A direct correlation was identified between the advanced maternal age with the incidence of trisomy 21, due to the maternal meiotic nondisjunction (35). The same correlation has been observed regarding the paternal age. A 70-year-old is about eight times more likely to pass mutations than a 20-year-old man, due to the accumulation of mutations during the cellular cycle of spermatogonia. In contrast to autosomal aneuploidies, in which the maternal factor is higher, the father contributes more frequently to sex chromosome aneuploidies (36, 37). Aneuploid conceptuses involving autosome are paternally derived in 5-10% (12, 38).

1.3. Fertilization and Embryo Development

Human development begins at fertilization, the moment whereas occurs the fusion of spermatozoon with a mature oocyte and originates the zygote (figure 1.3.1.). During fertilization, the spermatozoon must penetrate a dense barrier: the corona radiata, perivitelline space, zona pellucida and oocyte cytoplasmic membrane (1). The main consequences of this process are the restitution of human cellular ploidy and the determination of the sexual identity. This diploid, totipotent, and unicellular zygote has the capacity to divide, migrate, growth, differentiate and, ultimately, become a multicellular human being (22).

Regardless of the millions of spermatozoa deposited in the vagina by a single ejaculation, only hundreds of them succeed to encounter with the oocyte. The spermatozoon that will fertilize the oocyte must be capacitated, in which stage the sperm cell tail gains a hyperactivated movement and the cytoplasmic membrane and the outer membrane of the acrosome are rearranged, preparing it to release the enzymes stored (6, 21, 39). The acrosome, by releasing enzymes to digest the corona radiata and zona pellucida, facilitates the linkage spermatozoon-zona pellucida (26). The first sperm cell to stablish the connection, merge its cytoplasmic membrane of the head with the oocyte membrane and crosses, entering within the cell (40). To prevent polyspermy, the membranes fusion activates a block pathway. The sum up of these events lead to the harden of zona pellucida and inactivation of the oocyte's ligands, and at the same time, also signalizes the oocyte to continue the cellular division from metaphase II (27). While oocyte finish meiosis, the nuclear membrane of the sperm cell desegregates and the chromatin decondense, forming the male pronucleus. Both male and female pronuclei migrate to the center of the cell and simultaneously start to replicate their DNA. The two pronuclei fuse and form a single diploid nucleus, the zygote, and fertilization ends (8).

At about 30 to 36 hours later, the zygote's chromosomes align and are pulled apart by the mitotic spindle from the male cell, and the first mitotic division of the cleavage phase begins (figure 1.3.1.). The embryo divides at 18 hour intervals thereafter (41). The resulting cells (blastomeres) of each mitosis contain half the cytoplasmic volume of the mother cell, being sequentially smaller (figure 1.3.1.). Only around day 4, these 16- to 32-cells acquire intercellular junctions and adhere between themselves, giving rise to the morula state. Usually, at this state the formed cluster of blastomeres already reached the uterus. The continuous cellular reorganization and compaction leads to the membrane polarization with an increase of the sodium-potassium pumps in the morula poles. These pumps allow the transport and regulation of metabolites, and the entry of water by osmosis, creating a cavity full of aqueous fluid. It can be identified a certain cellular organization with an outer cellular layer (trophoblast) that encloses the cavity (blastocele) underneath the zona pellucida, and an inner cell mass (embryoblast) that stands out from the rest of the cells in the center, it is now called a blastocyst (6, 9, 21, 27).

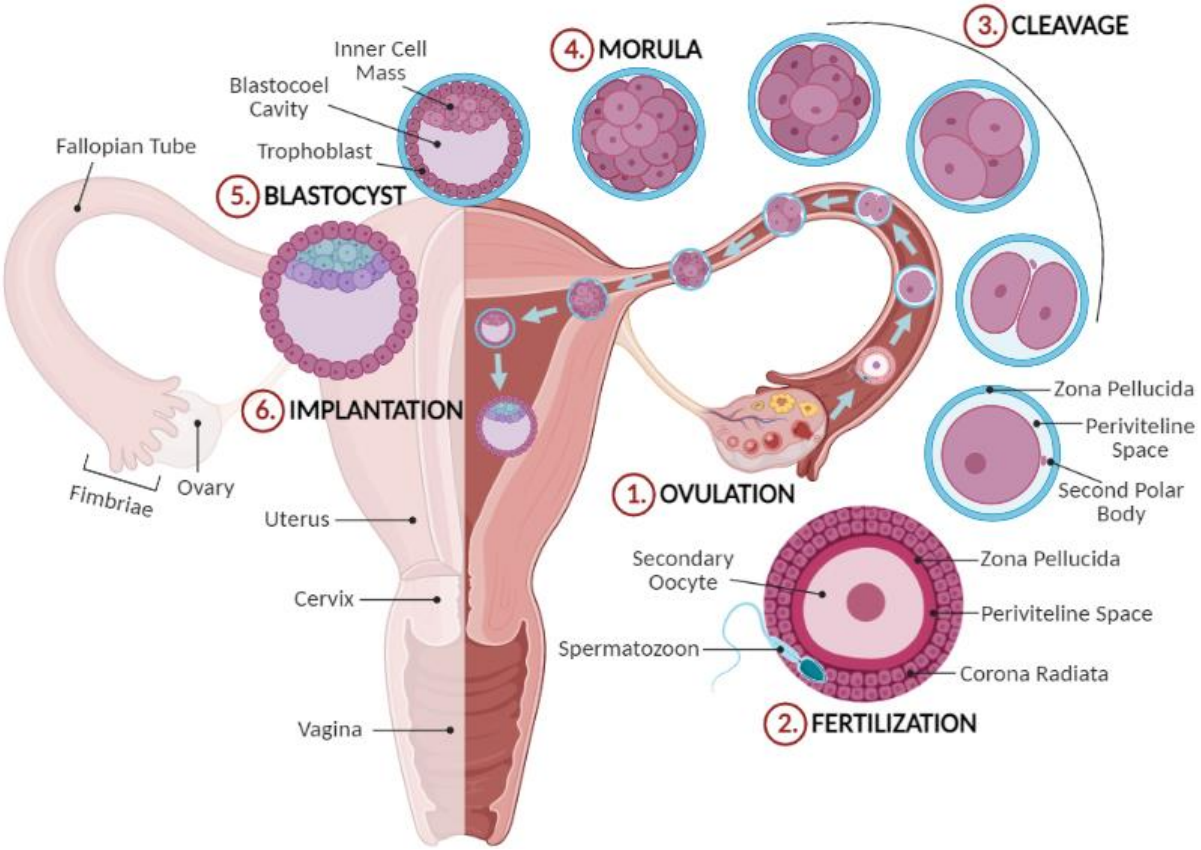

Figure 1.3.1. Early embryo development. The spermatozoa encounter the ovulated oocyte in the fallopian tube, and after fertilization the male and female nuclei unite and give rise to the zygote. The zygote then undergoes multiple cellular divisions (cleavage stage). When it reaches the morula stage, the cells begin to differentiate and form three main structures of the blastocyst: inner cell mass, blastocoel cavity and trophoblast. At this time, the embryo hatches from the zona pellucida, and is able to implant within the endometrium. This early functional and morphological development is associated with its migration from the fallopian tube until the uterus. (Created using Biorender.com)

Implantation is preceded by the secretion of enzymes by trophoblast, that bores a hole in the zona pellucida and releases the blastocyst. Implantation begins with the contact and ligand- mediated attachment between the blastocyst to the endometrial cells and ends with trophoblast cells invading and migrating through the uterine wall by the enzymatic digestion. The implanted embryo induces the formation of a vascular network, ensuring the required nutrition for its development (40, 42). The pregnancy, usually, lasts about 38 to 40 weeks (1). The road to fertilization that leads to a viable pregnancy is very challenging and it is not surprising that even with regular sexual intercourse the chance to achieve a pregnancy is very low.

1.4. Birth Defects and Spontaneous Abortions

Despite the tremendous difference in cellular size and chromatin organization between the sperm cell and the oocyte, the nuclear genome of the offspring results equally of them. To ensure the formation of an embryo with a normal number of chromosomes, the thorough meiotic process has to be completed successfully in both female and male gametes (1).

The majority of aneuploidies are indeed lethal, and even when they are not, they lead to severe developmental abnormalities and decreased lifetime. It is estimated that about 50% of pregnancies end in spontaneous abortion, and that around 35% of these abortions are due to aneuploidies (21, 43), and even after birth the average life expectancy and quality is very low (20). The data regarding aneuploidy in humans are, however, clearly underestimated, once it does not include information from spontaneous abortions during the first weeks of gestation. Aneuploidy is a fairly common feature among human embryos. Trisomies of small gene-poor chromosomes appear to be the most compatible to a normal karyotype development. On the other hand, autosomal monosomies are linked to early miscarriages. Withal, an abnormal number of sex chromosomes has a milder impact in the lifespan of the individual (20, 29). It is estimated that at least 5% of all human conceptions are aneuploid (16), mainly involving errors in the chromosomes 13, 18, 21, X and Y (33, 44), and the most common in spontaneous abortions are sex-chromosome monosomy, accounting for nearly 10% (16).

The genetic analysis of embryos following IVF have point it out that about 70% of embryos had mosaic chromosomal imbalances (figure 1.4.1.). Mosaicism is a biological phenomenon which describes an individual comprised of multiple cell lineages derived from a single fertilized egg. Can be originated due to the high rate of mitotic errors during embryo cleavage, and/or the error reparation system of the embryo. At the cleavage stage 15 to 75% of embryos are mosaic, and at the blastocyst this rate decreases to 5 to 30%. Approximately one-half of the mosaic embryos has the capacity to self-correct by elimination of the abnormal cells and, thereby, becoming euploid (45). Mosaic aneuploidies are less serious and less common than constitutional aneuploidies, usually with a milder phenotype. Furthermore, aneuploidies are frequently associated with a low life compatibility identified with a higher prevalence among the aneuploidy mosaicism occurrences (44, 46). Scott *et al*. reported that around 30% of the cleavage stage embryos analyzed are diagnosed as mosaic (47). Other genetic analysis, reported an incidence of segmental aneuploidies of 10 to 15% of incidence. Segmental aneuploidy is the loss or gain of chromosome fragments and is not compatible with fetal development, resulting in spontaneous abortion, fail implantation or if viable would cause congenital abnormalities in the affected offspring. It is noteworthy that segmental aneuploidies are a result of mitotic-errors and to be meiotic errors are twice as likely to be paternally, rather than maternally derived (12).

The high frequency and clinical relevance of human aneuploidy is undoubtedly, but currently the knowledge about the risk factors of meiotic nondisjunction is scarce. The only factor indubitably and regularly linked as a direct cause of human aneuploidy is the biological advanced maternal age (34). Significant changes in sperm aneuploidy ratio can occur at single time points in some individuals, strongly supporting that aneuploidy frequency can suffer oscillations by the effect of transient factors, lifestyle changes and/or environment exposures (12). Despite the several years of scientific research,

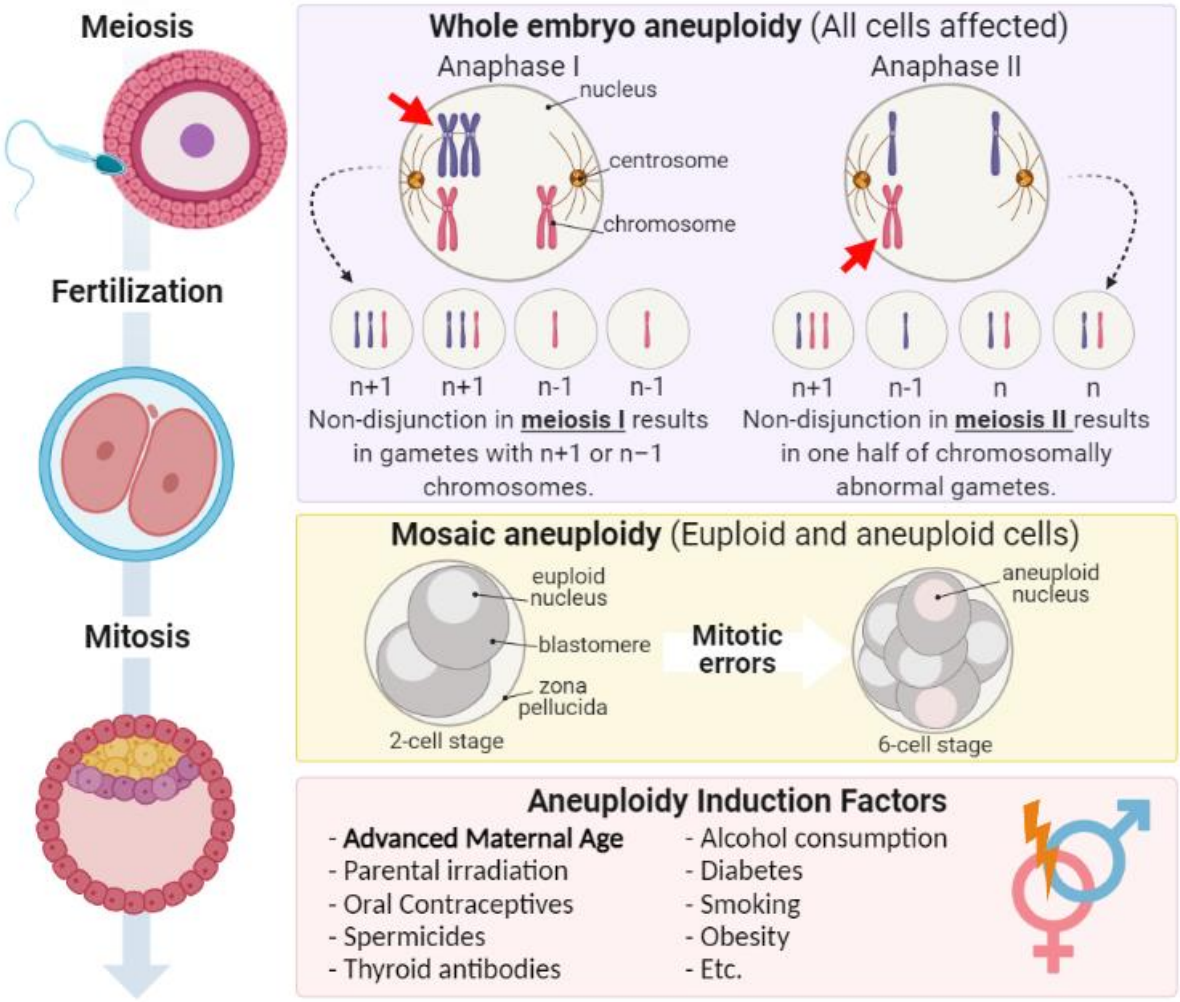

Figure 1.4.1. Emergence of aneuploidy in embryos. Aneuploidy in embryos can be caused by errors in gamete's meiosis or by post fertilization mitotic errors, which leads to dysbalanced chromosomes. Meiotic errors in the gamete involved in fertilization will affect all the organism cells. Mitotic errors after fertilization give rise to mosaicism, in which the organism is constituted by both aneuploid and euploid cells. (Created using Biorender.com)

there have been some other suggestions of possible aneuploidy-inducing agents, including: parental irradiation, oral contraceptives, spermicides, thyroid antibodies, alcohol consumption, maternal diabetes, smoking, obesity, folic acid deficiency, consanguinity, allelic combinations at specific loci and the presence of certain chromosomal polymorphisms, resumed in figure 1.4.1. (16, 20).

Aiming a normal cellular division, the first requirement is for a close pairing of homologous chromosomes, followed by recombination between non-sister chromatids. Recombination plays a crucial role in maintaining the association of paired chromosomes until the onset of anaphase I, to ensure proper arrangement on the spindle at metaphase I and an accurate chromosome segregation (19). Reduced levels of recombination and aberrant meiotic recombination have been shown to be associated with aneuploidies in humans. Crossovers that occur in the proximity of the centromere are similarly associated with nondisjunction in humans, perhaps for locking the homologous too tightly. This failure of recombination between chromosomes that suffer nondisjunction account for 20 to 25% of trisomies, in specific for nearly 50% of trisomy 21 and sex chromosomes trisomies occurrences (24, 28, 29). A

third factor is that cohesion must be maintained between the homologous chromosomes and sister chromatids, until they are arranged on the meiotic spindle. An improperly attached kinetochore to the meiotic spindle, creates a differential tension between the centromeres, which preclude anaphase to take place. If this problem in the tension-sensitive checkpoint persist the cellular meiosis is arrested and, ultimately, could end triggering apoptosis. The chromosome congression is even more error-prone in older oocytes. After all chromosome pairs be aligned at the metaphase plate, the cohesin linkage, protein complex that hold the pair together, is destroyed by a separase, allowing their separation. If this breakage occurs before the fine alignment of all chromosomes, anaphase begins with nondisjunction, providing aneuploidies (43, 46, 48). Once again, the advanced maternal age was linked with a weakened cohesion, which likely contributes to the well-documented increased incidence of aneuploidy (23).

The differences in gametogenesis between female and male are critical to understand the susceptibility of gametes to DNA damage and which are the DNA repair mechanisms inherent. Both gametes have the capacity to repair genetic errors, which is substantially compromised in the spermatozoa given their highly condensed state. The oocyte has the capacity to repair the DNA damage of the inseminated sperm cell, even though a low and limited one. Early DNA repair mechanisms in the zygote are considered a maternal trait, since it uses the maternal machinery until the embryonic genomic activation occurs at 4-cell stage (49-51). Considering the faster cell cycle in early embryonic cells than in adult somatic cells, the DNA replication needs to be faster with low inaccuracies. Overall, the genome integrity is in danger during this early stages, whereby the efficiency of DNA repair system is of extreme importance (52). During embryo cleavage stage, the maternal transcripts that encode to DNA repair mechanisms are used and their amount decrease over time. At the same time, the embryonic genome is activated, as the embryonic repair proteins accumulate and apoptotic systems that eliminate damaged blastomeres are activated. However, recent findings suggest that embryo checkpoint response is inefficient and error-prone, tolerating DNA damages and ChA (46, 50, 53).

For most of inherited genetic diseases, there isn't a reliable and efficient treatment. Therefore, prevention of genetic disorders is still one of the best approaches. A genetic screening and diagnosis of both parents and the control of the processes behind a pregnancy (oocyte maturation, fertilization, implantation, selection and transfer of only chromosomally normal embryos) will allow the achievement of an unaffected pregnancy and a healthy baby (54).

1.5. In vitro fertilization

The role of clinical ART is to assist couples in difficulty establishing a successfully pregnancy resulting in a healthy baby. Depending on the cause of infertility, the couples may resort to a panoply of different ART approaches: in vitro manipulation of gametes and embryos; artificial insemination; hormonal therapies; embryo transfer; IVF; intracytoplasmic sperm injection (ICSI); gamete intrafallopian transfer; and zygote intrafallopian transfer (6, 40, 41). The scientific advances in ART field allowed the optimization of embryo culture media and incubation conditions to the specific embryo

metabolic requirements. The zygotes can be incubated in vitro until the sixth day. During all this development their morphological kinetic is assessed (55). Depending on the clinic practice, at the third, fifth or sixth day, if necessary, a cellular biopsy is performed to assess possible genetic abnormalities by laser-assisted hatching, or similar approaches, and the embryo is vitrified (1, 40). Embryo transfers in the blastocyst stage are associated with improved implantation rates due to the better assessment of the embryo, since the blastocyst presents a complex and well-defined structure. Also, only 40 to 60% of the fertilized embryos succeed to reach the blastocyst stage, per which the transfer in this stage prevents an unviable transfer (55-57).

Despite important scientific and technological breakthroughs, during the last decades, almost half of these couples remain childless, even after multiple cycles of ART (3). Additionally, the efficiency of embryo implantation is low, ranging from 4% to 40% (58). Even today, a major limitation in clinical IVF is the inability to predict which is the most viable embryo of all with the higher odds to implant and develop to a live and healthy baby. Consequently, 2 of 3 ART cycles are unable to result in a pregnancy, and 8 of 10 transferred embryos do not implant, which lead to low implantation and ongoing pregnancy rates per embryo transfer. To enhance the odds of a pregnancy, clinics through time have chosen to perform multiple embryos transfer (15). In a matter of fact, the frequency of having a multiple pregnancy in couples following ART is 15- to 20-fold greater than in couples with spontaneous conceptions (13). A multiple pregnancy constitutes not only a health risk to the mother, but also to the babies. Preterm delivery, infant deaths and physical and cognitive disabilities, as cerebral palsy, are more frequent in these children (57, 59). The main goal of ART is focused on the birth of healthy neonates rather than solely on increase pregnancy rates. For pregnant women a multiple pregnancy is also a health risk; it could lead to preeclampsia, heart failure, and pulmonary edema (57). To decrease complication risk, it has been a change of guidelines worldwide restricting the number of embryos allowed per transfer and a single embryo transfer (SET) is encouraged. Since the implantation of ART, the key challenge remains the refinement of parameters for selection of a SET with the highest success probability. The success of ART relies on the synchronization between the transfer of a genetically normal human embryo and the optimal endometrial receptivity to which it will implant. However, this success rate remains relatively low, which highlights the importance of the embryo quality and viability to transfer (13, 60). In this manner, it is of extreme importance the development of more consistent and noninvasive techniques for prioritize the transfer of the most viable embryo, and to overcome the abovementioned hurdles to achieve a viable pregnancy.

1.6. Non-invasive Morphological Assessment of Embryo Quality

The first implemented and the most conventional embryo evaluation criteria involves manual grading based on morphological analysis by highly trained embryologists. This assessment allows the identification of any delay in the embryo development, which can be associated with the activation of the cellular cell cycle checkpoints due to DNA damages and/ or malfunctions. Throughout the cleavage

stage (day 1 to 3 after fertilization) the number of blastomeres and the degree of fragmentation is evaluated. During the blastocyst stage (day 3 to 6 after fertilization) is assessed the expansion of the blastocele and the development of the inner cell mass, as well as the trophoectoderm (58, 61). Many studies have been demanding an alternative and/ or coupled method for embryo selection criteria, since morphology is not completely reliable, nor reflective of its physiology and was shown that human embryos of similar morphology have very different outcomes (58, 62). This type of selection is crude, lacks of precision, subjective and inconsistent among different medical centers (63).

Nowadays it is possible to accompany all the morphological changes due to the innovative time-lapse imaging system which takes periodically live pictures of the incubated embryos. All the pictures taken during the incubation are combined to show the dynamics and continuity of the embryonic development at every critical stage. This new approach ensures the optimal physiological conditions during all the incubation and provides more parameters to assess the embryo quality. Previous, it was necessary to remove the embryos from the incubator, which would lead to a variation of the incubation conditions in each observation (64, 65). The duration of the cell cycles and features of the cells during all the process may indicate that some cellular process is not occurring correctly. Prolonged cell cycles are likely to reflect that the embryo does not have sufficient energy to maintain its metabolic activity, DNA damages or ChA that activates cell cycle checkpoints (58). Wong *et al.* actually identified a correlation between atypical long cytokinesis during the first three divisions in the cleavage phase to fail in reaching the blastocyst stage (66). Lately, observations of embryos development by time lapse sytems, as EmbryoScope®, have revealed that the time of division to the 5-cell stage and the time of morula formation are more reliable in predicting developmental potential (67, 68). The EmbryoScope incubator is currently used by a significant number of ART clinics throughout the world. Time-lapse systems should attenuate the subjectiveness of morphological criteria, due to the greater information and the possibility of watch the images several times, with no harm inflicted to the embryo. A study from 2017 identified that the major inter-laboratory variability occurred in events that take longer to happen (30-45 min), for instance appearance of pronuclei and extrusion of the second polar body. Bigger the complexity of the system observed, namely after the 8-cell stage, higher is the inter-laboratory variability associated. While faster events, such as cell division and disappearance of pronuclei, have significantly lower inter-laboratory variability (69).

The morphological grading system assessment has several parameters: the embryo cell number at each developmental stage, blastomere size, symmetry and fragmentation, cleavage rate, nucleate scoring of zygotes (65). The *Asociación para el Estudio de la Biología de la Reproducción* (ASEBIR), in an attempt to respond to the lack of consensus in embryo morphology assessment and selection, created a grading system for embryo classification. The current scoring system, includes all the parameters that are clearly related to the best embryo implantation changes and divides the embryos in categories: A, B, C and D. Embryos graded with A quality are the ones with an excellent and defined structure and are associated with the higher chances of developing; while embryos of D quality present

several morphological cellular dysfunctions and have a low prospect of develop. They strongly advise for a morphological assessment using time-lapse imaging systems, beginning with oocyte until right before transfer. All the parameters briefly mentioned above are considered to be associated with abnormal embryo development, low rate of blastocyst formation, high percentage of biochemical pregnancies and obstetric complications in pregnancies (56, 70).

The data collected from the time-lapse imaging systems approach of morphokinetic parameters during embryo incubation should be analyzed and modeled, together with the molecular and genetic features, so the embryo morphological selection criteria became lesser subjective (71). At 2019, Khosravi *et al.* presented an artificial intelligence (AI) approach using time-lapse microscopy embryo images, with the purpose of an accurate and automated embryo quality assessment (63). The models created by this selection approach would be unbiased and do not suffer from low concordance rates between high qualified embryologists (72). Unfortunately, until the moment their model could not successfully predict the chances of pregnancy. Auspiciously, in the 37th edition of the Meeting of the European Society of Human Reproduction and Embryology of 2021 Bori *et al.* reported that embryos with different ploidy cloud be distinguished by AI determination of a longer time to blastulation and higher cell activity, with an accuracy above 70% (73, 74).

1.7. Preimplantation Genetic Testing for Aneuploidy

A most recent criteria for SET relies on the genetic analysis of a small amount of the embryo cells allowing the identification of the embryo ploidy, designed preimplantation genetic testing for aneuploidy (PGT-A) (41). In fact, PGT-A has been shown to improve IVF efficiency. The ability to identify euploid embryos will, hypothetically, lead to reduced rates of miscarriages, reduced time to achieve a pregnancy and increase the rates of clinical pregnancies (75). Needless to mention, the identification of aneuploid embryos will also reduce the financial burden, emotional stress and the complications associated with a multiple pregnancy. However, it was proposed by the European Society of Human Reproduction and Embryology (ESHRE) that this technique can only be performed if the couples have a known genetic condition; after RIF; couples with normal karyotypes who have experienced recurrent miscarriage; severe male factor; and couples with advanced maternal age (women older than 38 years old) (45, 76-78).

These methods have been used in clinical practice and have shown to be useful in prediction of embryos with ChA, however many problems still remain to be overcomed (54). PGT-A involves embryo biopsy, per which is an invasive approach that could compromise the embryo integrity. Depending on the embryo development phase in which the procedure is done it can be more or less prone to cause detrimental effects. During clevage phase all the blastomeres share the same differentiation status. So the collection of random cells has a direct impact on its viability. The collection before the embryo reaching the blastocyst stage highly influences the establishment of cellular polarity and fetal programming, inducing irreversible developmental damage and impairs blastocyst implantation (47,

79). Trophoectoderm and inner cell mass has a different differentiation status and will give rise to different cellular populations, (extraembryonic appendixes and to the embryo, respectively) (47, 80). Therefore, collecting a random sample of trophoectoderm cells during the blastocyst phase obviates detrimental effects on the embryo (81-83). The current standard practice for PGT-A involves the analysis of the whole cellular genome of 5 to 10 trophectoderm cells biopsied at the blastocyst stage. This also makes the genetic diagnosis more reliable and less prone to false positive results, since the embryo is able to repair genetic abnormalities during the clevage phase (84). Nevertheless, if the biopsy technique is performed poorly it can compromise the embryo development, blastocyst implantation and lead to a inconclusive PGT-A diagnosis (31, 85).

Furthermore, a day 5 biopsy requires frozen-thawed embryo (45), it is a labor-intensive technique that involves around 8 cumulative hours of labor for the embryology team per each biopsy case and between 2-3 weeks to obtain the results. Only unaffected embryos are then implanted. In Portugal, the legislation and the *Conselho Nacional de Procriação Medicamente Assistida* (CNPMA) only allow the transfer of a maximum of 2 embryos per cycle (20, 33).

For that, it is of extreme importance the development and optimization of non-invasive techniques to predict the embryo quality. The non-invasive approaches include time-lapse microscopy; and the metabolomic, transcriptomic and proteomic profiles of spent embryo culture media (SECM). The morphological criteria were proved as a not very reliable method, once about 70% of the morphological normal embryos are aneuploid, and about 37% of the embryos with trisomy reach the blastocyst stage (33, 65). While Kirkegaard *et al.* concluded that the only parameters with predictive value on correlating morphokinetic parameters to embryos ploidy were related to the events that occur faster, such as the first cytokinesis (86). Chawla *et al.* have reported a differential morphokinetic analysis of cleavage stage between euploid and aneuploid embryos (87), Rienzi *et al.* describe that they did not found evidence that supported the same correlation (88). At the present moment, different research groups agree that the partnership between time-lapse microscopy and PGT-A can improve embryo selection, but there is no consensus between what are the parameters to follow (76, 89).

In clinical practice the PGT-A is challenging. Recent biotechnological advances, increase the accuracy and precision of its diagnosis. Many clinics also moved from day-3 blastomere biopsy to day-5 trophectoderm biopsy, which minimize the detrimental impact on the embryo (12). However, not all embryos survive in vitro to the blastocyst stage, though hypothetically they could have resulted in a healthy live birth if they were transferred early (90). PGT-A is further limited by a lack of standardization due to the multiple available technological platforms. Also, each clinical center have its own manipulation protocols that lead to a low procedure standardization (45). Nevertheless, PGT-A improves the reproductive outcomes by reducing spontaneous abortions, the time until achieving a successful pregnancy and potentially increasing live birth rates. PGT-A is nowadays the most reliable method for selecting euploid embryos for transfer, however is an highly invasive technique and costly (31). Given its importance further investigation is urgent to develop a non-invasive technique for embryo

ploidy determination before implantation. Currently, there are three main trending research areas for this purpose: associate the time-lapse microscopy with AI-based models; analysis of cell-free DNA released by the embryo into the medium; and correlate metabolomic/ proteomic markers present in the spent embryo culture media (SECM) to embryo viability (91). In 2018, Rubio *et al.* started the study of the cell-free DNA present in SECM as a substitute for PGT-A. Their data shown to be as accurate, precise, sensitive and robust as the classic PGT-A at detecting aneuploidies. This technique is being at the moment validated in many ART clinics and can be used by any couples resorting ART, per which can decrease the total cost of the infertility treatment. However, it remains expensive, time-consuming, and prone to handling errors that can lead to unconclusive results. Altogether, it is now unquestionable that the SECM provides important information about the health, namely the ploidy of the embryo (92, 93).

1.8. Metabolomics in the Reproduction Field

Exploring the early embryo metabolism is an interesting approach to discover biomarkers for aneuploidy diagnosis and to develop a new non-invasive prioritization criteria for SET. In the last two decades, emerged new high-throughput approaches, generally grouped as Omics. Their main goal is to offer a global view of the complex biological system after the analysis of the molecules involved in the processes, under specific circumstances. In theory, the embryo selection for transfer can be decided by data obtained at the genomic, transcriptomic, proteomic, and/or metabolomic analysis (figure 1.8.1.). Altogether the field of Omics provide the general understanding of the cellular activity. Beginning with the cellular genotype, following to its transcripts and proteins, finishing with the metabolome, which reflects ultimately the output of gene expression (42, 94). Genomics, as seen above, represented a huge breakthrough in the ART field by allowing the development of PGT-A techniques. Currently, the other fields (proteomic, transcriptomic and metabolomic) are receiving attention from the researchers to create a new non-invasive system to identify the best single embryo to transfer (85, 95).

Unlike the human genome, which is relatively stable and static throughout the human body cells, the cellular metabolome and secretome are dynamic and differs as a response to a variety of genetic, nutritional, and environmental conditions. Moreover, human metabolome is estimated to include around 3000 metabolites, a value significantly lower when in comparison with over than 250,000 genes, approximately 200,000 transcripts, and 1 million proteins (62, 96). In the clinical field of human reproduction different studies tried to identify non-invasive biomarkers to help predict embryo competency by analysis of human embryos secretome using metabolic footprinting (15, 32, 42, 64). Both the uptake of nutrients present in the media and the export of metabolites by the embryo can play an important part in providing information about the embryo competence.

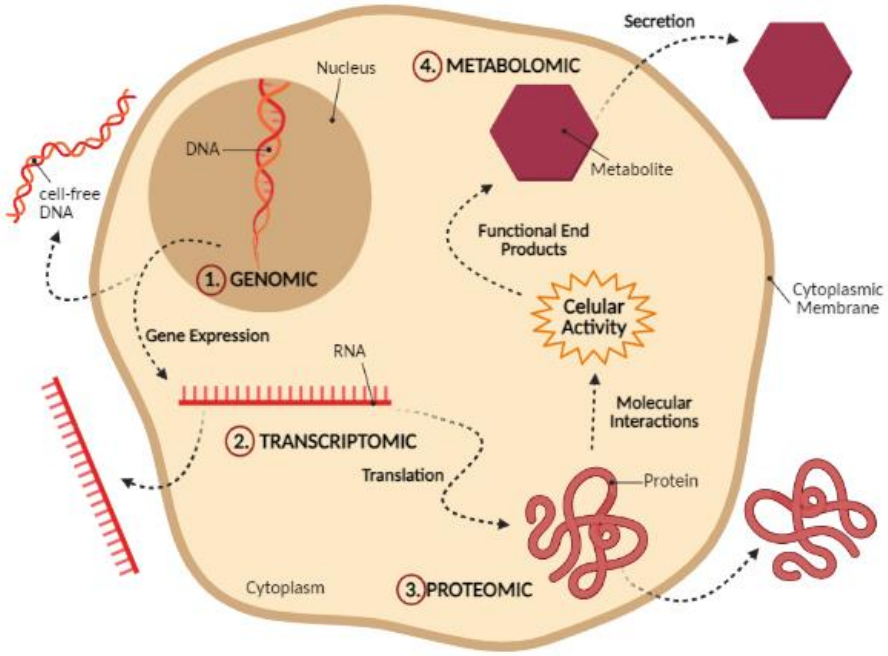

Figure 1.8.1. Omics. A schematic representation outlining the complexity behind the biological systems. The omics cascade describes the flow of biological activity in an organism. The term of omics includes the fields of (**1**) genomics, (**2**) transcriptomic, (**3**) proteomic and (**4**) metabolomic. The goal of genomics is to identify the coding and non-coding genes and genetic variants associated to a specific condition. Transcriptomics aims to identify the mRNAs, small non-coding, and long non-coding RNAs. Proteomics evaluates the structural, non-structural, and functional proteins, and enzymes. Metabolomics studies the functional end products of the cellular activity, as lipids, amino acids, carbohydrates, and nucleotides. Together these fields allow the holistic view of all the cellular pathways conditioning the cellular phenotype and physiological state. Note: this figure is not at scale. Abbs.: DNA - deoxyribonucleic acid; RNA – ribonucleic acid. (Created using BioRender.com)

Metabolomic is the scientific field involved in the systematic analysis of metabolites present in the biological samples and its concentrations and fluctuations in a defined environment (94). The metabolites represent the final outcome of cellular regulatory processes to genetic and environmental factors, varying fastly in a diverse amount range (57, 62). The analysis using high-throughput platforms coupled with statistical tools allows the correlation of a specific metabolic profile with embryo characteristics, offers avenues of development of new methods for pathological diagnosis and managing. It can be used to identify specific biomarkers for a determined health status (92, 97).

Metabolites are the downstream products of genes, transcripts, and proteins which reveal the response of the biological systems to a variety of genetic, environmental, and nutritional conditions. Those metabolites are in dynamic balance with the ones present in the biofluids that perfuse the cells. Then, a pathological state should influence biofluid composition. It is expectable that the metabolism of a chromosomally normal embryo be different of an abnormal embryo, and that influences the metabolites present in external media (96, 98, 99).

Nuclear magnetic resonance (NMR) spectroscopy is an important approach to study embryo metabolism using SECM. The basic principle of NMR involves the atomic nucleus. An atom with a non-zero nuclear spin (an odd atomic number), when placed in an external magnetic field can absorb

and re-emit radiofrequency with a frequency, which is specific of the magnetic field acting up on the nucleus (100, 101). Not only the external magnetic field affects the magnetic field of each nucleus present in the sample, but also by the weak magnetic fields created by each nucleus in the neighborhood. Thus, the chemical environment affects significantly the spin of a nucleus and its resonance at a specific frequency, that ultimately allows the identification and quantification of molecules. The hydrogen isotope (^{1}H), the most sensitive NMR nuclei, is the most often used nuclei in metabolomic studies. ^{1}H is present in most of metabolites and has a natural abundance of almost 100%, that makes perfect as target for NMR analysis. Furthermore, NMR spectroscopy is a highly reproducible even between different spectrometers and/or operators. It is also a very versatile technique, making it possible to analyze intact tissues or biofluids, in most of the cases, with minimal sample preparation and the sample is not consumed during the analysis. The main drawback is the relative NMR low sensitivity, specially when compared with Mass Spectrometry tecnhiques. In recent years, the development of ultra-high-field NMR spectrometers, and advancements made in NMR probes, such as cryogenically cooled probes and microprobes, have enhanced sensitivity and reduced the required sample size (64, 97, 98).

1.9. Biomarkers of Interest

Over the last three decades, researchers are trying to predict the embryo potential using quantification of specific metabolites, such as carbohydrates and amino acids, used in embryo metabolism (42). When embryo culture media was first introduced by ART its composition was fairly simple, containing only glucose, lactate and pyruvate in a physiological salt solution supplemented with patient's serum. The study of the SECM composition, allowed the media optimization, with the understanding of the embryonic metabolic requirements, like several amino acids, different energy substrates, growth factors and vitamins. At the present moment, it is recognized that embryo has different metabolic requirements along the maturation process, from zygote to the compaction stage and to the blastocyst stage (figure 1.9.1.). Thus, the incubation of the embryo in the same media along all the process has been progressively replaced for sequential systems used in different phases (55, 64).

The metabolomic profiling of SECM of human embryos represent a unique opportunity to obtain insights of the biochemical events displayed during embryonic development that could be correlated to its quality. This is the case of the lactate and tyrosine uptake by the embryo, only at day-2 after fertilization occurs the embryonic genome activation associated with the consumption of the lactate present in the media. Until there, the metabolic activity is maintained by the maternal machinery with an increased pyruvate uptake, as summarized in figure 1.9.1. (62, 102). Around day-5 begins the tyrosine uptake, indicative of the beginning of blastocyst stage. At the blastocyst stage, the production of alanine, glutamate and glutamine increases, and the production of pyruvate decreases. A recent study indicated that during in vitro development, glucose and lactate consumption are not significant (95). Even though it has been pointed it out that during early cleavage stage, pyruvate is the predominant energy substrate, switching to glucose at the morula stage (61, 62, 103). During the early embryo development its genomic

activity is initiated to sustain its biological activity. Therefore, the uptake of exogenous amino acids increases both to protein synthesis and to be metabolized. Glutamic acid, isoleucine, methionine, and phenylalanine are the amino acids particularly required to improve embryo development during the cleavage phase (62, 104).

Since metabolites reflect the downstream of gene expression, genetic variance would have a repercussion on the metabolite embryo profile. However, further and consistent research is required to ensure this hypothesis (64). The embryo metabolism is critical for its development and viability, and, thereby, that makes SECM metabolomics an interesting approach for aneuploidy diagnosis (55, 85). The metabolic embryo profiles can be measured non-targeted and non-invasively using the SECM to identify the compounds both exported and consumed. Nevertheless, the limiting and small volumes of SECM available and low-abundance metabolites could be challenging (95).

Vergouw *et al.* had compared the accuracy of the predicted embryo viability by morphological criteria versus via metabolomic profiling, and the the second present a higher accuracy, 53.6% against 38.5% (105). Highlighting that morphology does not necessarily convey all of the biological information related to the embryo physiology. Metabolomics appears to be a method for the selection of the embryo with the higher implantation rates, which might increase the success rates of SET in the near future. Several studies identified metabolites related with embryo health and viability (65, 94). Pudakalakatti *et al.* identified a correlation between embryo with high-implantation potential with higher consumption of pyruvate present in media, at day-3 by NMR analysis (106). Those embryos converted pyruvate into lactate or into alanine, in order to avoid the toxic accumulation of ammonia, leading to an alanine increase in the SECM. For this reason, it was also correlated with increased alanine in the SECM. The authors purposed the low pyruvate to alanine ratio in the SECM as a potential biomarker for selection of the SET (106). Brison *et al.* at 2004 evaluated the amino acid turn over using the SECM by HPLC and found elevated asparagine and decreased glycine and leucine levels in successful implantations (107). Other study, using the amino acid profile, identified that day-2 embryos which consume less glutamine and arginine and produce alanine and threonine in significantly lower quantities were correlated with a higher viability (104). To our knowledge the first global metabolomic study to assess human embryo viability using ^{1}H-NMR spectroscopy to analyze the metabolomic profile of SECM was in 2007 (15). It was possible to identify increased glutamate levels and alanine to lactate ratio in embryos that resulted in a pregnancy (15). A study from 2015 indicated that the human blastocysts which have produced mainly lactate, alanine, aspartate, pyroglutamate and glutamate resulted in a pregnancy (95). In 2014, Wallace *et al.* did an NMR-based metabolic profile of SECM and observed an increase of formate to glycine ratio and a decrease of citrate to alanine ratio of embryos that resulted in a viable pregnancy (108). A recent study suggests that a discriminant analysis construct with SECM amino acid concentrations predict the embryo implantation potential with an high accuracy (90.4%) (109). The turnover of amino acids and carbohydrates appear to be predictive key biomarkers in assess embryo competence. Some studies point out that combining metabolomic profiling with morphology may

significantly improve the implantation rates (61). The change of amino acid turnover observed is in agreement with the quiet embryo hypothesis, stating that *in vitro* embryos maintain low levels of metabolism minimizing the energy consumption, and at the same time, decreased levels of reactive oxygen species that could be toxic (58, 110). These studies are very promising, and some are in agreement with each other. Nevertheless, the cohort used was small and are preliminary studies, per which further analysis is necessary before applying it to the clinical field.

Regarding specifically the embryo aneuploidy, even though the limitations mentioned, the morphokinetic criteria for embryo selection is still the most used worldwide. Some data reported that giant oocytes (>200 µm) and enlarged second polar bodies (>30 µm) are often assotiated with aneuploidy (111, 112). Zygotes with more than 2 pronuclei have around 50% of chances to develop aneuploidy (70). During day-2 to 3, the slower and faster duration of the cellular division were correlated with lower implantation rates and higher aneuploidy rate, when compared to the optimal cleavage rate (113). An observational and qualitative study identified that compacted morula cells have higher incidence of aneuploidy (114). Recently, was found that embryos that took longer than 13 hours for blastocele expansion had reduced euploidy rates and displayed significantly higher glucose uptake (65).

Among the new possible systems proposed to substitute the conventional PGT-A it is the assessment of the SECM, as non-invasive, non-time consuming, less expensive and subjective, sensitive, and clinically applicable, by NMR spectroscopy. The conceptual basis of this approach has been proved in previous studious (96), which demonstrated that embryos with and without ChA have different metabolic patterns, that reverberate into SECM composition variations (42). The most recent studies have focused in this new avenue of possible approach to develop a new non-invasive diagnosis. Picton *et al.* at 2010 shown the association between metabolic activity and cytogenetic composition of human embryos in vitro. That study identified a significant different amino acids turnover at day-3 and day-5. At day-3, the asparagine and valine turnover were decreased and the glycine turnover was increased in euploid embryos, when compared to aneuploid embryos. At day-5, euploid embryos shown a significant decreased turnover of serine, leucine and lysine (115). In 2012, Sánchez-Ribas *et al.* have evaluated the metabolic signature of trisomy 21 human embryos at day-3 by NMR spectroscopy and MS and identified an increase of caproate, a short chain fatty acid, and of androsterone sulfate, endogenous sex steroid, in SECM of trisomic 21 embryos. These results corroborate the hypothesis that aneuploidy could reverberate into metabolic alterations, such as in lipid energy intake and hormone secretion. They have also observed a non-significative increase of several aminoacids, such as proline, threonine, alanine, methionine, valine, and arginine (32). Similarly, other study showed that embryos derived from sperm carrying high genetic abnormalities presented reduced pyruvate uptake and alanine export and lower levels of glutamine in SECM (116). A recent study advocates that ultra-fast and fully automated screening of an embryo ploidy is possible based on multiple combinations of specific mass spectral peak signatures. With that approach, it was identified 12 characteristic peak signatures for euploid and 17 for aneuploid embryos, with a sensitivity of 84% (117).

In the past years our understanding of human aneuploidy has increased dramatically (118-120). However, the knowledge behind its molecular mechanisms it is still unclear. Metabolomics offers a unique and comprehensive insight of embryo metabolic activity in a non-invasive and non-targeted manner, thus opening the door for the discovery of potential biomarkers which correlates with the embryo quality and its ability to establish within the uterus and develop. As mention previously, several studies had profiled human SECM and correlated it with pregnancy outcomes, ChA, and embryo development. It is anticipated that an improved knowledge in this area would lead to the identification of a non-invasive biomarker with the ultimate goal of developing a technology that is suitable for both prognostic and diagnostic purpose in ART. The validation of predictive biomarkers in a large cohort population will support the development of new technologies in the clinical setting and will help increase

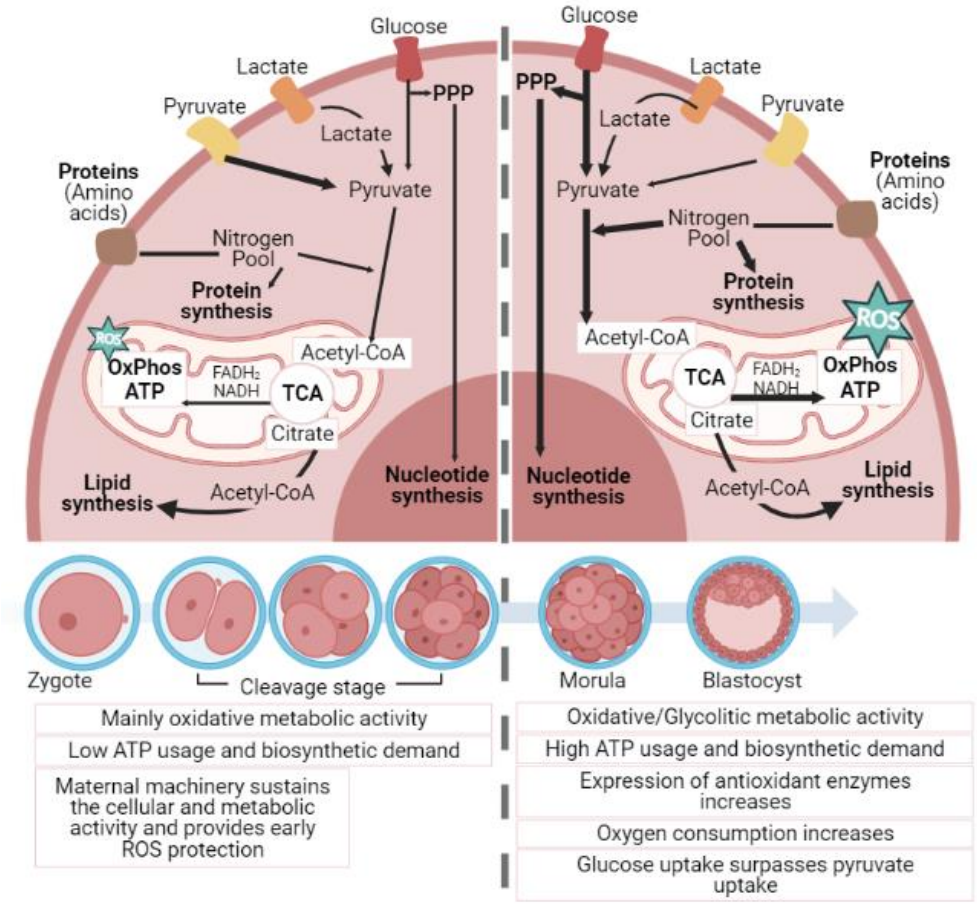

Figure 1.9.1. Metabolic changes during human preimplantation development. During the first two days after fertilization, the embryo metabolism and defenses are sustained by the maternal machinery (e.g., mRNA and proteins). After that, the embryonic genome is activated and begins to produce its own cellular machinery and slowly activating specific pathways. Until around day-3, pyruvate is the predominant substrate used among some amino acids through tricarboxylic acid (TCA) cycle and oxidative phosphorylation. At morula stage, the embryo increases its energetic demands, intimately associated with cellular proliferation and differentiation. At this time, glucose uptake surpasses pyruvate, which allows a higher ATP production by the glycolytic pathway and an efficient biomass and nucleotide generation through the pentose phosphate pathway (PPP). Abbs.: ATP-adenosine triphosphate; ROS-reactive oxygen species; NADH- nicotinamide adenine dinucleotide; $FADH_2$- flavin adenine dinucleotide (Created using BioRender.com)

live birth rates using SET. Even though the morphological assessment has limited predictive value, they are quick, safe, economical and well-stablished in ART practice, per which other techniques are unlikely to replace them, but could certainly complement them and together overcome each weakness (65). It will be important to continue and expand the scientific research on embryos metabolome. Non-invasive quantitative techniques are the key in studying the embryonic development capacity and its metabolic status, and will be the future of ART.

2. Objectives

Several studies have kindled the interest on the continuous development of the biotechnology behind assisted human reproduction addressed to an efficient selection criterion for prioritizing the embryo with implantation best outcomes to transfer. Therefore, and for everything previously stated, it is hypothesized that embryos ploidy is correlated with embryonic metabolic activity. In the present study, we investigated the molecular constituents of SECM using quantitative ^{1}H-NMR to find if there is a correlation between the SECM metabolic profile and embryo aneuploidy and if this approach can contribute to the definition of biomarkers for an early prediction of embryo's ploidy. The majority of previous studies have evaluated the composition of SECM on day 3. Evidence points out that embryos are able to correct chromosomal errors found by day 3, whereby a different diagnosis could be obtained on day 5. On this present study we purpose to evaluate SECM from two sequential periods of embryo in vitro incubation (day 3 and day 5, after fertilization).

The ultimate goal is to begin the validation of SECM analysis by NMR spectroscopy as a PGT-A system substitute in clinical practice. Using SECM would be a non-invasive and non-damaging approach accessible to every couple resorting to ART, opposite to the current PGT-A, and still improving the ongoing SET pregnancy rate.

3. Materials and Methods

3.1 Study Design and Participants

In this study, 5 couple patients that underwent in vitro fertilization (IVF), specifically intracytoplasmic sperm injection (ICSI), followed by preimplantation genetic testing for aneuploidy (PGT-A) for a single-center prospective sibling case-control pilot study were recruited. The patient inclusion and exclusion criteria are summarized in table 3.1.1. Despite no alteration of overall procedures towards the IVF/PGT-A be necessary, an additional consent to donate the spent embryo culture media (SECM) for scientific research purposes was requested (supplementary data 1). A total of 62 SECM samples were collected from day 3 and 5 and were analyzed by ^{1}H-NMR spectroscopy (SECM from euploid embryos, n=28; SECM from aneuploid embryos, n=24; and a matched control embryo-free media, n=10). During the study the following variables were also documented: female and male subject age (in years); female body mass index (in Kg/m^2); menstrual cycle length (days); number of preceding ART cycles; duration of infertility (in years); infertility diagnoses; anti-Müllerian hormone (AMH) (ng/mL) and antral follicular count (AFC) baseline levels; number of follicles >11 mm on the day of ovulation triggering; total dose of exogenous gonadotropins (IU/day); total duration of ovarian stimulation (days); number of oocytes retrieved; number of fertilized oocytes; number of embryos available for biopsy at day-5; number of embryos available for biopsy at day-6; quality of each embryo available for biopsy; and number of euploid and aneuploid embryos. In order to minimize potential confounding variables, were assigned paired-sibling embryo culture media (1 to 3 pairs of samples derived from the same patient and treatment cycle), after the results of the PGT-A procedure were known. Pairing was performed using the samples from embryos with similar developmental staging and morphological grading.

Table 3.1.1. Study inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
▪ Age: ≥18 and <49 years old. ▪ Body Mass Index (BMI) ≥18.5 Kg/m^2 and <30 Kg/m^2. ▪ Planned for IVF followed by PGT-A. ▪ Oocytes retrieval and fertilization by ICSI ▪ Six follicles over 14 mm on the day of the triggering. ▪ Signed and dated informed consent.	▪ Previous history of poor ovarian response (<4 oocytes retrieved) with a maximal dose of ovarian stimulation (≥300 IU/day). ▪ Presence of a medical condition which is known to affect ART outcome (e.g. thyroid dysfunction). ▪ Current use of anti-depressants, anti-psychotics, steroids, antiepileptics or chemotherapy. ▪ Those unable to comprehend the investigational nature of the proposed study.

3.2 Ethical Approval

The study was conducted in conformance with Good Clinical Practices (GCP). All experiments were approved by the ethical committee of Local Ethics Committee of the Instituto Valenciano de

Infertilidad – Lisbon and by Institutional Review Board/ Independent Ethics Committee (IRB/IEC). Every patient included in the present research gave a freely written consent and were properly informed of all aspects of the study relevant to their decision to participate, such as the aims, methods, anticipated benefits, and potential hazards.

3.3 Infertility Treatment, Embryo Culture and Biopsy

All the infertility treatment, IVF and PGT-A procedures were performed according to normal IVI RMA Lisboa clinical practice. Firstly, women induced ovarian stimulation using either recombinant follicle-stimulating hormone (FSH) (Gonal F, Puregon, Pergoveris, Bemfola, Ovaleap or Elonva) or highly purified human menopausal gonadotrophin (Menopur). Around the same time, women underwent daily administrations of either cetrorelix or ganirelix (GnRH antagonist) or oral progestogens (desogestrel) in either a fixed or flexible protocol. The progression of every cycle was monitored by vaginal ultrasound scans and serum quantification of estradiol and progesterone. Oocyte maturation was triggered with a GnRH agonist (0.3 mg decapeptyl), after 3 follicles of ≥17 mm was observed. After 35-36 h of hCG administration, the cumulus-oocyte complexes were collected by transvaginal aspiration and the oocytes were washed in gamete medium (Cook Medical). Then, the oocytes were cultured in Fert™ medium (Origio; Cooper Surgical) at 5% CO_2, 5% O_2, and 37° C. Oocytes were denudated right before ICSI, 4 hours after their retrieval, using mechanical and chemical procedures (pipetting in 40 IU/mL hyaluronidase in FertiCult Flushing medium, FertiPro. Semen samples were collected by masturbation, after 2 to 4 days of sexual abstinence, then left to liquefy for 10 min at 37° C in 6.5% CO_2 in a Heracell™ incubator (ThermoFisher Scientific) and the initial semen quality parameters are evaluated. To obtain the most viable and motile spermatozoa, the sample was submitted to a discontinuous gradient centrifugation (Sil-Select Plus™ Set 45% and 90%, FertiPro). After centrifugation at 350 g for 15 min at 37° C, the pellet was collected, thereafter washed in 5 mL of FertiCult™ Flushing medium (300 g for 15 min at 37° C). To obtain a purified population of the highly motile spermatozoa, they are also subjected to a swim-up in FertiCult Flushing medium, during 20 min at 37°C. After which 1 mL of the uppermost is removed, washed as mentioned before, resuspended in 0.5 mL of Fert™ medium (Origio; Cooper Surgical), which will trigger capacitation, and the semen quality parameters were re-assessed. Finally, this final fraction of sperm cells was put in 10% PVP (polyvinylpyrrolidone) in FertiCult Flushing medium, and a single spermatozoon is selected to insert within the oocyte. ICSI was only performed, after the collection and handling of all mature oocytes, as well as sperm cells are capacitated, using an Olympus IX71 microscope at ×400 magnification. Fertilized oocytes were cultured in a preequilibrated EmbryoSlide culture dish (Vitrolife), using Cleav™ sequential media and the EmbryoScope Plus Time-Lapse System (vitrolife). Beyond maintaining the idyllic embryo development conditions, this system captures live images at every 10 min in seven focal planes for morphological assessment, analyzed using EmbryoViewer software. Each microwell was

filled with 30 µL of sequential-step media and 1.6 mL Hypure™ Oil Heavy (Kitazato Corporation) per dish. During the first 3 days they are incubated in ORIGIO® Sequential Cleav™. Once they reached the morula stage, a specific section of the zona pellucida was compromised by laser pulses, using an Olympus IX71 microscope at ×400 magnification, in order to stimulate embryo hatching. Then, they were incubated in ORIGIO® Sequential Blast™ until they reached the expanded blastocyst stage, at day 5/6. Later, the blastocysts were assessed and selected by applying the hierarchic classification according to the ASEBIR embryo morphologic grading (56). Embryos were graded from A (high morphologic quality) to D (low morphologic quality) by high skilled senior embryologists. The embryos classified as high quality (A and B) were selected to follow to biopsy. Using laser pulses and the Olympus IX71 microscope at ×400 magnification some trophoectoderm cells, preferably hatching ones, were removed. After biopsy, embryos were vitrified and the trophoectoderm cells collected were sent to Juno Genetics to be analyzed by next generation sequencing to identify aneuploidies.

3.4 Spent Embryo Culture Media (SECM) Collection and Preparation

The culture media whereas each embryo developed were collected with the goal to evaluate the metabolomic profile and compare it with the chromosomic analysis. The SECM collected samples were categorized in three groups (embryo-free culture, day-3 and day-5). At day-3 and day-5, after the removal of the embryo, 30 µL of culture media were collected from the EmbryoSlide culture dish (Vitrolife) into 0.2 mL tube (VWR PCR tube), within a microscope environmental chamber, heat controller with CO_2 probe, at 37° C and 6.5% CO_2 (Smart Station, Astec). The samples collected were centrifuged in a clean bench (Thermo Scientific, Heraguard) at 2,000×g in the microcentrifuge (Labnet International, INC.) during 2 min to form an interface between the paraffin oil, the medium and the cellular debris. Then 25 µL from the middle section was collected and mixed with 30 µL of sodium phosphate buffer (NaH_2PO_4 and Na_2HPO_4) 100 mM at 7.2 pH, 3-(trimethyl-silyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP) 0.29 mM and sodium azide 0.95 mM diluted in D_2O solution into a new 0.2 mL tube (VWR PCR tube). The samples were immediately anonymized, frozen in liquid nitrogen and stored at -20° C, at IVI RMA Lisboa, until analyzed.

3.5 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy is a quantitative and nondestructive technique that requires minimal sample volume and preparation, representing a powerful and reproducible approach for noninvasive metabolic analysis of biological systems. In this project NMR assay was conducted to characterize the metabolic profile of SECM from embryos with different ploidy.

SECM samples were thawed at room temperature and then follow to be centrifuged during 2 min at 3.5×g in a Fresco™ 21 microcentrifuge (Thermo Scientific™). The 55 µL solution were placed in a 1.7 mm NMR capillary tube (Brucker MATCH™ holder) using a 100 µL microliter® syringe (Hamilton). The NMR spectra were acquired in a BrukerAvance II 500 spectrometer (BrukerBiospin)

equipped with a prodigy TCI cryoprobe. All experiments were acquired at 298 K. For each sample, were obtained a one-dimensional (1D) ^{1}H-NMR spectra using a noesypr1d pulse program, which generates an unedited spectrum with improved solvent peak suppression and facilitates the observation of signals near the water resonance enabling the obtainment of an improved baseline. In 1D-NOESY experiments, 256 scans were accumulated for each experiment, free induction decay (FID) size of 48 K points, using a sweep width of 11.7616 ppm window centered at 4.70 ppm. A relaxation delay of 3 sec and mixing time of 10 msec was incorporated between FIDs, during which was applied a continual water presaturation radio frequency field of 35Hz.

The spectra will be processed by a Fourier Transform and multiplied by an exponential function using TopSpin 4.1 software (Bruker). Phase correction, baseline correction and spectra calibration were performed before further analysis.

3.6 Metabolomic Profile and Analysis

TSP present in the sample's solution was used as a standard reference compound. The metabolites assignments and quantification were conducted by resorting to spectral databases: Human Metabolome (HMDB) and Chenomx Nmr Suite 8.11. A total of 18 and 23 metabolites were assigned on day 3 and day 5, respectively.

3.7 Statistical analysis

Metabolite concentration of SECM from aneuploid and euploid embryos and embryo-free media groups are used for a univariate statistical analysis of data using analysis of variance (ANOVA) followed by Tukey post-hoc test for multiple comparisons using GraphPad Prism 6.0 (GraphPad software, San Diego, CA, USA). Outliers were removed using Grubbs' method, alpha=0.2. Values of $p < 0.05$ were considered as statistically significant.

Multivariate statistical analysis of NMR spectroscopy data was performed on SIMCA 13.0.3 software (Umetrics, Umea, Sweden). Metabolite concentration data and the patient-, treatment-, and embryo-specific related variables, such as embryo morphological quality, age, BMI, cause of infertility and other, were subjected to unbiased metabolic profiling using principal component analysis (PCA), as possible confounders.

Further analysis was performed with MetaboAnalyst 5.0 (121, 122) and scaled using auto-scaling. The primary multivariate analysis was a PCA . Supervised methods to create a discriminative and predictive model were also performed, namely partial least squares discriminant analysis (PLS-DA) and orthogonal PLS-DA (OPLS-DA) and evaluated the hierarchical clustering by Heatmap analysis. Outliers were identified and removed using PCA score-plots. To outlier exclusion was preferred a conservative approach, in which only samples that experimental procedures could explain the behavior were removed. Predictive models were evaluated by the area under (AUC) the Receiver Operating Characteristic (ROC) curve and by prediction of samples with unknown embryo's ploidy. Metabolites

with AUC>0.85 were noted as “promising candidates” for biomarkers differentiating aneuploid from euploid embryos and used in the predictive diagnosis analysis. The power of this study was calculated using the MetaboAnalyst tool.

4. Results

4.1. Cohort characterization

This research can be divided into two phases. Phase 1 consisted of day 3 and day 5 collection of the spent embryo culture media (SECM) from embryos developing in vitro that would be subjected to a preimplantation genetic testing for aneuploidy (PGT-A); and of the analysis and profiling of the SECM of embryos with different ploidy by ^{1}H-NMR spectroscopy. Phase 2 consisted of the development of a predictive model for a potential criterion to embryo selection to transfer. The predictive power was measured with the use of the area under the receiver operating characteristic (ROC) curve (AUC).

During this thesis, a total of 24 couples were enrolled for the study that fitted the inclusion criteria. However, 5 of them were excluded due the retrieved oocytes did not meet the requirements; for 1 couple there was no free-space on the embryoscope incubator; 6 couples could not be included due to the lack of paired embryos on day 5; and for 7 couples the results of PGT-A did not arrive at time to be included. Thus, only 5 couples were inserted in the present study. A total of 26 embryos were analyzed, of which 12 and 14 were aneuploid and euploid, respectively. The 3 mosaic embryos were not considered for the metabolic analysis. After the preprocessing of the dataset, 7 paired embryos (at least, 1 aneuploid and 1 euploid embryos of the same couple) were considered to develop the predictive model and 12 unpaired embryos were considered to test the model.

The morphological quality grading and ploidy of the embryos assessed and the infertility treatment and patient related data in the present study are presented in table 4.1.1. The average retrieved oocytes after trigger ovulation were 16.20±6.61, obtaining 11.60±2.97 successful fertilized eggs, and only 6.20±2.17 achieved the blastocyst stage and were biopsied. Most of the blastocysts inserted in the study had good morphological quality (B quality – ASEBIR grading system 2015 (56)). The morphological quality grading was quite homogeneous among euploid and aneuploid embryos. As observed in figure 4.1.1, 10 and 2 aneuploid embryos were graded B and A, respectively. While in euploid embryos, 3 were identified as A, 10 as B, and 1 with C grade.

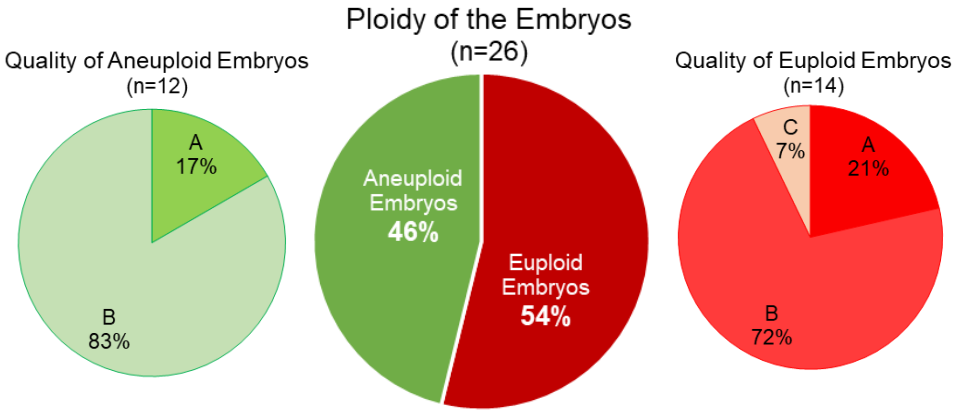

Figure 4.1.1. Ploidy and quality of the embryos analyzed in the study.

Table 4.1.1. Data of the treatment and patient's characteristics that could influence the clinical outcome, morphological quality and ploidy of the embryos included in the study. Continuous data are expressed as mean ± SD.

Variables	Patients Recruited (n=5)
Female	
Age (years)	37.60 (1.67)
BMI (kg/m^2)	22.13 (2.14)
Menstrual cycle (days)	28-35
Ovarian Stimulation Protocol	GnRH antagonist
AMH (ng/mL)	2.58 (1.39)
AFC	19.25 (12.37)
Dosage of Gonadotropins (IU/day)	1310.0 (475.8)
Ovarian Stimulation Duration (days)	12.2 (1.3)
Male	
Age (year)	37.80 (3.03)
BMI (kg/m^2)	25.40 (1.74)
Sperm Quality Parameters	
Normozoospermia	3
Oligoasthenoteratozoospermia	2
Infertility Diagnosis	
Idiopathic	1
Implantation Failure	2
Recurrent Abortion	1
Insemination Failure	1
Duration of Infertility (years)	2.25 (1.85)
Medical Conditions	
Low Ovarian Reserve	1
Uterine Malfunctions	1
Advanced Maternal Age	1
Previous IVF Treatment Cycles	1.20 (0.83)
Recruited Follicles >11mm	14.20 (3.27)
Retrieved Oocytes	16.20 (6.61)
Fertilized Oocytes	11.60 (2.97)
Embryos Biopsied on day 5	6.20 (2.17)
Embryos Biopsied on day 6	0.60 (1.34)
Embryo Quality	
A	5
B	20
C	1
Embryo Ploidy	
Aneuploid	12
Euploid	14
Mosaic	3

4.2. ^{1}H-NMR Metabolomic Analysis of the SECM

Regarding the intra-specific embryos characteristics it was analyzed how the samples clustered considering its quality. It was taken in consideration the following parameters: female and male donors age and body mass index (BMI), antral follicular count (AFC) baseline levels, anti-Müllerian hormone (AMH) baseline levels, infertility diagnosis, medical conditions, infertility duration time and sperm quality. The infertility treatment specificities taking into account were the total dosage of gonadotropins, number of previous IVF cycles and duration of ovarian stimulation (OS). Unbiased multivariate analysis with principal component analysis (PCA) of the metabolite's levels obtained from the ^{1}H-NMR spectra and of the specific patient's and embryo's characteristics and treatment procedures showed no significant clusters among variables with strong overlaps, observable in the supplementary figures 8.1. to 8.15. This data suggests that all the observable metabolic differences are due to the influence of embryo's ploidy and not patient-, treatment-, nor embryo quality- specificities derived.

In this study, the embryos were incubated in a sequential step media. After fertilization until day 3, embryos were incubated in cleavage media. Since day 3 until day 5 they were incubated in blastocyst media. The composition of both media is different and optimized for the specific metabolic early requirements of each phase. It was analyzed the media from the two timepoints, that otherwise would be discarded, to evaluate the metabolic activity of embryos with different ploidy.

The metabolic profile was obtained by identification and quantification of the metabolites using ^{1}H-NMR spectra. Representative ^{1}H-NMR spectra of SECM from day 3 and day 5 are presented in Figure 4.2.1. and 4.2.2, respectively. At day 3, it was possible to identify 18 compounds, and 23 at day

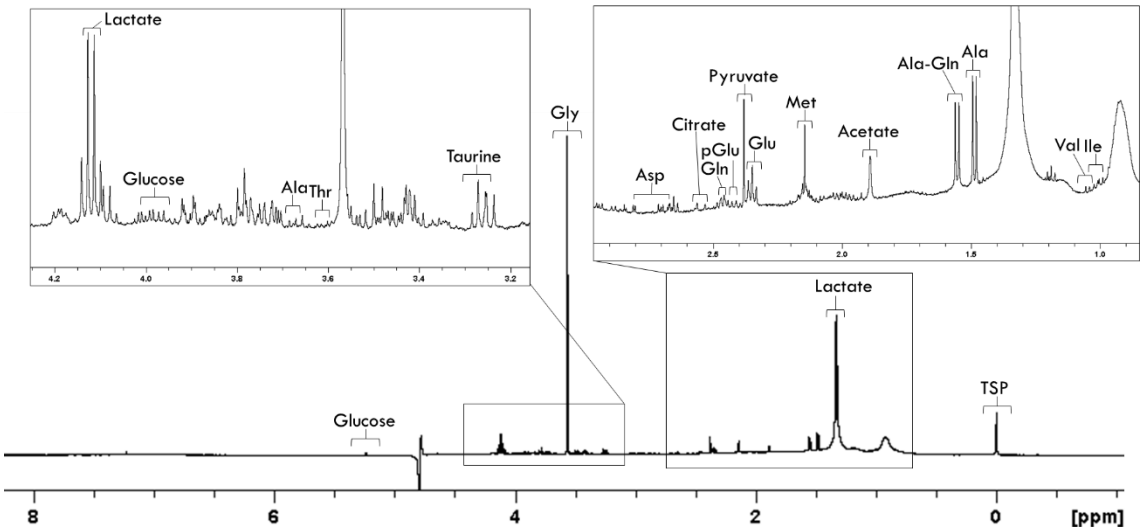

Figure 4.2.1. Representative ^{1}H-NMR spectra of cleavage SECM samples (day 3) measured at 298K, using TSP as a reference compound. Abbreviation: Ala, alanine; Ala-Gln, L-alanyl-L-glutamine; Asp, aspartate; Gln, glutamine; Glu, glutamate; Gly, glycine; Ile, isoleucine, Met, methionine; pGlu, pyroglutamate; ppm, parts per million; Thr, threonine; TSP, Trimethylsilylpropanoic acid; Val, valine.

5, as listed in table 4.2.1. Not only the blastocyst media has more metabolites, but they are also present at higher concentrations. Between SECM of aneuploid, euploid embryos and embryo-free media in

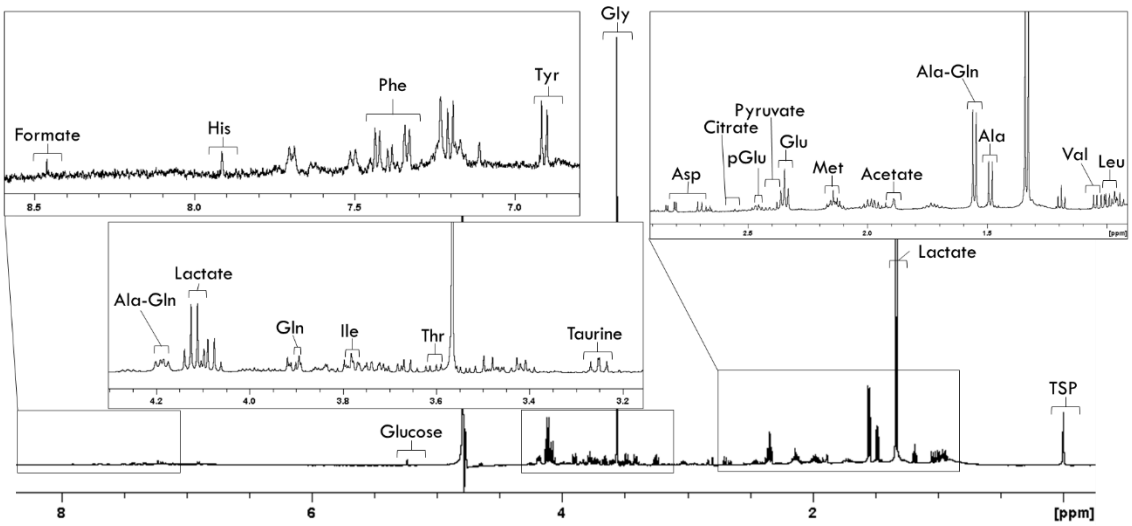

Figure 4.2.2. Representative ^{1}H-NMR spectra of blastocyst SECM samples (day 5) measured at 298K, using TSP as a reference compound. Abbreviation: Ala, alanine; Ala-Gln, L-alanyl-L-glutamine; Asp, aspartate; Gln, glutamine; Glu, glutamate; Gly, glycine; Ile, isoleucine, His, Histidine; Met, methionine; pGlu, pyroglutamate; Phe, Phenylalanine; ppm, parts per million; Thr, threonine; TSP, Trimethylsilylpropanoic acid; Tyr, Tyrosine; Val, valine.

Table 4.2.1. Metabolites identified from cleavage and blastocyst media using ^{1}H-NMR spectra.

Compounds	Cleavage Media	Blastocyst Media
Acetate	×	×
Alanine	×	×
Aspartate	×	×
Citrate	×	×
Formate	×	×
Glucose	×	×
Glutamate	×	×
Glutamine	×	×
Glycine	×	×
Histidine		×
Isoleucine	×	×
Lactate	×	×
L-Alanyl-L-Glutamine	×	×
Leucine		×
Lysine		×
Methionine	×	×
Phenylalanine		×
Pyroglutamate	×	×
Pyruvate	×	×
Taurine	×	×
Threonine	×	×
Tyrosine		×
Valine	×	×

equal timepoints were only observed differences regarding the compounds' levels and not in composition.

In figure 4.2.3. are represented the levels of all the metabolites identified directly involved in the glycolytic and oxidative bioenergetic pathways. All of them were present in cleavage and blastocyst media, before and after embryo incubation. Glucose (0.782±0.001 mM and 1.665±0.003 mM on day-3 and day-5 embryo-free ECM, respectively) and lactate (3.477±0.057 mM and 6.085±0.617 mM in day-3 and day-5 embryo-free ECM, respectively) were the substrates present in higher concentrations. During the cleavage phase, the chromosomally different embryos had a similar uptake pattern. While

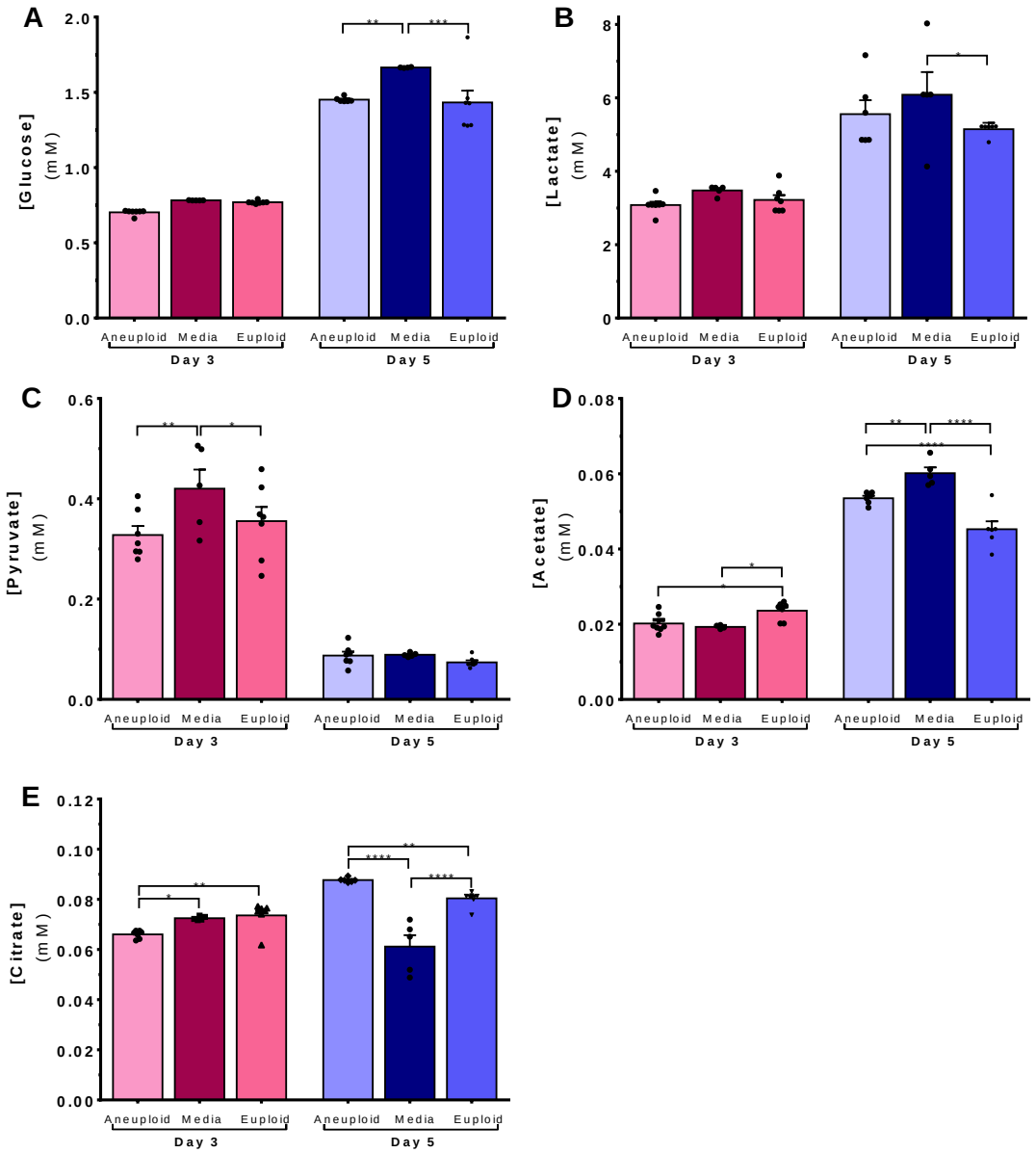

Figure 4.2.3. Metabolites associated with glycolytic and oxidative pathway: (A) Glucose, (B) lactate, (C) pyruvate, (D) acetate, (E) citrate. Results are expressed as mM and presented as mean± standard error of the mean. SECM from euploid embryos n=7; aneuploid embryos n=7; embryo-free media n=5. Significant differences between groups are denoted as: *(p<0.05), **(p<0.01), ***(p<0.005) and ****(p<0.0005).

during the blastocyst phase, there is a tendency for an increased uptake of euploid embryos of glucose and lactate (1.433±0.078 mM of glucose and 5.146±0.071 mM of lactate) when compared with aneuploid embryos (1.451±0.007 mM of glucose and 5.557±0.377 mM of lactate). Pyruvate exhibited a more preponderant role during cleavage phase, howsoever the two experimental groups had a similar behavior. No significant differences were observed on day 5. Euploid and aneuploid embryos had a differential uptake of acetate, observable on day 3 and 5. SECM acetate levels from euploid embryos displayed are 0.024±0.001 mM on day 3 and 0.045±0.002 mM on day 5. The levels in aneuploid samples from day 3 was 0.020±0.001 mM and on day 5 was 0.054±0.001 mM. Aneuploid embryos during cleavage phase tended to uptake citrate (0.066±0.001 mM) and euploid embryos displayed no difference comparing to embryo-free cleavage media (0.074±0.002 mM); while in the blastocyst phase, the embryos secreted citrate (0.080± 0.001 mM in euploid and 0.877±0.001 mM in aneuploid embryos).

Amino acids are used by cells in protein synthesis or in bioenergetic pathways. Some amino acids are not sufficiently synthesized by human cells, but are still necessary for the cellular activity, denominated as essential amino acids (EAA). Only half of the essential amino acids identified in blastocyst media were also present in cleavage media. Aneuploid and euploid embryos showed a significantly different behavior regarding the metabolization of the majority of EAA during blastocyst stage, apart from valine in which no difference was observed. Histidine, phenylalanine, threonine and methionine levels were increased in SECM from aneuploid embryos (0.155±0.006 mM, 0.214±0.001 mM, 0.420±0.026 mM and 0.145±0.002 mM, respectively) and decreased in euploid embryo's samples (0.082±0.003 mM, 0.158±0.001 mM, 0.291±0.007 mM and 0.084±0.009 mM respectively). Euploid embryos tended to uptake higher amounts of leucine (0.196±0.003 mM), lysine (0.218±0.002 mM) and isoleucine (0.242±0.006 mM), in comparison to aneuploid embryos (0.228±0.002 mM, 0.243±0.004 mM and 0.307±0.006 mM, respectively). While during cleavage phase, only methionine uptake was significantly higher by aneuploid embryos (0.148±0.002 mM) when compared to embryo-free media (0.164±0.002 mM).

The denominated non-essential amino acids (NEAA) are synthesized by human cells, and all NEAA were present in both timepoints (figure 4.2.5.). During cleavage phase, the only significant differences identified were regarding the higher uptake of pyroglutamate (0.085±0.004 mM), when compared to embryo-free media (0.112±0.012 mM). The levels of aspartate in day 5 were decreased in SECM from euploid embryos (0.816±0.033 mM), when compared to embryo-free media (0.949±0.099 mM). During blastocyst phase, euploid embryos uptake pyroglutamate and alanine (0.136±0.008 mM and 0.775±0.024 mM, respectively), while aneuploid embryos secrete (0.162±0.007 mM and 0.915±0.034 mM, respectively).

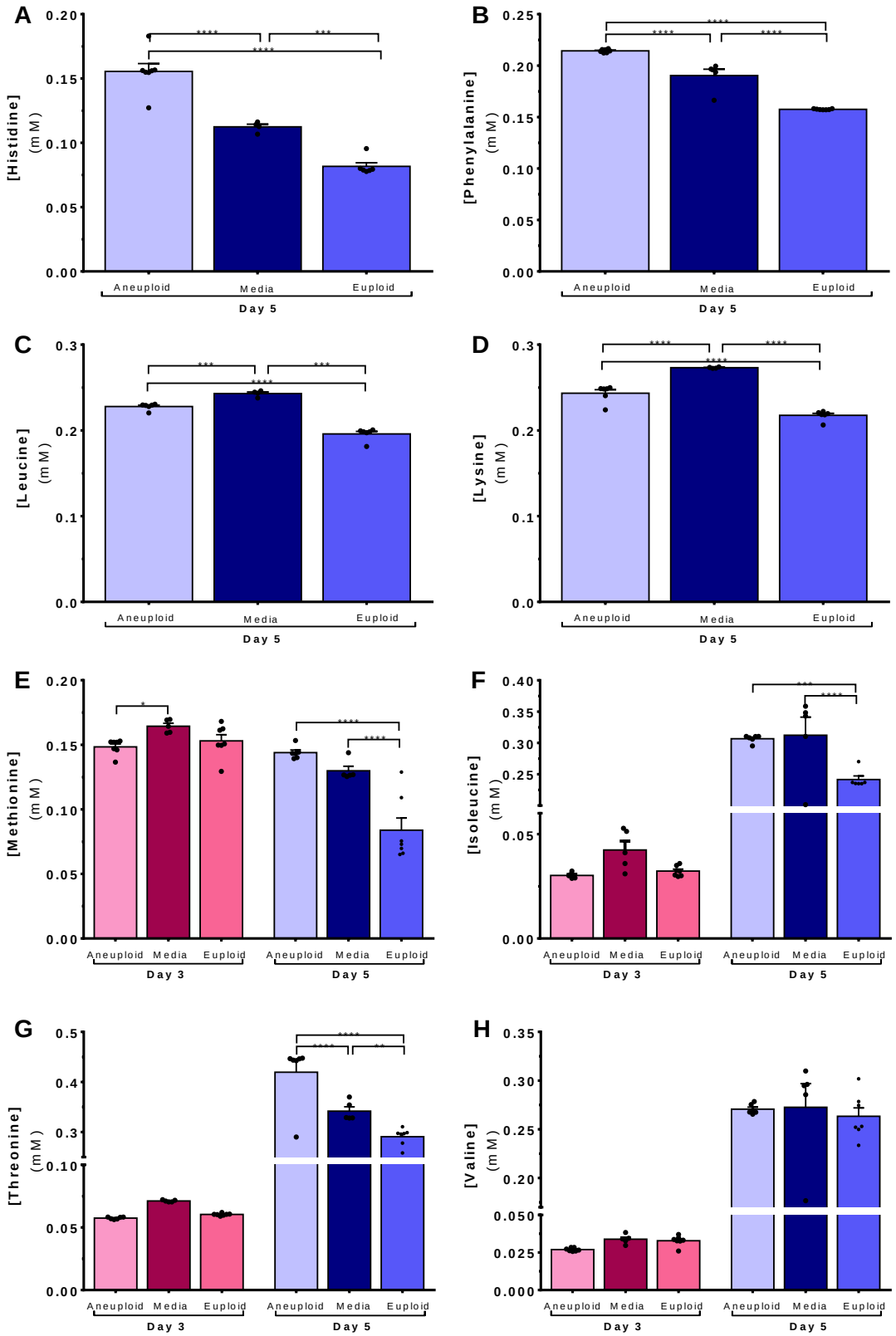

Figure 4.2.4. Essential amino acids present in SECM: (A) Histidine, (B) phenylalanine, (C) leucine, (D) lysine, (E) methionine, (F) isoleucine, (G) threonine, (H) valine. Results are expressed as mM and presented as mean± standard error of the mean. SECM from euploid embryos n=7; aneuploid embryos n=7; embryo-free media n=5. Significant differences between groups are denoted as: *(p<0.05), **(p<0.01), ***(p<0.005) and ****(p<0.0005).

Conditionally non-essential amino acids (CNEAA) are amino acids only not synthesized under specific pathophysiological conditions, represented in figure 4.2.6. Of the CNEAA identified in day 5, only tyrosine was not present in cleavage media. On day 3, was not identified any significant differences among euploid and aneuploid embryos, but embryos tended to uptake taurine and secrete glutamine from embryo-free media. During blastocyst stage, euploid embryos absorbed higher amounts of glutamine and tyrosine (0.366±0.001 mM and 0.148±0.006 mM, respectively) when compared to aneuploid embryos (0.479±0.001 mM and 0.204±0.001 mM, respectively).

Formate was present in both sequential step-media, and its levels are represented in figure 4.2.7. A. This compound is a precursor of nucleotide synthesis. On day 3, was identified an uptake of formate by aneuploid embryos (0.024±0.005 mM) and its secretion by euploid embryos (0.029±0.004 mM), being significant different in the SECM. On day 5, both groups behaved similarly with a non-significant tendency for its uptake.

L-Alanyl-L-Glutamine (Ala-Glu) is a dipeptide commonly associated with ammonia removal, which in case of accumulation can cause cellular damage. This compound was found in cleavage and blastocyst media (levels represented in figure 4.2.7. B). It was observable a non-significant uptake of this metabolite by aneuploid and euploid embryos.

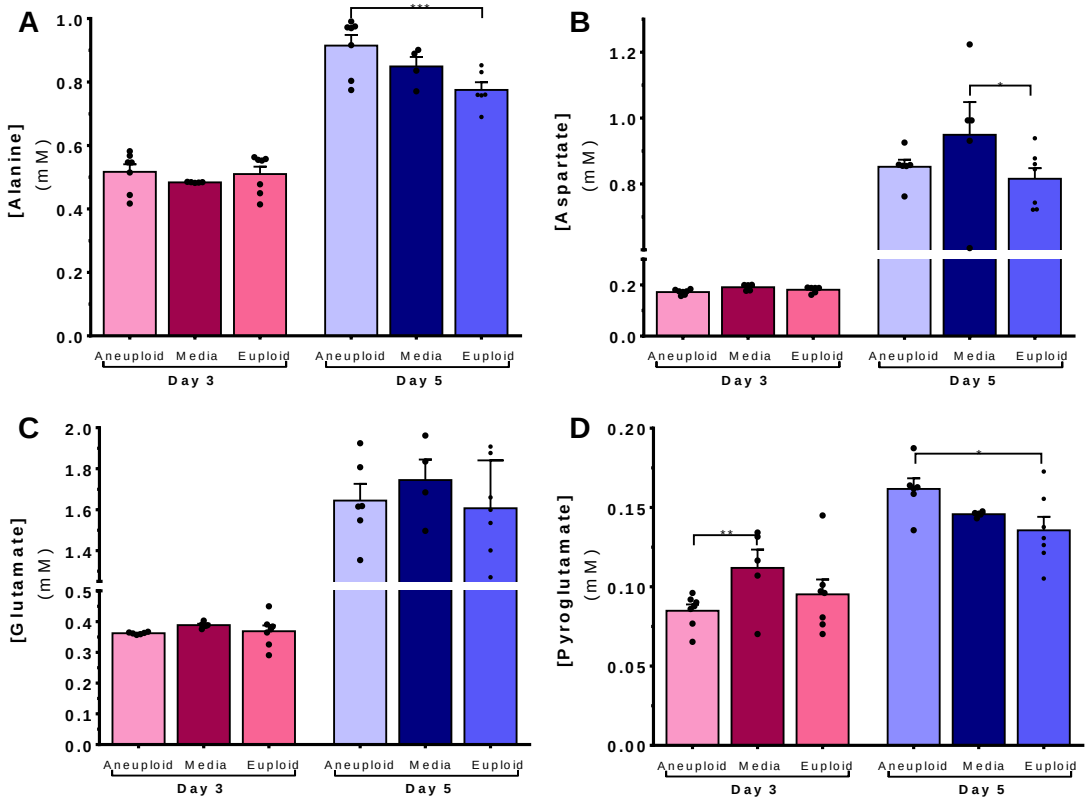

Figure 4.2.5. Non-essential amino acids present in SECM: (A) Alanine, (B) aspartate, (C) glutamate, (D) pyroglutamate. Results are expressed as mM and presented as mean± standard error of the mean. SECM from euploid embryos n=7; aneuploid embryos n=7; embryo-free media n=5. Significant differences between groups are denoted as: *(p<0.05), **(p<0.01), ***(p<0.005) and ****(p<0.0005).

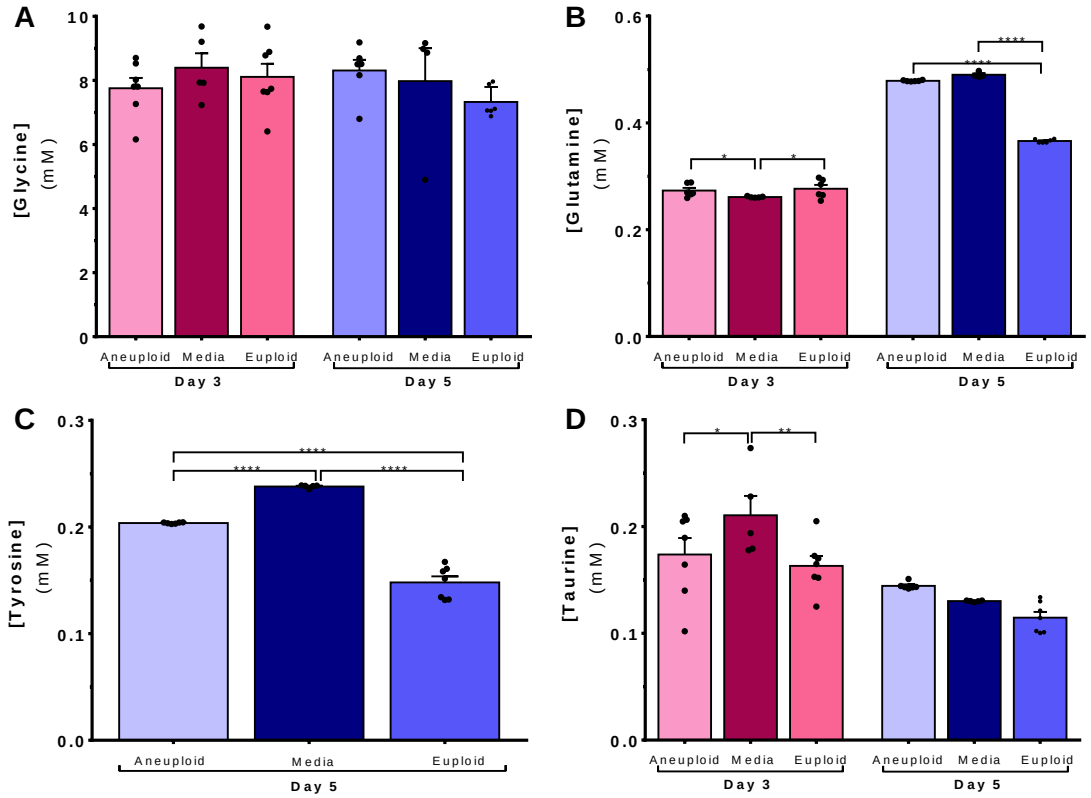

Figure 4.2.6. Conditionally non-essential amino acids present in SECM: (A) Glycine, (B) glutamine, (C) tyrosine, (D) taurine.Results are expressed as mM and presented as mean± standard error of the mean. SECM from euploid embryos n=7; aneuploid embryos n=7; embryo-free media n=5. Significant differences between groups are denoted as: *(p<0.05), **(p<0.01), ***(p<0.005) and ****(p<0.0005).

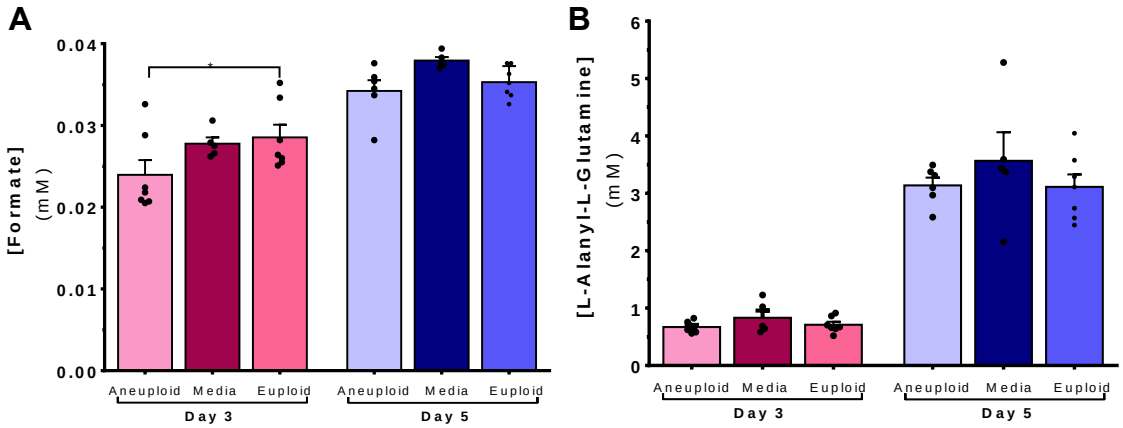

Figure 4.2.7. Levels of Formate (A) and L-Alanyl-L-Glutamine (B) in SECM. Results are expressed as mM and presented as mean± standard error of the mean. SECM from euploid embryos n=7; aneuploid embryos n=7; embryo-free media n=5. Significant differences between groups are denoted as: *(p<0.05), **(p<0.01), ***(p<0.005) and ****(p<0.0005).

4.3. Multivariable Analysis of Metabolite Concentration in SECM

A univariate and multivariate data analyses were performed aiming find if the SECM metabolic profile allow the discrimination between aneuploid and euploid embryos. Metabolite levels were subjected to scalling and to a multivariate analysis using the MetaboAnalyst. A univariate data analysis (Student's t-test) was used to look for metabolites that are significant different between euploid and aneuploid embryos, the important features identified are shown in Figure 4.3.1. and in table 4.3.1. On day 3, the levels of 3 of the 18 metabolites were significantly different (glucose, valine, and citrate). On day 5, 6 of 23 metabolites were significantly different: phenylalanine, histidine, tyrosine, glutamine, threonine, and methionine.

Table 4.3.1. Important features of SECM metabolic profile identified by Student t-test analysis. n=7 paired SECM

Compound	*p*. value	-log10 (*p*)	FDR
SECM of day 3			
Glucose	1.81E-6	5.74	3.26E-5
Valine	1.10E-3	2.96	9.91E-3
Citrate	3.86E-3	2.41	2.32E-2
SECM of day 5			
Phenylalanine	8.20E-11	10.1	1.89E-9
Histidine	6.12E-4	3.21	7.04E-3
Tyrosine	1.93E-3	2.72	1.48E-2
Glutamine	5.14E-3	2.29	2.95E-2
Threonine	7.44E-3	2.13	3.42E-2
Methionine	9.92E-3	2.00	3.80E-2

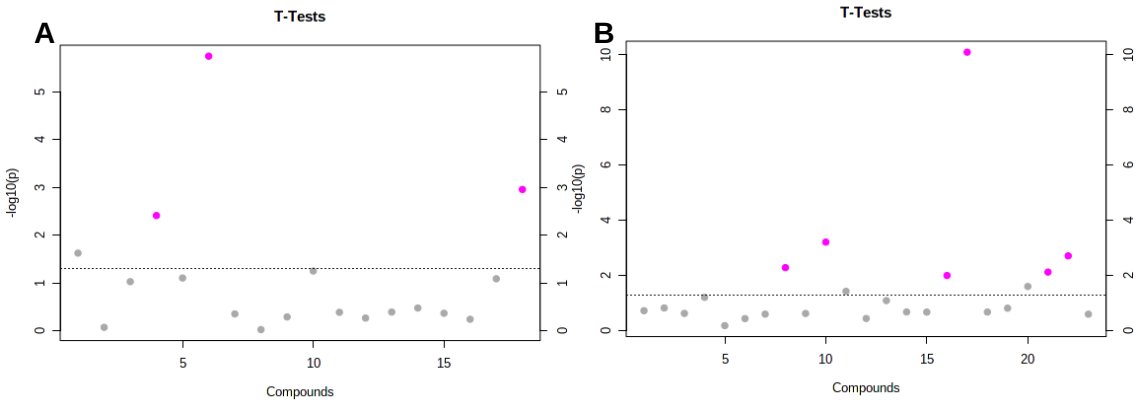

Figure 4.3.1. Important features selected by Student t-test analysis with threshold 0.05 on day 3 (A) and on day 5 (B). The pink circles represent features with corrected *p* values above 0.05. Note that *p* values are converted by -log10, in order to plot significant features with smaller *p* values on the graph.

To evaluate the differences between aneuploid and euploid embryos, the metabolic profile of the SECM were analyzed through PCA score plot. All samples scattered in the figure 4.3.2. fell into the 95% confidence interval. The aneuploid and euploid groups were clustered together and separated from each other, despite a minor overlap, thus indicating a significant metabolic footprinting in SECM from

embryos with different ploidy and suggested that the involved metabolites were perturbed in the aneuploid groups. In both timepoints, the first component (PC1) is mainly responsible for the variation (58.9% and 74.4% in day 3 and day 5, respectively). Noteworthy, on day 5, sample A from aneuploid embryos presents a higher variability when compared with samples from other aneuploid embryos. The large majority of its metabolites are in higher concentrations, such as glucose, valine and lysine, this increased levels are clearly noticed in figure 4.3.6. as it will be analyzed further on. It was chosen kept this sample in the analysis, since no laboratorial handling reason was found that explain this behavior.

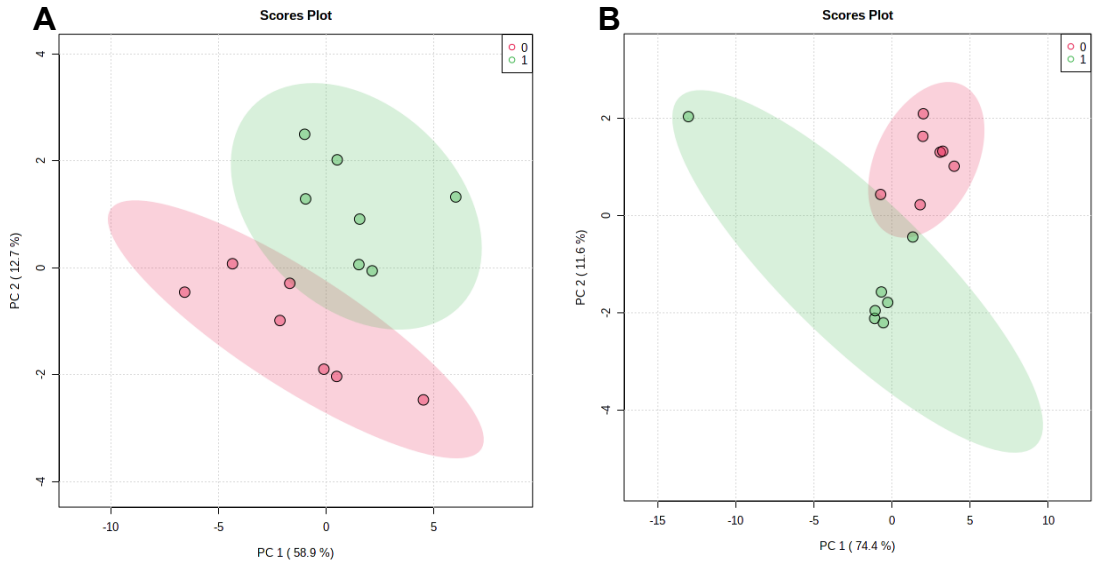

Figure 4.3.2. PCA score plots of SECM from day 3 (A) and day 5 (B). PCA score plot depicts the clustering patterns with 2 principal components of the metabolite concentration data from SECM on day 3. Shaded ellipses represent the 95% confidence interval region with a minor overlap. The SECM from aneuploid and euploid embryos are colored in green and red, and represented by 1 and 0, respectively. Data were analyzed using MetaboAnalyst 5.0. n=7 paired SECM from aneuploid and euploid embryos.

To create a discriminative and predictive model between the aneuploid and euploid groups, it was performed a PLS-DA and orthoPLS-DA with the metabolite levels. Regarding day 3, the metabolites responsible for the discrimination of groups both using PLS-DA and orthoPLS-DA are glucose, valine, citrate, acetate, isoleucine, formate, threonine, and aspartate (figure 4.3.3. B and supplementary figure 8.16. B). The robustness of the PLS-DA model for day 3, further used to identify biomarkers able to predict embryo ploidy, was evaluated by cross-validation (supplementary figure 8.16. C). By using tenfold cross-validation procedure the model presented a goodness-of-prediction parameter (Q^2) of 0.709 (supplementary figure 8.16 C). Further validation was performed by permutation analysis (p=0.0795), as seen in supplementary figure 8.16. B. The score plot in the orthoPLS-DA showed better clustering tendency between the aneuploid (green) and euploid group (pink) in day 3 as shown in figure 4.3.3. A. The percentage of extracted variance related to class information (T score) was 22.4% and explained 52.7% of the variance of class variables (R^2Y=0.527) (figure 4.3.3.). Permutation analysis corroborated the model predictive goodness (Q^2=0.402), with $p=5\times10^{-4}$.

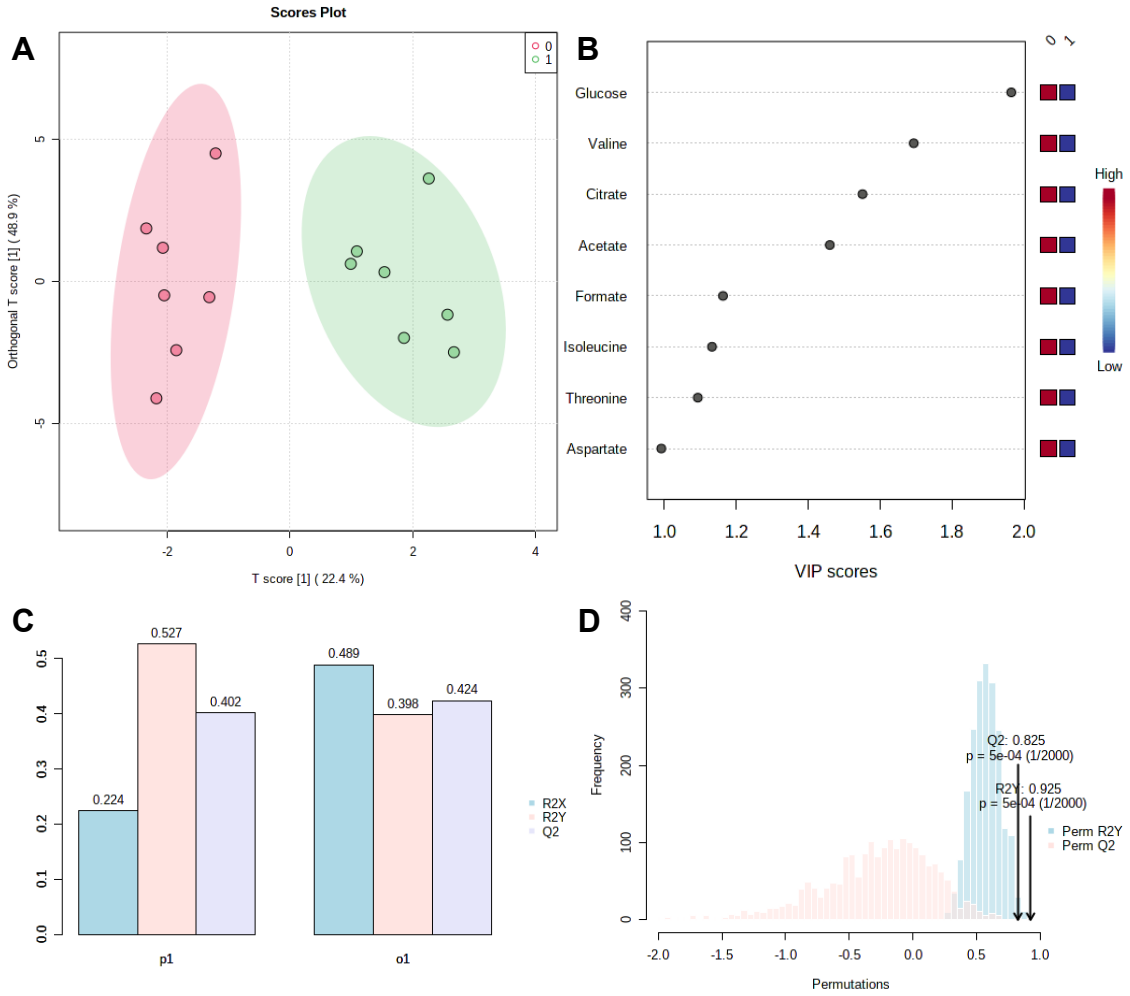

Figure 4.3.3. Summary of orthoPLS-DA of day 3 data set. (A) OrthoPLS-DA score plot depicts the clustering patterns from the metabolite concentration data of SECM on day 3. The SECM from aneuploid and euploid embryos are colored in green and red, and represented by 1 and 0, respectively. T score [1] is responsible for 22.4% of the clustering, when orthogonal T score [1] represents 48.9% of it. Shaded ellipses represent the 95% confidence interval region with no overlap. **(B)** The orthoPLS-DA VIP scores indicates that 8 metabolites are significantly responsible for the group discrimination. Red, high levels of metabolites in SECM; blue, low levels of metabolites in SECM. **(C)** The orthoPLS-DA score plot discriminate both groups with a model including 18 metabolites with a performance statistic of R^2Y of 0.527, a Q^2 of 0.402 at VIP threshold of 1.0. **(D)** Permutation test indicates that discrimination of the two groups is correct (*p* value for both R^2Y and Q^2 are under 0.05). Altogether, indicates a significative and robust predictive clustering model. Data were analyzed using Metaboanalyst. n =7 paired SECM from aneuploid and euploid embryos.

Regarding day 5, the main metabolites responsible for the discrimination of the PLS-DA and orthoPLS-DA models are phenylalanine, histidine, tyrosine, threonine, glutamine, methionine, isoleucine, and taurine (supplementary figure 8.18 and figure 4.3.4.). Once more, the cross-validation procedure shown that the model has a good predictive ability (Q^2=0.783) and the permutation analysis shown that the model was acceptable (*p*=0.078) (supplementary figure 8.18). The corresponding orthoPLS-DA highly discriminate the groups, as seen in figure 4.3.4. The percentage of extracted variance related to class information was 29.9% and explained 50.2% of the variance of class variables

(R^2Y=0.502) (figure 4.3.4.). The permutation analysis confirmed the model predictive capacity (Q^2=0.206), with p=5×10^{-4}.

Altogether, these multivariate analysis of SECM both in day 3 and day 5 showed significant metabolic differences between groups and a good clustering and prediction.

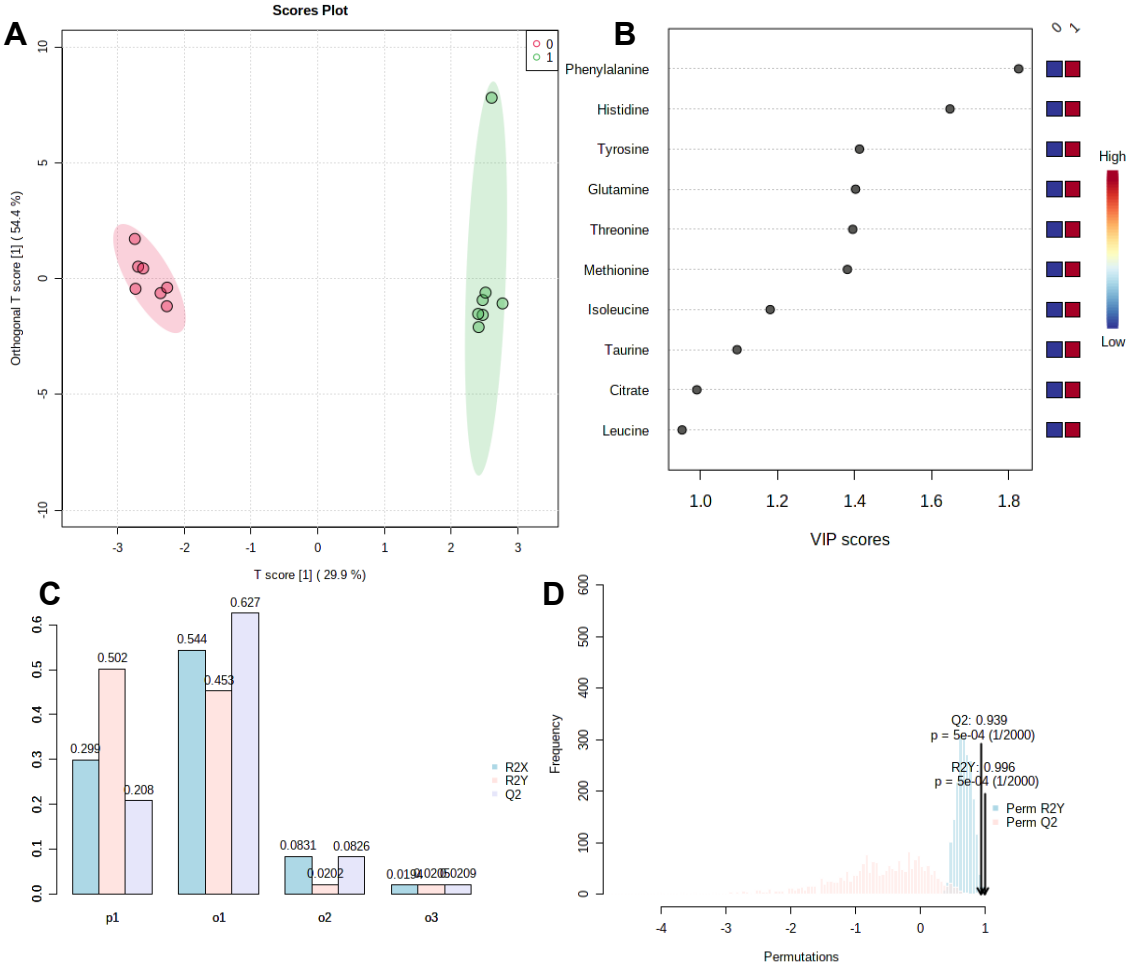

Figure 4.3.4. Summary of orthoPLS-DA of day 5 data set. (A) OrthoPLS-DA score plot depicts the clustering patterns from the metabolite concentration data of SECM on day 5. The SECM from aneuploid and euploid embryos are colored in green and red, and represented by 1 and 0, respectively. T score [1] is responsible for 29.9% of the clustering, when orthogonal T score [1] represents 54.4% of it. Shaded ellipses represent the 95% confidence interval region with no overlap. **(B)** The orthoPLS-DA VIP scores indicates that 10 metabolites are significantly responsible for the group discrimination. Red, high levels of metabolites in SECM; blue, low levels of metabolites in SECM. **(C)** The orthoPLS-DA score plot discriminate both groups with a model including 23 metabolites with a R^2Y of 0.502, a Q^2 of 0.206 at VIP threshold of 1.0. **(D)** Permutation test indicates that discrimination of the two groups is correct (p value for both R^2Y and Q^2 are under 0.05). Altogether, indicates a significative and robust predictive clustering model. Data were analyzed using Metaboanalyst. n =7 paired SECM from aneuploid and euploid embryos.

In figure 4.3.5. and figure 4.3.6. are shown the hierarchical clustering of the day 3 and day 5, respectively, samples in columns, the metabolites in rows and each cell is colored based on metabolites' levels. Using 18 and 23 metabolites, both heat maps presented an acceptable clustering. Once again, the sample A from day 5 aneuploid group shown a very different pattern from the rest. The same behavior is obtained if the hierarchical clustering analysis is performed based only in the significantly different

metabolites for each day. SECM samples from day 3 clustered based on the glucose, valine, and citrate metabolic behavior (supplementary figure 8.17); while samples from day 5 were clustered based on a higher metabolite number, using: glutamine, phenylalanine, histidine, threonine, tyrosine, methionine, isoleucine, pyruvate, taurine, acetate, citrate, and leucine (supplementary figure 8.19). Following the tendency previously noted, data from day 3 aneuploid samples suggest a lower metabolite levels on SECM (blue), while data from day 5 aneuploid samples indicate higher metabolite levels (red).

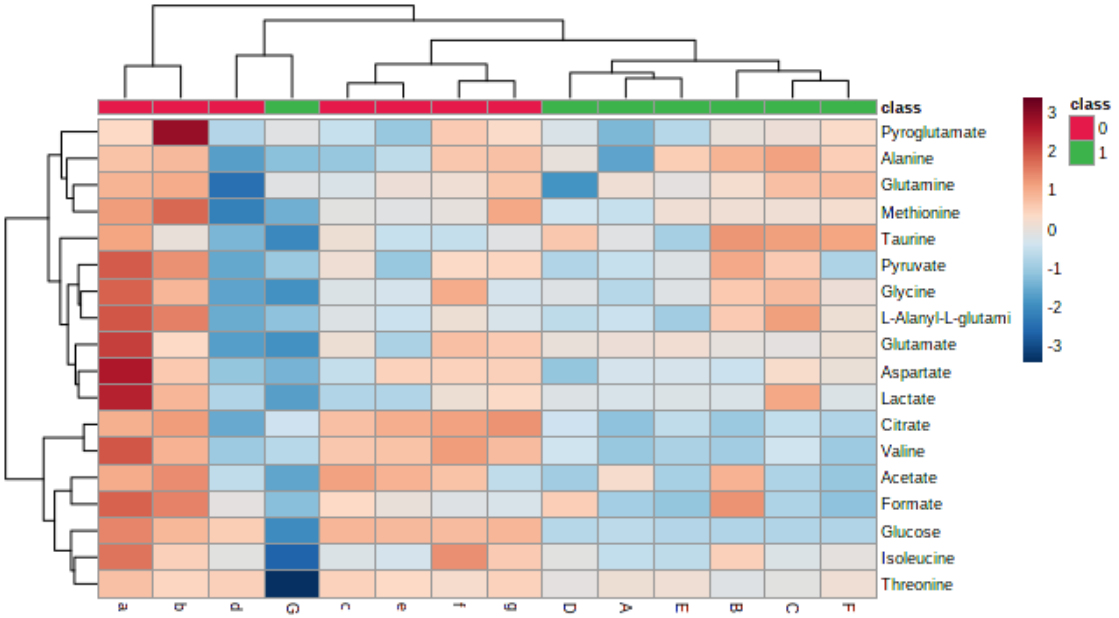

Figure 4.3.5. Heat map of 18 altered metabolites in day 3 SECM from aneuploid compared to euploid embryos. Red, increased metabolites levels on SECM; blue, decreased metabolites levels on SECM. The SECM from aneuploid and euploid embryos are colored in green and red, and represented by 1 and 0, respectively. Data were analyzed using Metaboanalyst. n =7 paired SECM from aneuploid and euploid embryos.

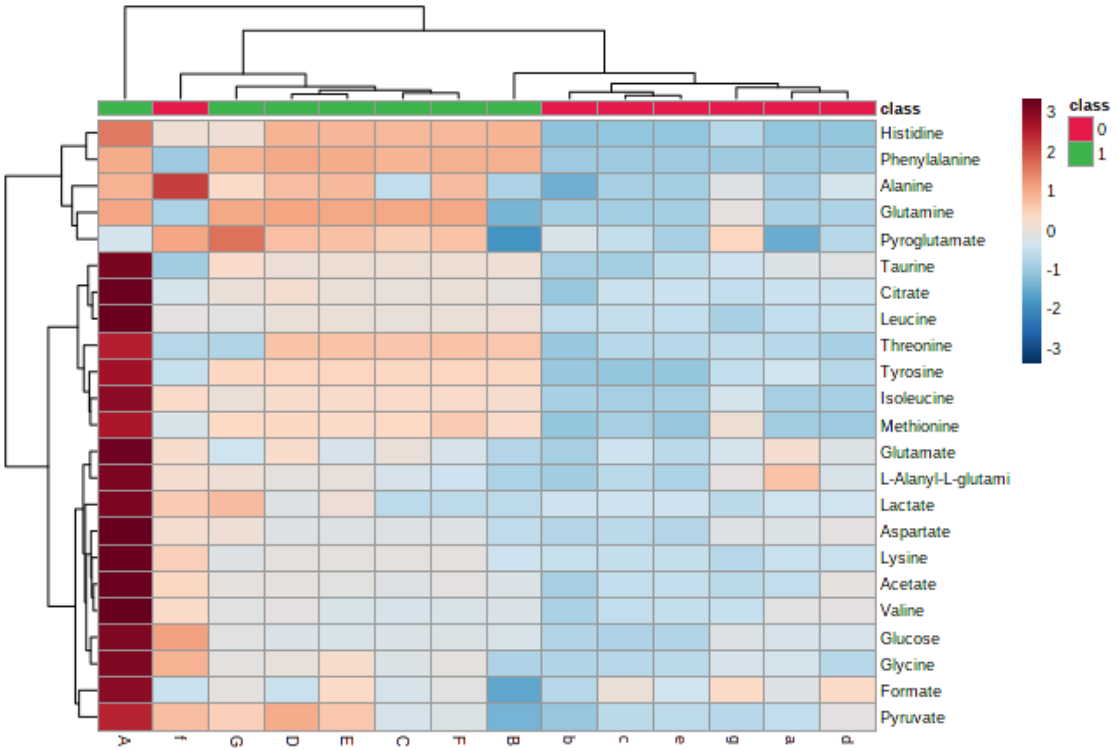

Figure 4.3.6. Heat map of 23 altered metabolites in day 5 SECM from aneuploid compared to euploid embryos. Red, increased metabolites levels on SECM; blue, decreased metabolites levels on SECM. The SECM from aneuploid and euploid embryos are colored in green and red, and represented by 1 and 0, respectively. Data were analyzed using Metaboanalyst. n =7 paired SECM from aneuploid and euploid embryos.

4.4. Predictive Power of the SECM Biomarkers

A receiver operating characteristic (ROC) curve analysis of potential biomarker using metabolite levels for differentiating between aneuploid and euploid embryos were construct in MetaboAnalyst (table 4.4.1). From the 18 and 23 metabolites quantified, 5 and 11 metabolites from day 3 and 5, respectively, showed an area under the curve (AUC) higher than 0.85 when SECM from aneuploid and euploid embryos were compared.

For the predictive model of day 3 SECM was used 5 metabolites (glucose, threonine, acetate, valine, and citrate). In figure 4.4.1. A is represented the AUC value of ROC for logistics regression model of day 3. The AUC value was 0.973 (95% CI=0.75-1). The predictive model of day 5 SECM used 11 metabolites (phenylalanine, histidine, tyrosine, methionine, taurine, citrate, leucine, threonine, isoleucine, lysine, and glutamine). In figure 4.4.1. B is represented the AUC value of ROC for logistics regression model of day 5. The AUC value was 1.000 (95% CI=1). The predictive models indicate a sensitivity of 83% and 100% and specificity of 100% and 78% for identification of aneuploidy on day 3 and day 5, respectively.

The prediction capacity of the created models is shown in table 4.4.2. and table 4.4.3. For data set from day 3 the model predicted correctly 11/12 and 10/12 in day 5.

Table 4.4.1. List of AUC determined for the different metabolites to discriminate between aneuploid and euploid embryo, using MetaboAnalyst 5.0.

	Day 3		Day 5	
Compounds	**AUC**	**t-tests**	**AUC**	**t-tests**
Acetate	0.888	0.024	0.796	0.148
Alanine	0.510	0.835	0.776	0.219
Aspartate	0.776	0.093	0.653	0.234
Citrate	0.857	0.004	1.000	0.046
Formate	0.796	0.078	0.520	0.650
Glucose	1.000	1.813E-6	0.776	0.341
Glutamate	0.714	0.437	0.633	0.314
Glutamine	0.541	0.931	0.857	0.002
Glycine	0.571	0.506	0.756	0.223
Histidine	---	---	1.000	8.417E-6
Isoleucine	0.786	0.056	0.878	0.013
Lactate	0.571	0.405	0.592	0.301
L-Alanyl-L-Glutamine	0.633	0.534	0.612	0.368
Leucine	---	---	0.980	0.057
Lysine	---	---	0.878	0.176
Methionine	0.612	0.400	1.000	0.002
Phenylalanine	---	---	1.000	2.083E-18
Pyroglutamate	0.643	0.329	0.714	0.255
Pyruvate	0.592	0.424	0.735	0.142
Taurine	0.612	0.565	1.000	0.025
Threonine	1.000	0.081	0.898	0.002
Tyrosine	---	---	1.000	0.001
Valine	0.888	0.001	0.684	0.254

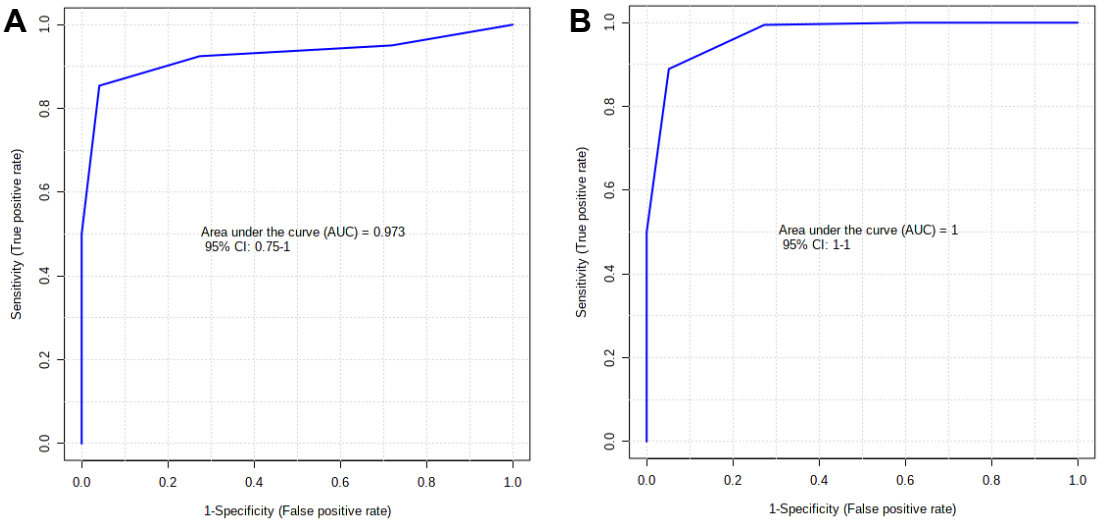

Figure 4.4.1. AUC of ROC curve for logistics regression model with 8 latent variables (PLS-DA only), using liner Support Vector Machine, for SECM on day 3 (**A**) and on day 5 (**B**). For the predictive model, it was chosen the metabolites with AUC>0.85. The numerical value of the AUC would be close to 1 if the prediction was excellent and close to 0.5 if it was poor. The average accuracy based on 100 cross validations is 0.973 and 1.000, on day 3 and day 5, respectively. These predictive models display a high sensitivity and accuracy.

Table 4.4.2. Class prediction of training set samples based on day 3 ROC.

Sample	Ploidy	Probability	Class Prediction	Accuracy
M	Aneuploid	0.95697	Aneuploid	Yes
N	Aneuploid	0.99985	Aneuploid	Yes
O	Aneuploid	0.99475	Aneuploid	Yes
P	Aneuploid	0.96556	Aneuploid	Yes
Q	Aneuploid	0.99500	Aneuploid	Yes
R	Euploid	0.83098	Euploid	Yes
S	Euploid	0.90339	Euploid	Yes
T	Euploid	0.60354	Aneuploid	No
U	Euploid	0.96224	Euploid	Yes
V	Euploid	0.94614	Euploid	Yes
X	Euploid	0.90664	Euploid	Yes
Z	Euploid	0.99530	Euploid	Yes

Table 4.4.3. Class prediction of training set samples based on day 5 ROC.

Sample	Ploidy	Probability	Class Prediction	Accuracy
M	Aneuploid	0.84555	Aneuploid	Yes
N	Aneuploid	0.87795	Euploid	No
O	Aneuploid	0.63560	Aneuploid	Yes
P	Aneuploid	0.83895	Aneuploid	Yes
Q	Aneuploid	0.77481	Euploid	No
R	Euploid	0.62266	Euploid	Yes
S	Euploid	0.63634	Euploid	Yes
T	Euploid	0.88455	Euploid	Yes
U	Euploid	0.90241	Euploid	Yes
V	Euploid	0.89090	Euploid	Yes
X	Euploid	0.85618	Euploid	Yes
Z	Euploid	0.86585	Euploid	Yes

4.5. Power Analysis

Due to the pandemic-imposed conditions, there was a low number of couples who meet the study requirements resorting to IVF treatments. Moreover, the low efficiency of embryo development, low probability of obtaining a pair of, at least, 1 euploid and 1 aneuploid embryo per couple, lack of free-space in the clinical embryoscope incubator and the decision of some couples to not participate in the study, the number of samples analyzed was smaller than the expected for the thesis time. Thus, it was performed a power analysis aiming to identify the ideal sample number required to have accurate, precise, and robust set-data for proposing non-invasive preimplantation genetic testing for aneuploidy metabolite biomarkers present in the SECM. The results are shown in figure 4.5.1. Data suggest that in both day 3 and day 5 SECM would reach a maximum plateau at 24 paired samples. Using day 3 SECM has only a maximum predictive power of 0.13, while day 5 SECM has a predictive power of around 1.0; but with 15 paired samples is obtain a predictive power of 0.92.

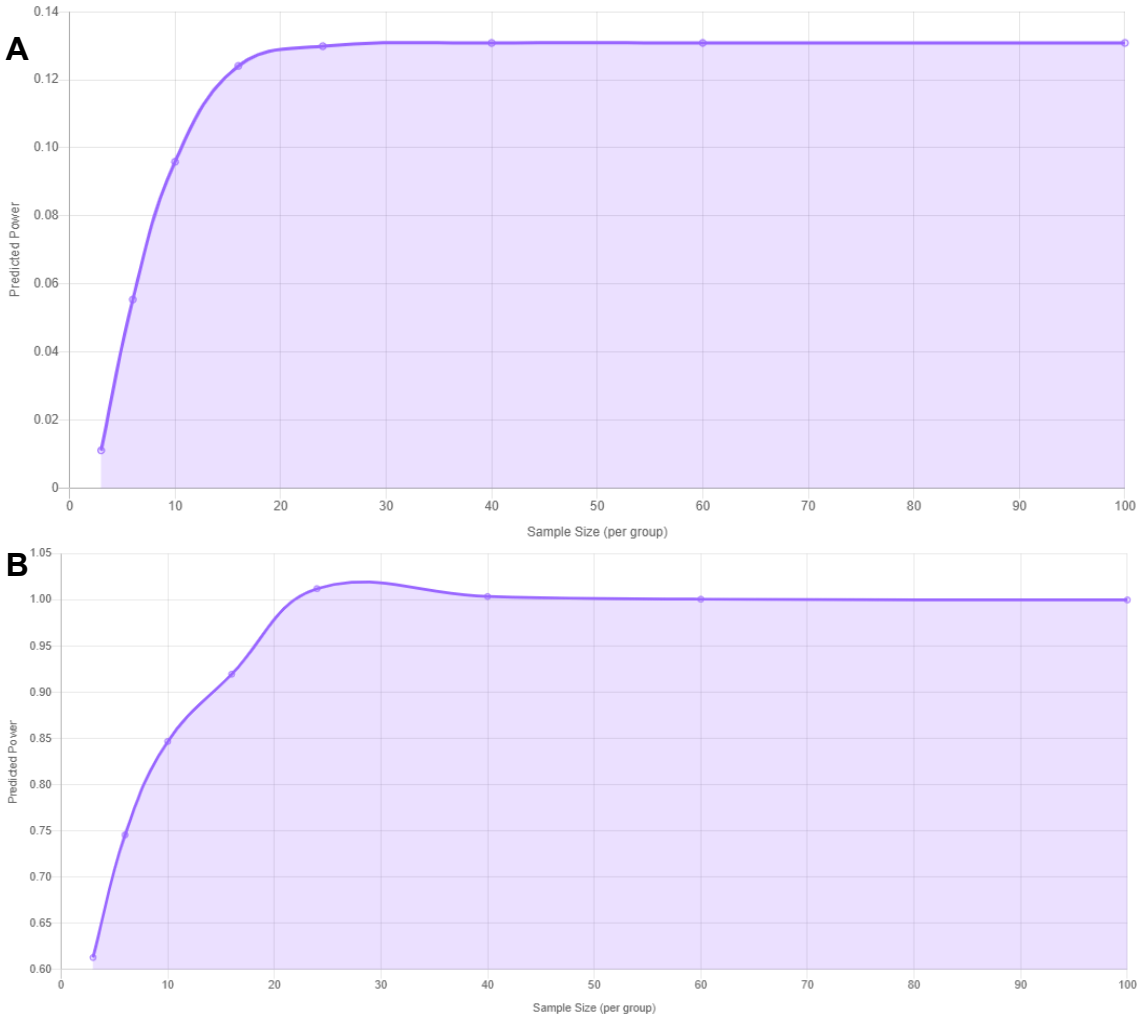

Figure 4.5.1 Power analysis and sample number (n), using a false discover rate of 0.05 and a maximum sample size per group of 100. Both on day 3 (**A**) and day 5 (**B**) reach the plateau of maximum predictive power of 0.13 and 1.0, respectively, with n=24. It was used SECM from 7 aneuploid and 7 euploid embryos.

5. Discussion

5.1. The Ploidy of the Embryo Influences Its Metabolic Activity

Transferring embryos that are most likely to implant and develop in a successfully pregnancy is the holy grail in the ART field. Aneuploidy is a genetic condition that mostly conditionate embryo development and cannot be identified using the classic morphological criteria (123, 124). Although day 5 trophoectoderm biopsies are less likely to dramatically compromises the implantation and pregnancy rate, if this invasive technique is performed poorly it can severely compromise both the embryo and the PGT-A results (82, 83). There is an active development of new selection procedures that met different criteria: be safe for the embryo, accurate, and effective. In this thesis, we present a preliminary study of use the determination, by ^{1}H-NMR, of SECM metabolomic profile to search for aneuploidy diagnosis. This approach does not affect the embryo development and do not need any other supplementary embryo invasive techniques beside those already performed in ART practice. A better understanding of SECM metabolome of aneuploid embryos will help improve embryo selection to transfer criteria and, similarly, will open a new door for the development of a new non-invasive preimplantation genetic testing for aneuploidy.

The present research has the main aim to study the SECM profile of aneuploid and euploid embryos and identify if numerical chromosomal abnormalities reflect in a differential SECM composition. The couples that choose to make a PGT-A as a single embryo selection criteria prior transfer are advised to resort specifically to ICSI. The embryos were incubated in a sequential step-media, after fertilization until the third day in cleavage media and then until the fifth day in blastocyst media. In this study, SECM from both timepoint samples were analyzed by ^{1}H-NMR spectroscopy.

As presented in table 4.1.1, the number of retrieved oocytes, successful fertilized ones and healthy embryos that reach day 5 decreased along the in vitro culture. This is an old problem in ART procedures, since it diminish the treatment successful rate (34, 125). To overcome this problem, it is necessary the development of new techniques to handle and diagnose embryos in an automatic, non-invasive and harmless way. The ratio of aneuploid/euploid embryos inserted in the study and previously presented is slightly homogenous. This may had happened because of the chosen inclusion criteria of only use paired embryos and does not represent the usual rate of aneuploid/euploid embryos resultant of IVF treatments. On the contrary, the aneuploidy is quite common among IVF embryos, recent studies have reported that it affects more than half of them (34, 79). This was one of the factors that justifies such a small cohort in this study. From the 11 couples that met all the study criteria, opted to join the study, and had the PGT-A results be delivered at time to be analyzed, 6 did not have at least 1 aneuploid and 1 euploid embryo, per which the SECM from 17 embryos were not considered. In a matter of fact, only 39% of all the embryos from the 11 couples were diagnosed as euploid (data not shown).

To be sure that the effects on SECM were exclusively originated by embryos ploidy, the effect of other variables on SECM was also assessed. Hence, we verified the influence of age and BMI of the

mother and father to be, sperm quality and menstrual cycle periodicity. Considering that infertile couples that resort to IVF treatments have a different infertility background and can undertake different hormonal drugs and dosages, we considered the impact of ovarian stimulation (OS) protocol, OS duration, AMH and AFC baseline, dosage of exogenous gonadotropins, infertility couple diagnosis, infertility length, number of previous IVF cycles and other medical conditions. Morphological embryo quality is a classic approach for prediction embryo development after transfer and is used in every IVF clinic worldwide (126). Thus, we analyzed if embryo quality grading, specifically if a different morphological early development, could have an influence in the SECM metabolic profile. As previously showed, none of the conditions studied explained the data cluster that we obtained. The only characteristic that correlates with the data clustering is the different ploidy state of the embryos, rather than another pseudo-plausible variable that have been correlated to embryonic aneuploidy, such as advanced maternal age (34, 127) or sperm quality (128, 129).

After ICSI, the outcome of fertilization is verified 17-19 h later with the presence of the second polar body. From there, the zygote starts the process of cleavage. For yielding ATP, embryonic cells are capable of resort to glycolysis and to oxidative phosphorylation. For both cleavage and blastocyst stage, it was analyzed if there were preferable different metabolic pathways between aneuploid and euploid embryos. For that we evaluated the export and import of the metabolites identified in SECM.

By day 3, the zygote evolved to a structure with a small number of cells per which we expected their metabolite turnover from the media to be substantially small. When we compare the metabolite composition of day 3 embryo-free media and SECM, we can identify 8 compounds with a significant variation. Surprisingly, it was even possible to recognize a differential metabolic activity between euploid and aneuploid embryos in such early stage. Despite small, euploid embryos secreted higher amounts of acetate than aneuploids. Acetate is a pivotal metabolite, that can be enzymatically interconverted to acetyl-CoA, which may enter directly in tricarboxylic acid (TCA) cycle or in the fatty acid synthesis, but also synthesized from pyruvate resulting from glycolysis (130). Acetate export to the SECM, could be related with a higher acetate production of euploid embryos, that cannot be accumulate intracellularly since is toxic for the cell. In 2013 Nadal-Desbarats *et al.* evaluated the SECM differences after 24h incubation between the third and fourth day and identified that embryos that secreted higher amounts of acetate were able to progress until the blastocyst stage, while the others arrested their development (131). Even though, in our study it was evaluated a different period and both embryos reached the blastocyst stage, this could be an indicator of cellular deregulation in embryos with poor prognosis. Aneuploid embryos tended to uptake from the media citrate in a highly extent, and since citrate is an intermediate of TCA cycle (130), it may suggest an higher ATP demands in this case, that led an increase of oxidative phosphorylation. During cleavage phase has been described that embryo metabolism relies mainly on pyruvate via oxidative phosphorylation (132). Its metabolism is oxidative, even though their mitochondria are poorly developed with a spherical structure yielding a low ATP production. Only on the blastocyst phase, mitochondria start to differentiate and to replicate themselves

(132, 133). The uptake of pyruvate was not significantly different between the groups, but the mitochondrial activity seems to be dysregulated by chromosomal abnormalities. SECM from aneuploid embryos had decreased glucose, valine, and citrate levels, indicating a higher uptake of these metabolites. Citrate uptake is in concordance with a higher oxidative phosphorylation metabolism. This high turnover of glucose and citrate by aneuploid cells corroborate the hypothesis that aneuploidy may have an early stimulating impact in embryo metabolic activity. It is observable that aneuploid embryos had a higher uptake of formate, and once it is a precursor of purine nucleotide de novo synthesis (134), this can indicate that aneuploidy promotes nucleotide synthesis dysregulation and induce an early nucleotide turnover. Beside valine, no other amino acid level is significantly different between aneuploid and euploid SECM. In contrast, Huo, *et al.* have reported in 2020 that serine, aspartate, histidine, and alanine were significantly different among SECM collected in day 3 from embryos that resulted or not in pregnancy. They have used HPLC and the amino acids concentration were in µmol/ml (109). In our data, we did not observe differences in any of those amino acids on day 3. However, these metabolites variations were under the lower limit detection of our spectra resolution (mM) and may be a limitation of our methodology.

When the biological system reaches the morula stage its cellular number greatly rises, as well as the process of cellular differentiation and the embryo genome is fully activated. From there, the embryo develops into a high defined structure, the blastocyst, with different metabolic requirements and higher requests for energy. By day 5, we anticipated that the high cellular structure would lead to higher changes on SECM composition. We found 16 metabolites significantly different between embryo-free media and SECM. When we compare this state with the previous one, we can verify indeed the higher dependence for the media substrates. Of this, aneuploid and euploid SECM present 13 metabolites with significantly different levels. The main ploidy impact was observed among essential amino acids turnover. They were associated with smaller but very important variations. In general, the SECM from aneuploid cells had higher amounts of essential amino acids (EAA), except for valine which no difference was identified. Euploid cells competently uptake them from the media, while aneuploid cells were uncapable of import them and in certain cases, such as with threonine, methionine, histidine, and lysine, occurs the release to the media. Human cells are unable to synthesize enough EAA and are completely dependent of extracellular sources for a viable physiological cellular activity (130, 135). This behavior was observed by Sánchez-Ribas *et al;* an increase of some amino acids, including the previously EAA, in SECM of aneuploid embryos (32). We also observed that there is a higher uptake of tyrosine and glutamine by euploid embryos. Glutamine is a key cellular osmoregulator, often associated with nitrogen removal, antioxidant defenses, an important precursor of nucleotide synthesis and significantly improves blastocyst formation (104, 130, 136). The fact that euploid embryos uptake more efficiently this amino acid indicates that they are also more able to control oxidative cellular damages and to synthesis DNA in a high rate, as crucial it is during the blastocyst phase. It has been suggesting that tyrosine uptake could indicate that the embryo reached the blastocyst stage (95).

Aneuploid embryos export alanine and pyroglutamate to the media, while in euploids occurs its uptake. Pyruvate can be converted to alanine and this process is associated with ammonia removal (104). Some studies had pointed out that this conversion can be an important route for intercellular ammonia removal, that could be harmful, even being correlated with an increased implantation potential (135). Until the moment it was not possible to perform this correlation, however our results suggest that aneuploid embryos have an higher ammonia production and alanine export could a way of to eliminate that excess (106). On the other hand, Seli *et al* had reported that embryos which resulted in a successful pregnancy had lower amounts of alanine in the SECM (15). In the future, it would be very important to correlate the SECM metabolome with transfer outcomes. Pyroglutamate is an intermediate substrate involved in the synthesis of glutathione (antioxidant defenses) (137). Once again, this data proposes that euploid embryos beneficiates the antioxidant cellular defenses. Feasibly, the poor prognosis of aneuploidy in embryos could be related to an inefficient antioxidant defense, while the glycolytic and oxidative phosphorylation pathways are active, producing high amounts of reactive oxygen species with little cellular damage control. Euploid embryos uptake higher amounts of phenylalanine, histidine, glutamine, threonine, and methionine, concordant to the higher amino acid turnover discussed above. Overall, this results that euploid embryos metabolism have a higher demand of amino acids present in the media for protein anabolism and energy metabolism. Also at day 5, acetate and citrate levels are significantly different in aneuploid and euploid embryos SECM. Euploids showed a higher uptake of acetate, while aneuploids increased citrate export. During this late phase of embryos in vitro development, seems that in euploid blastocysts acetate is re-routed for lipid synthesis, with a lower mitochondrial activity, occurring citrate export to the media (133). In contrast, aneuploid blastocysts uptake a small amount of acetate and export high amounts of citrate, indicating a smaller role of oxidative phosphorylation. Lipid metabolism regulates embryo development, implantation and pregnancy outcome (138), per which a dysregulated lipidic pathway could be on the basis of aneuploid embryos low viability.

Kirkegaard *et al.* have reported no significant differences among viable and no viable, while using a similar experimental approach with a larger cohort (148 infertile patients that resorted to SET, of which 47 embryos resulted in a clinical pregnancy and 101 did not), but a smaller amount of SECM highly diluted (139). Despite no possible direct comparison between their sample group with ours, we argue that with our approach, using a higher volume of less diluted SECM and 1.7 mm NMR capillary tubes, it was possible to observe the small differences, but significant ones in the embryos metabolite's levels.

In the beginning of the 21 century, it was proposed "The Quiet Embryo Hypothesis" which states that the embryos most likely to develop, implant and result in a successful pregnancy presents a relatively low overall metabolism level, such as lowest glycolytic rates and amino acid turn over (104, 110, 140-142). During the cleavage phase, our results corroborated this hypothesis, since the secretome suggested that aneuploid embryos were more active. Although, during blastocyst stage, euploid embryos had a higher amino acid turnover. Some recent published studies suggested the most viable blastocysts

had higher levels of glucose consumption and amino acid turnover (143). The lack of consensus regarding the embryo metabolic activity can be explained by the different laboratorial approaches used, different timepoints of media collection and by methodologies and handling variability. It is necessary to perform future studies, with a bigger cohort and a follow up after embryo transfer, which would support to confirm if this theory is corrected or the correlation between the embryos metabolic profile and a successful pregnancy is more complex than the "Quiet Embryo Hypothesis" states.

Altogether, our study indicates that ploidy can influence considerably the embryo metabolic activity, especially noticed during the blastocyst stage.

5.2. Accuracy and Robustness of the Predictive Model

With untargeted ^{1}H-NMR metabolomic analysis it is possible to identify and quantify diverse metabolites from a single spectrum, reflecting the profile signature of several metabolites in a complex sample. Performing multivariate statistical analysis, such as PLS-DA, on the data generated by this approach, it is a powerful tool for biomarkers identification, which can further be translated to a reliable model for diagnosis. In this study, the metabolites quantification by ^{1}H-NMR spectra of SECM samples were used to construct a predictive model for aneuploidy diagnosis based on a supervised analysis. Our PLS-DA and orthoPLS-DA models both for SECM on day 3 and day 5 has a good predictive ability, as assessed by the cross-validation and permutation tests. Even more significantly, the goodness of the model was further attested by the correct classification of the embryo ploidy. The accuracy of the day 3 and day 5 predictive models were 92% and 83%, respectively.

The human embryos only have their genome activation after the four-cell stage (day 2) during cleavage phase (138). Before that, all their metabolism and defenses rely on maternal cellular machinery and is a reflex of its mother's oocyte features (62, 102). This might indicate that using day 3 SECM samples would not add much relevant metabolic information concerning the ploidy and be more dependent of the oocyte donor. However, it is not what we observed, the metabolic profiles of day 3 SECM samples are clustered based on ploidy despite the oocyte donor characteristics (supplementary figure 8.5. and 8.6.). When we look at the accuracy of each predictive model, day 3 rate is slightly higher than in day 5. By and large, coupling both timepoints would complement each other and error-proof the early aneuploidy diagnosis. In 2020, Rubio *et al.* validated the use of cell-free DNA in SECM as a biomarker for aneuploidy. Theirs predictive model concordance rate with the classical PGT-A was of 78.2% on day 6 of embryo development (92). Ours concordance rate is higher, and our methodology does not require any change in the clinical regular procedures, as period, type and quantity of media used in embryo incubation, unlike theirs. However, their cohort of 1301 human embryos warrant a more robust and valid model. Pais *et al.* analyzed SECM from 149 euploid and 165 aneuploid embryos on day 5 and 6 using MALDI-ToF mass spectrometry (117). They identified 12 characteristic peak signatures for euploid and 17 for aneuploid embryos, with a sensitivity of 84% and a false positive rate of 18%. Even with a bigger cohort their ploidy predictive model accuracy is comparable to ours on day

5. Using HPLC, Huo *et al.* identified aspartate, serine, alanine and histidine significantly different between SECM from day 3 embryos that resulted in a successful pregnancy and those that did not (109). Their predictive model for implantation potential has an accuracy of 90.4%, which is similar to ours on day 3 (109). All together we corroborate the applicability of SECM metabolic fingerprint as a preimplantation criterion for prioritizing the embryo to transfer.

The major limitation of this study is indeed the small cohort, presently consisting of 7 SECM from aneuploid and 7 SECM from euploid embryos per timepoint, to analyze from different embryo's ploidy and build the predictive model. As mentioned before, this was due to the low percentage of both embryo development and pairing of at least 1 aneuploid and 1 euploid embryo per couple. A way to increase the cohort, it was considered for the study embryos with mosaicism or SECM from embryos that reached the blastocyst status on day 6. However, by other hand consider those samples it adds other variables that could be confounders. Also, it would be interesting to collect more data about the different types of aneuploidies, to look if different aneuploidies lead to different metabolic profile. Looking at the power analysis, it suggests that in day 3 the model predictive power will not rise above 0.14, even if we increase substantially the sample population. In contrast, the day 5 model predictive power quickly hits its maximum even with a smaller cohort. This data highlights the fact that a small increase of the sample cohort would be enough and lead to a more robust model.

Nevertheless, this study corroborates previous studies and provides proof of concept of the application of ^{1}H-NMR metabolomics in the diagnosis of aneuploidy and euploidy in early embryo development, where these markers may have a significant impact on the embryo selection criteria and pregnancy outcome. The performance of the metabolic fingerprint identified by our modelling approach using ^{1}H-NMR spectra is very reproducible and robust, per which using different NMR spectrometers in the same experimental conditions should result in similar results. In sum, this research addresses a gap in knowledge that has been bedeviling ART community for years. Before our findings can be applied to the ART clinical field, further studies involving larger sample groups, adding the detailed aneuploidy nature, as well as ascertain the SECM behavior of the mosaic embryos are necessary.

6. Conclusion

In conclusion, this pilot research underscores the power of ^{1}H-NMR in metabolic-based phenotyping of SECM as a tool to discriminate embryos on the basis of their ploidy. We also validate the analysis by ^{1}H-NMR of a small sample volume of SECM (25 µL), that is important since the application of this approach do not lead to a change in current ART clinical procedures. Application of multivariate statistics resulted in a PCA capable of discriminate euploid and aneuploid embryos, both in day 3 and 5. Therefore, it can be deduced that an euploid embryo metabolism differs from that of an aneuploid embryo, and it corroborates the conclusion of several previous studies (91). Specifically, euploid embryos showed a lower metabolic activity by day 3, but an increased one by day 5, specially related to the amino acid turnover. It can be deduced that some metabolic pathways highly contribute to the differences detected. Particularly, these results seem to point out that euploid embryos favor the uptake of metabolites associated with osmoregulation, ammonia removal and with antioxidant functions, which are major factors in the embryo health. The use of predictive models to analyze the metabolic profiles showed that the most predictive model was made up of 5 and 11 variables described, in day 3 and 5 respectively. PLS-DA models proved to be highly accurate in embryo's ploidy prediction, striking 92% and 83% in day 3 and day 5 models, respectively. The initial proposed hypothesis that embryo ploidy has a direct impact on their media composition was validated. Combining the metabolic profile of SECM is effective as input data for a ploidy predictive model based on SECM metaPLS-DA.

This preliminary study shows some fragilities. Most of the metabolites quantified are in close levels to the lower limit detection of our ^{1}H-NMR spectra approach, which increases the error associated with its quantification. The cohort is smaller than the proposed, we were only able to analyze 7 paired embryos. However, the power analysis proposed that would be only required at least 24 paired embryos, and not the 50 paired samples initially planned.

So, the next step will be increasing the cohort, in order to construct a more robust, accurate, specific, and highly ploidy predictive model and identify a non-bias set of biomarkers linked to the embryo's ploidy. With the cohort extent it will be also possible to study if the different types of aneuploidies had a different impact in the early metabolic activity. Furthermore, the inclusion of embryos with mosaicism would enrich, not only the knowledge regarding the impact of chromosomal imbalances in embryo metabolism but would also shape the applicability of the SECM metabolome as a reliable non-invasive preimplantation genetic diagnosis. Since the long-term goal of this research is to reduce time-to-pregnancy and associated costs, it would be essential to follow-up the embryo development and pregnancy outcome after single embryo transfer and analyze a possible correlation with its early metabolic activity.

The omics field, namely genomics, has changed the ART outcomes when introduced the PGT-A to the clinical practice. The other omics branches have been considered in several studies to develop a new, non-invasive, robust, and accurate approach for the diagnose of chromosomally abnormal

embryos (91, 144). Genome sequencing of cell-free DNA present in SECM (92), the SECM proteome (145), Raman and near-infrared metabolic footprint analysis (99, 105, 146), and even using automated artificial intelligence that analysis time-lapse morphokinetic (73, 74). Even though these methodologies promise to revolutionize ART field in a few years, their clinical implementation and robustness is still problematic. Through combining our predictive models with a non-invasive SECM collection method, we are a step closer from that nearby future. From the very beginning of IVF clinical practice, embryo morphology has been the criterion par excellence preferable to evaluate its development and to select the embryo to transfer. In the long run, it would be interesting coupling the time-lapse systems with the metabolic profile of SECM, which would constitute a more error-proof embryo selection criteria to optimize live-births after transfer.

ART specialists are in great need of an updated, accurate and precise technique to support the selection of the most viable embryo for transfer and increase the chances of reaching a successful pregnancy. For all the evidence pointed out throughout this thesis we strongly suggest the metabolic profile of SECM by ^{1}H-NMR spectroscopy as a reliable option for a non-invasive method to detect aneuploid IVF embryos. Looking forward, these novel findings deserve further research for the development of the new generation preimplantation genetic testing for aneuploidy.

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8. Supplementary Data

8.1. Copy of the information brochure for the participant and informed consent for recruiting and participation in the present study.

FOLHETO DE INFORMAÇÃO PARA O PARTICIPANTE E CONSENTIMENTO INFORMADO PARA PARTICIPAÇÃO NO ESTUDO

Título do estudo:

Seleção de embriões euploides/aneuploides por ressonância magnética nuclear: uma técnica rápida, económica e não invasiva para aumentar o sucesso de procriação medicamente assistida

Código do estudo:

2004-LIS-037-SN

Promotor:

IVI Lisboa

Investigador Principal:

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Centro:

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INTRODUÇÃO

Este folheto de informação tem como objetivo apresentar um estudo de investigação, para o qual a convidamos a participar. Este estudo foi aprovado pela Comissão de Ética para a Saúde do IVI Lisboa e está de acordo com a regulamentação portuguesa e comunitária aplicável de estudos clínicos, nomeadamente com o disposto na Lei n.º 21/2014, de 16 de abril, na sua redação atual (Lei da Investigação clínica), o Regulamento (EU) 2016/679 (Regulamento Geral sobre a Protecção de Dados), a Lei n.º 12/2005 (Lei da informação genética pessoal e informação de saúde).

Para isso pedimos que leia com atenção este documento de forma a receber a informação correta, necessária e suficiente para que possa decidir se deseja ou não participar neste estudo.

Para qualquer esclarecimento adicional de dúvidas que possam surgir pode consultar um colaborador do estudo ou quem entenda conveniente, mesmo que seja externo ao estudo. Após sentir que está esclarecida, se decidir participar neste estudo, por favor assine e date a página de assinatura deste documento.

Este estudo será financiado pelo IVI Lisboa.

PARTICIPAÇÃO VOLUNTÁRIA

A sua participação neste estudo é voluntária. Este documento contém informação sobre diversos aspetos do estudo, que a ajudarão a decidir se quer ou não participar no estudo, designadamente sobre os seus riscos e benefícios previsíveis, e os métodos utilizados.

Pode decidir não participar neste estudo ou mudar a sua intenção e revogar o consentimento em qualquer momento sem que esta decisão afete a sua relação com o seu médico, nem prejudique o seu tratamento. No caso de revogar o consentimento da sua participação, nenhum dado novo será inserido na base de dados e poderá exigir a destruição de todas as amostras previamente recolhidas.

Se decidir participar estará também a autorizar o Promotor do estudo a utilizar e partilhar a sua informação de saúde, a qual estará sempre codificada.

DESCRIÇÃO GERAL DO ESTUDO

1. Em que consiste este estudo?

A procriação medicamente assistida (PMA) é um conjunto de técnicas e de tratamentos médicos que favorecem a gravidez em casais inférteis ou com fertilidade reduzida. Apesar dos avanços alcançados até à data, a seleção do embrião capaz de gerar um bebé saudável constitui um passo crítico na PMA. A seleção dos melhores embriões baseia-se na avaliação morfológica, contudo cerca de 70% dos embriões com aparência normal apresentam um número de cromossomas anormais, incompatíveis com a vida. Nesse sentido, o Teste Genético Pré-Implantacional de Aneuploidias (TGPI-A) constitui uma importante estratégia de seleção embrionária, pois avalia os desequilíbrios cromossómicos dos embriões antes da sua transferência para o útero materno. Esta análise é realizada no estádio blastocisto, quando o embrião se encontra no 5º ou 6º dia de desenvolvimento. O procedimento consiste numa biópsia, através da qual são extraídas células sem que se comprometa o seu desenvolvimento normal. Depois da biópsia, os embriões são criopreservados até que sejam analisados os resultados para serem tomadas decisões de forma fidedigna. Esta técnica tem a desvantagem de ser dispendiosa e de requer vários dias até que sejam conhecidos os resultados.

Este estudo propõe a utilização da técnica de ressonância magnética nuclear (NMR) para determinação do perfil dos metabolitos (pequenos compostos) presentes no meio de cultura dos embriões antes da sua transferência para o útero materno. Os embriões resultantes das fertilizações dos óvulos são colocados num meio de cultura (funciona como o "alimento") que proporciona o seu desenvolvimento. Alterações na composição do meio de cultura dos embriões cultivados em laboratório poderão ser úteis para identificar os embriões com número normal (euploides) ou anormal (aneuploides) de cromossomas. Pretendemos, assim, avaliar a utilidade da técnica de NMR para distinguir de modo económico e rápido, os embriões não afetados (euploides) dos afetados (aneuploides) aumentando desta forma a probabilidade da conceção de um bebé saudável.

2. Qual é o objetivo deste estudo?

O objetivo deste estudo é avaliar se é possível distinguir os embriões euploides dos aneuploides antes da transferência e implantação no útero materno através da determinação do perfil de metabolitos no meio de cultura dos embriões que são cultivados no laboratório.

3. Quem pode participar?

A participação neste estudo é voluntária, mas é importante que, antes de aceitar participar, o compreenda perfeitamente e que esteja disposta a cumprir os procedimentos.

Este estudo não supõe nenhuma alteração ao protocolo laboratorial para a cultura e manipulação embrionária, nem ao procedimento de TGPI-A que lhe foi oportunamente explicado e para o qual já deu o seu consentimento de acordo com a prática clínica. No entanto, o estudo requer as seguintes modificações ao procedimento habitual:

- No caso de cumprir com os critérios do estudo, o meio de cultura dos embriões que estão a ser desenvolvidos no laboratório, e que no final do processo seria descartado, será analisado por NMR, e os resultados desta análise serão utilizados neste estudo.

4. Quanto tempo vou estar neste estudo?

Estará no estudo desde o momento da assinatura deste consentimento informado até que sejam conhecidos os resultados do TGPI-A. A estimativa da duração total do estudo é de 8 meses, e está prevista a avaliação de 50 embriões euploides e de 50 embriões aneuploides.

5. Em que consistem os procedimentos do estudo?

A equipa do estudo irá realizar os seguintes procedimentos:

- Recolha de informação relativa ao seu nome, ano de nascimento e história clínica relevante
- Colher o meio de cultura dos embriões para ser submetido a análise por NMR.

CONSENTIMENTO PARA A OBTENÇÃO DO MEIO DE CULTURA DOS EMBRIÕES

A. Objetivo da colheita de amostra:

Identificação de pequenos compostos capazes de diferenciar os embriões não afetados dos afetados.

B. Método de obtenção da amostra:

O meio de cultura dos embriões, que por prática clínica é descartado, será neste estudo preservado para posteriormente ser analisado por NMR.

C. Método de identificação das amostras:

Cada amostra será identificada de forma anónima com um código alfanumérico. A correspondência entre o código e a identidade do paciente será conhecida apenas pela equipa investigadora do estudo.

D. Avaliação e destruição das amostras:

A amostra do meio de cultura dos embriões será avaliada no laboratório no Instituto de Tecnologia Química e Tecnológica (ITQB). Todas as amostras serão destruídas após análise por NMR.

VÃO SER FEITAS ANÁLISES GENÉTICAS ADICIONAIS ÀS MINHAS AMOSTRAS?

Não.

BENEFÍCIOS E RISCOS PELA DA SUA PARTICIPAÇÃO NO ESTUDO

A participação neste estudo não irá trazer qualquer benefício adicional além do já previsto no processo do TGPI-A. No entanto, os dados recolhidos e o resultado do estudo poderão permitir otimizar os procedimentos de procriação medicamente assistida. Para além dos potenciais benefícios diretos supracitados, não será fornecido nenhum incentivo financeiro para a participação.

TRATAMENTOS ALTERNATIVOS

Não está previsto qualquer tratamento ou procedimento alternativo e diferente do processo de TGPI-A para o qual já deu o seu consentimento.

O QUE ACONTECE SE EU QUISER SAIR DO ESTUDO?

Se estiver a ponderar sair do estudo, por favor converse com o médico investigador do estudo para que ele possa ajudá-lo na decisão. Se decidir sair, não haverá qualquer tipo de penalização adicional, ou perda adicional de benefícios, aos quais já tivesse direito antes de ter entrado no estudo.

Se decidir revogar o consentimento para participar neste estudo, nenhum dado novo será adicionado à base de dados e poderá exigir a destruição de todas as amostras identificáveis previamente retidas para evitar a realização de novas análises.

SEGURO

O Promotor do Estudo, o Centro IVI Lisboa, certifica que dispõe de uma apólice de seguro de responsabilidade civil em vigor com cobertura para compensar e indemnizar qualquer prejuízo da saúde ou lesões dos participantes no estudo. Este seguro está de acordo com a lei vigente.

CONFIDENCIALIDADE E TRATAMENTO DOS DADOS

A sua privacidade será protegida de acordo com o previsto na legislação portuguesa e comunitária aplicável, designadamente no Regulamento (UE) 2016/679 (Regulamento Geral sobre a Proteção de Dados) do Parlamento Europeu e do Concelho de 27 de Abril de 2016 de Proteção de dados (RGPD), na Lei n.º 12/2005 (Lei da informação genética pessoal e informação de saúde), no Decreto-Lei n.º 131/2014, o qual regulamenta a Lei n.º 12/2005 no que se refere à proteção e confidencialidade da informação genética, e na Deliberação n.º 1704/2015 da CNPD – Comissão Nacional de Proteção de Dados, aplicável aos tratamentos de dados pessoais efetuados no âmbito de investigação clínica, bem como de acordo com todas as atualizações que a referida legislação venha a ter.

Os elementos da equipa de investigação necessitarão de utilizar alguns dos seus dados pessoais para conduzir este estudo, incluindo dados de saúde (por exemplo, o seu nome, ano de nascimento, dados da história clínica) conforme descrito neste documento de consentimento.

O acesso aos dados requeridos pelo estudo é necessário para poder participar. Não poderá participar neste estudo se não der autorização para o acesso e utilização dos seus dados. O Promotor deste estudo é o responsável pelo tratamento de todos os dados recolhidos neste estudo.

Participar no estudo significa que a equipa do estudo vai permitir que as seguintes entidades vejam os seus dados de saúde de modo a garantir que o estudo seja realizado corretamente:

- Autoridades de Saúde de todo o mundo (tais como o INFARMED, a Agência Europeia do Medicamento (EMA) ou a *Food and Drug Administration* (FDA) dos Estados Unidos da América, por exemplo);
- Comissões de Ética que supervisionam o trabalho de investigação clínica; e
- Representantes do Promotor (só serão partilhados com o Promotor e seus representantes dados de saúde codificados, com a exceção do monitor do estudo e do auditor do estudo, ambos representantes do Promotor).

As Autoridades de Saúde e a Comissão de Ética Competente poderão ter acesso direto aos seus dados de saúde. Quaisquer outras entidades terão apenas acesso aos dados de saúde codificados, exceto monitor do estudo e o auditor do estudo, ambos representantes do Promotor.

De forma a garantir que os procedimentos do estudo são seguidos, as Autoridades de Saúde, Comissões de Ética e os representantes do Promotor irão ter acesso aos seus dados de saúde no centro de investigação. Os representantes do Promotor designados por "monitores do estudo" terão acesso, por intermédio do médico investigador do estudo, aos seus dados de saúde para monitorizar o estrito cumprimento do protocolo do estudo e efetuar o necessário trabalho de monitorização. Os representantes do Promotor designados por "auditores do estudo" terão acesso, também por intermédio do médico investigador do estudo aos seus dados de saúde para verificar, de forma sistemática e independente, se as atividades relacionadas com o estudo clínico foram conduzidas de acordo com o protocolo, os procedimentos operacionais padrão do promotor, e de acordo com as boas práticas clínicas e Legislação aplicável.

Os dados de saúde que são enviados para o Promotor não serão identificados com o seu nome. Ao invés, a esses dados será atribuído um código, que só poderá ser relacionado com o seu nome através de um documento que estará na posse dos membros da equipa do estudo. Os dados enviados ao Promotor poderão incluir o seu ano de nascimento, valores e conclusões dos diferentes exames e procedimentos que serão feitos, entre outros.

O seu nome não constará em nenhum relatório publicado acerca deste estudo ou em qualquer outra publicação ou apresentação científica.

O Promotor poderá partilhar as suas informações de saúde codificadas com parceiros na investigação e com fornecedores de serviços que apoiam a condução do estudo e a análise dos resultados. Os dados do estudo poderão ser partilhados com autoridades competentes e conselhos de revisão. O Promotor poderá transferir dados de saúde seus, devidamente codificados, a partir de Portugal para outros países da União Europeia. Estas transferências vão ocorrer com base nas regras vinculativas aplicáveis às empresas

("binding corporate rules") do Promotor, que consistem num quadro de conformidade sobre privacidade empresarial composto por um acordo vinculativo, processos e políticas empresariais, formação e diretrizes que foram aprovadas na União Europeia.

Nenhum dado ou informação estão completamente seguros. Existe possibilidade de alguém conseguir obter as suas informações sem que o Promotor lhas dê. Nessa circunstância poderá haver uma utilização indevida, para fins diferentes dos que concordou através deste documento. O Promotor tem em vigor procedimentos muito apertados para evitar isso. Isto significa que a possibilidade de isso acontecer é muito baixa.

Poderá a qualquer momento retirar a sua autorização para a utilização e partilha dos seus dados pessoais, incluindo dados de saúde, contactando a equipa do estudo. Se o fizer, não poderá continuar a participar neste estudo. A partir dessa data nenhum dado novo de saúde que a identifique será recolhido. Contudo, os dados de saúde que já tenham sido recolhidos anteriormente a essa decisão poderão continuar a ser utilizados para manter a integridade do estudo e dos seus resultados conforme se exige pelas regras dos estudos, sem prejudicar a retenção dos dados que são necessários para satisfazer as obrigações legais aplicáveis. Tem o direito de discordar com tal utilização, dentro dos limites da lei. Se tiver alguma dúvida acerca da utilização e partilha dos seus dados de saúde pessoais, por favor contacte o investigador principal do estudo.

São-lhe reconhecidos, nos termos da lei, certos direitos relativamente à sua informação. São disso exemplos o direito de ver a sua informação, corrigir eventuais erros e limitar a utilização, o que poderá fazer através do seu médico investigador do estudo. Alguns destes direitos poderão estar limitados por lei. Para assegurar a integridade científica do estudo, poderá acontecer que o acesso a alguns dos seus dados relativos ao estudo só possa acontecer após o estudo terminar. Após a conclusão do estudo, poderá contactar o investigador principal do estudo para solicitar a consulta dos seus dados de saúde recolhidos durante o estudo e a correção de qualquer erro.

O Investigador e o Promotor estão obrigados a conservar os dados recolhidos para o estudo pelo menos durante 25 anos após a sua finalização. Posteriormente a sua informação pessoal só se manterá no centro para o cuidado da sua saúde e pelo promotor para outros fins de investigação científica se lhe der o seu consentimento expresso para isso e se assim a lei e os requisitos éticos aplicáveis o permitirem.

Para qualquer questão acerca do estudo, ou de alguma lesão relacionada com o mesmo, deverá utilizar o contacto de emergência indicado na primeira página.

Para qualquer questão/reclamação acerca da utilização da sua informação pessoal e de saúde, poderá contactar:

- A Autoridade de Controlo de Dados Pessoais em Portugal:

CNPD – Comissão Nacional de Proteção de Dados

Rua de São Bento n.º 148-3º

1200-821 Lisboa

Tel: +351 213928400

e-mail: geral@cnpd.pt

ou

- O Encarregado de Proteção de Dados do Promotor através do email: DPO@ivirma.com ou para o endereço postal: Calle Colón 1 - 4º, 46004 Valencia, Espanha, remetendo à sua atenção.

Quaisquer questões sobre os seus direitos e deveres como participante no contexto deste estudo clínico podem ser colocadas à seguinte entidade independente, que apreciou e emitiu parecer favorável para este estudo:

Comissão de Ética para a Saúde IVI Lisboa
Avenida Infante Dom Henrique 333 H 1- 9ª
1800-282 Lisboa
Tel: +351 21 850 3210
ces.lisboa@ivirma.com

A informação relativa ao estudo clínico estará disponível no Registo Nacional de Estudo Clínicos, nos termos e para os efeitos do disposto na Lei n.º 21/2014 na sua redação atual (Lei da investigação clínica).

COMPENSAÇÃO ECONÓMICA

Não está prevista nenhuma compensação económica pela participação no estudo.

OUTRA INFORMAÇÃO RELEVANTE

Todo o material relacionado com o estudo que lhe seja fornecido foi previamente revisto e aprovado pela Comissão de Ética para a Saúde do IVI Lisboa, que é o organismo independente cuja missão é proteger os direitos, a segurança e o bem-estar dos participantes em estudos clínicos.

Durante a sua participação qualquer informação referente ao estudo que seja relevante, de novo e que possa comprometer a sua elegibilidade para continuar a participar no mesmo, ser-lhe-á comunicada pelo investigador com a maior brevidade possível.

Quando assina a folha de consentimento anexada, compromete-se a cumprir com os procedimentos expostos do estudo. Não lhe será solicitado que abdique ou renuncie aos seus direitos legais contra a Instituição, o Investigador ou o Promotor, em caso de responsabilidade destes. Se decidir revogar o consentimento para participar neste estudo, nenhum dado novo será adicionado à base de dados. Também deverá saber que pode ser excluída do estudo se o promotor ou o investigador do estudo se assim o considerarem, seja por motivos de segurança se considerar que não está a cumprir com os procedimentos estabelecidos. Em qualquer dos casos, receberá uma explicação adequada do motivo da sua exclusão do estudo.

CONSENTIMENTO INFORMADO PARA PARTICIPAÇÃO NO ESTUDO

(a preencher pela participante)

Código: 2004-LIS-037-SN

Eu .....

com o N.º de Processo Clínico .....

- **Ao assinar este documento, declaro que:** Li este "Folheto de informação para o participante e consentimento informado para participação no Estudo".
- Tive tempo para refletir e oportunidade para colocar questões e fiquei esclarecido com as explicações dadas.
- Compreendo que a minha participação neste estudo é voluntária e aceito participar.
- Compreendo que em qualquer momento poderei retirar o meu consentimento e suspender a minha participação no estudo, comunicando tal decisão ao investigador do estudo. Compreendo que não serei penalizada nem perderei quaisquer benefícios, aos quais noutras circunstâncias pudesse ter direito.
- Compreendo que poderei ter de suspender a minha participação no estudo sem o meu consentimento caso não cumpra o plano do estudo, tenha sofrido algum dano de saúde resultante do mesmo, ou por qualquer outro motivo.
- Dou permissão para o acesso, utilização e partilha dos meus dados pessoais, dentro dos limites da lei, tal como descritos neste folheto.
- Compreendo que tenho o direito de apresentar uma reclamação à autoridade de controlo de dados pessoais em Portugal (CNPD – Comissão Nacional de Proteção de Dados).
- Os resultados da avaliação do meio de cultura de embriões limitam-se estritamente às descritas no conteúdo do estudo de investigação.
- Recebi informação suficiente sobre o estudo.
- Recebi uma cópia assinada e datada deste "Folheto de informação para o participante e consentimento informado para participação no Estudo".

Assinatura da **participante**

Nome da participante

Em maiúsculas ou letra legível

Data

DD/MM/AAAA

A assinatura e data têm obrigatoriamente de ser manuscritas pelo próprio participante.

Assinatura do **parceiro(a)**

Nome do **parceiro(a)**

Em maiúsculas ou letra legível

Data

DD/MM/AAAA

A assinatura e data têm obrigatoriamente de ser manuscritas pelo(a) próprio(a) parceiro(a).

Quando aplicável, o(a) parceiro(a) deverá assinar este Consentimento Informado.

Assinatura do **investigador do estudo**

Nome do Investigador do estudo que obteve o consentimento informado

Em maiúsculas ou letra legível

Data

DD/MM/AAAA

TESTEMUNHA IMPARCIAL

- **Ao assinar este documento, declaro que:** Li este "Folheto de informação para a participante e consentimento informado para participação no Estudo Principal".
- A informação constante no presente folheto de informação ao participante e consentimento informado foi explicada em detalhe e, aparentemente, compreendida pelo participante.
- O consentimento informado foi dado de forma livre.

Assinatura da **testemunha Imparcial 1**

Nome da testemunha Imparcial 1

Em maiúsculas ou letra legível

Data

DD/MM/AAAA

A assinatura e data têm obrigatoriamente de ser manuscritas pela própria testemunha.

Assinatura do **testemunha Imparcial 2**

Nome testemunha Imparcial 2

Em maiúsculas ou letra legível

Data

DD/MM/AAAA

A assinatura e data têm obrigatoriamente de ser manuscritas pela própria testemunha.

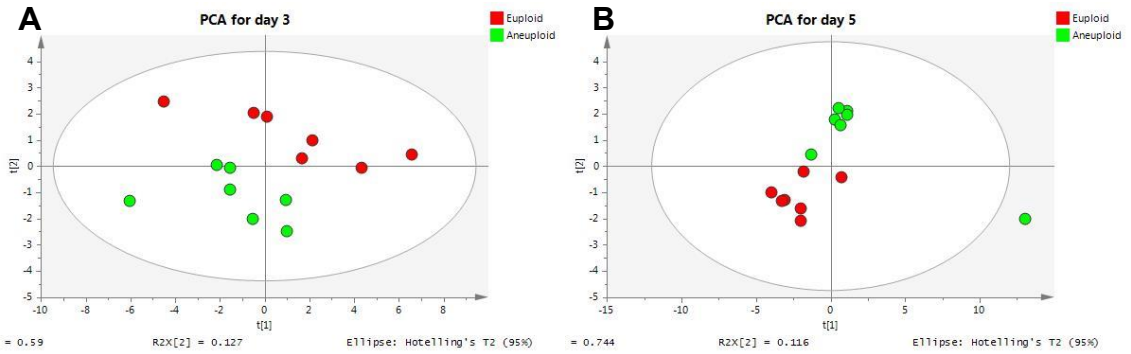

Figure 8.1. PCA score plots of SECM sample clustering from aneuploid and euploid embryos in (A) day 3 and (B) day 5 based on class variables. Green and red dots represent samples from aneuploid and euploid embryos, respectively. Data were analyzed using Simca. n=7 paired SECM from aneuploid and euploid embryos of 5 couples.

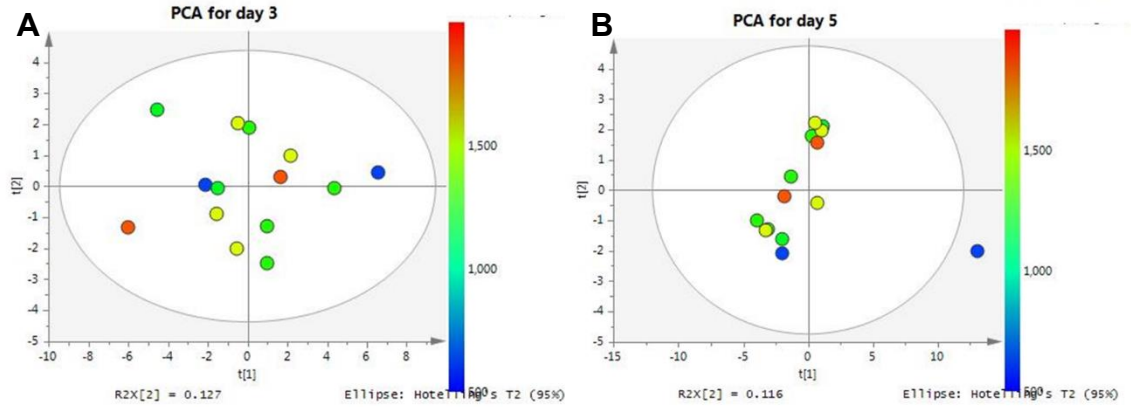

Figure 8.2. PCA score plots of SECM sample clustering from aneuploid and euploid embryos in (A) day 3 and (B) day 5 based on the total dosage of exogenous gonadotropins (IU) used per treatment. The dosage of gonadotropins used varied from 600 to 1875 IU, in the graphic lower values are represented in blue and higher levels are red. Data were analyzed using Simca. n=7 paired SECM from aneuploid and euploid embryos of 5 couples.

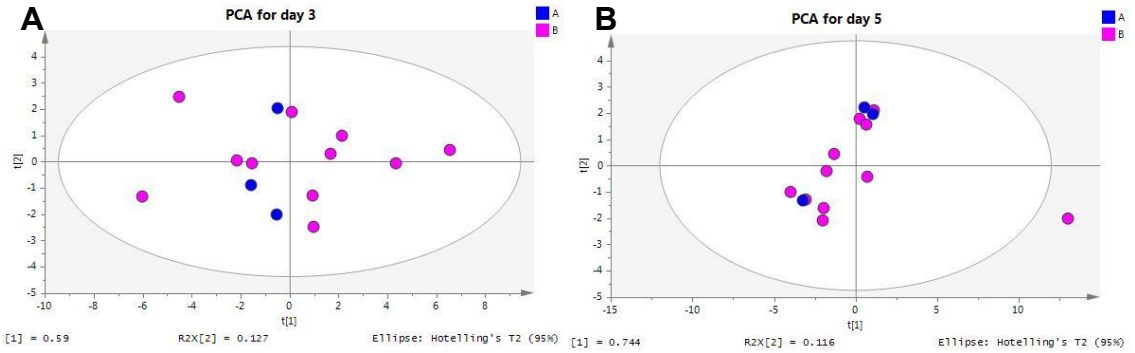

Figure 8.3. PCA score plots of SECM sample clustering from aneuploid and euploid embryos in (A) day 3 and (B) day 5 based on the embryo morphological quality grading. Blue and pink dots represent samples from A and B graded embryos, respectively. Data were analyzed using Simca. n=7 paired SECM from aneuploid and euploid embryos of 5 couples.

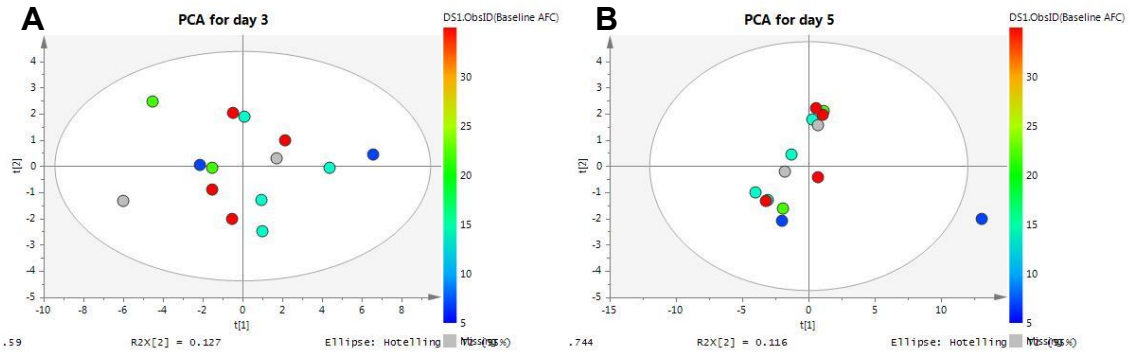

Figure 8.4. PCA score plots of SECM sample clustering from aneuploid and euploid embryos in (A) day 3 and (B) day 5 based on the Antral Follicle Count (AFC). Lower values are represented in the graphic in blue and higher levels are red. In grey is represented the samples with unknown AFC data. Data were analyzed using Simca. n=7 paired SECM from aneuploid and euploid embryos of 5 couples.

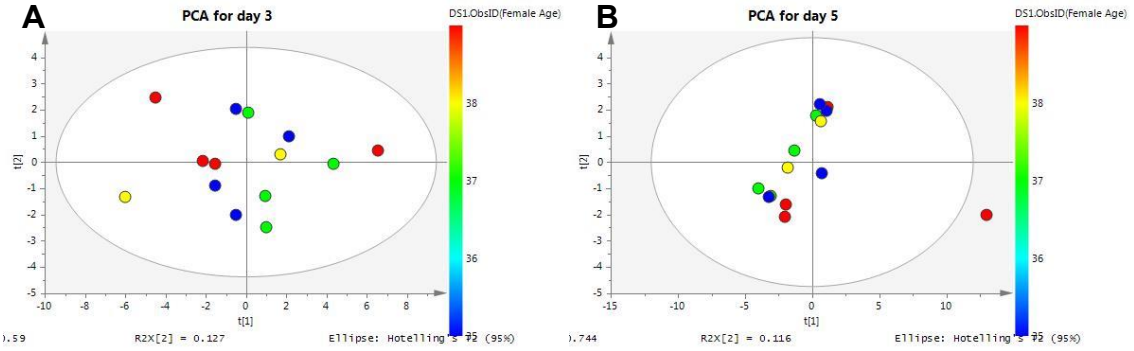

Figure 8.5. PCA score plots of SECM sample clustering from aneuploid and euploid embryos in (A) day 3 and (B) day 5 based on female age (years). Lower values are represented in the graphic in blue and higher levels are red. Data were analyzed using Simca. n=7 paired SECM from aneuploid and euploid embryos of 5 couples.

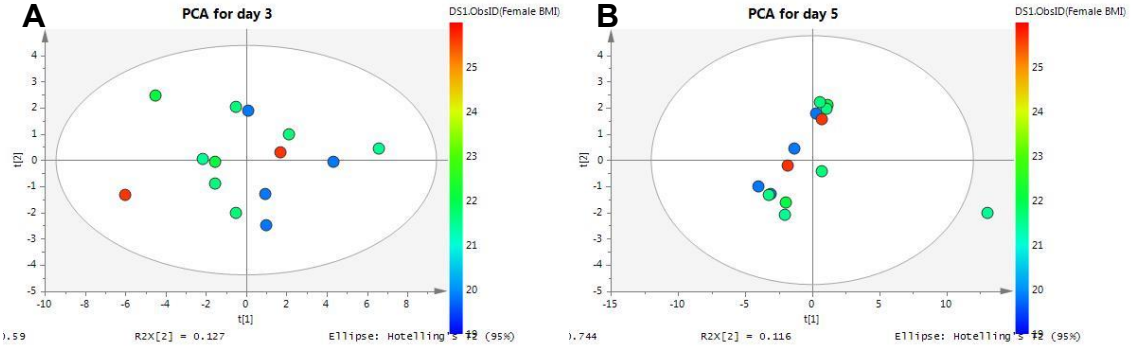

Figure 8.6. PCA score plots of SECM sample clustering from aneuploid and euploid embryos in (A) day 3 and (B) day 5 based on female body mass index (BMI) (kg/m^2). Lower values are represented in the graphic in blue and higher levels are red. Data were analyzed using Simca. n=7 paired SECM from aneuploid and euploid embryos of 5 couples.

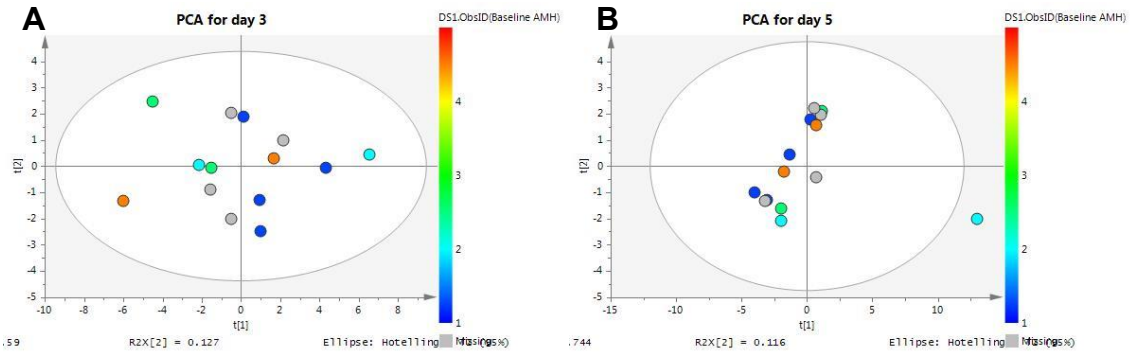

Figure 8.7. PCA score plots of SECM sample clustering from aneuploid and euploid embryos in (A) day 3 and (B) day 5 based on the Anti-Müllerian Hormone (AMH) (ng/mL). Lower values are represented in the graphic in blue and higher levels are red. In grey is represented the samples with unknown AFC data. Data were analyzed using Simca. n=7 paired SECM from aneuploid and euploid embryos of 5 couples.

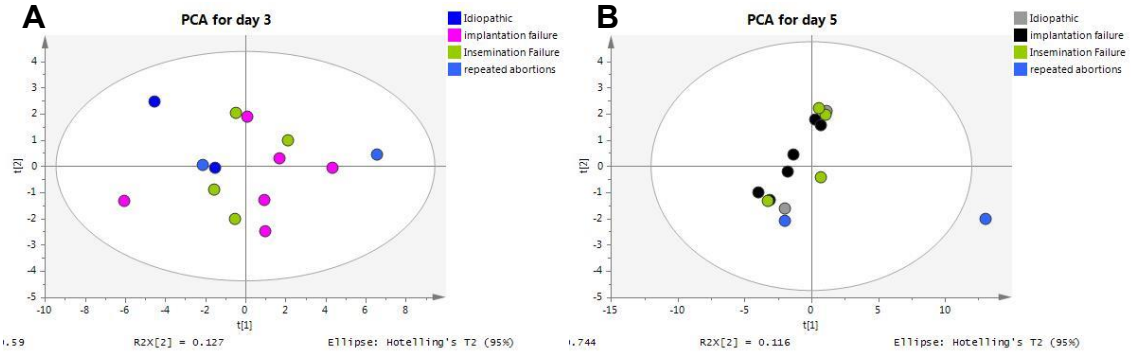

Figure 8.8. PCA score plots of SECM sample clustering from aneuploid and euploid embryos in (A) day 3 and (B) day 5 based on infertility diagnosis. Blue and grey dots represent idiopathic in day 3 and 5, respectively; pink and black dots represent implantation failure in day 3 and 5; green dots represent insemination failure; and blue dots represent repeated abortions. Data were analyzed using Simca. n=7 paired SECM from aneuploid and euploid embryos of 5 couples.

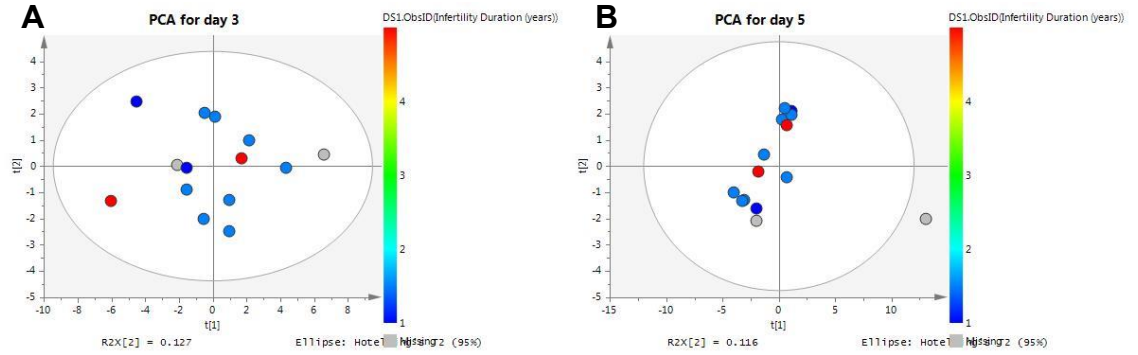

Figure 8.9. PCA score plots of SECM sample clustering from aneuploid and euploid embryos in (A) day 3 and (B) day 5 based on infertility duration (years). Lower values are represented in the graphic in blue and higher levels are red. In grey is represented the samples with unknown AFC data. Data were analyzed using Simca. n=7 paired SECM from aneuploid and euploid embryos of 5 couples.

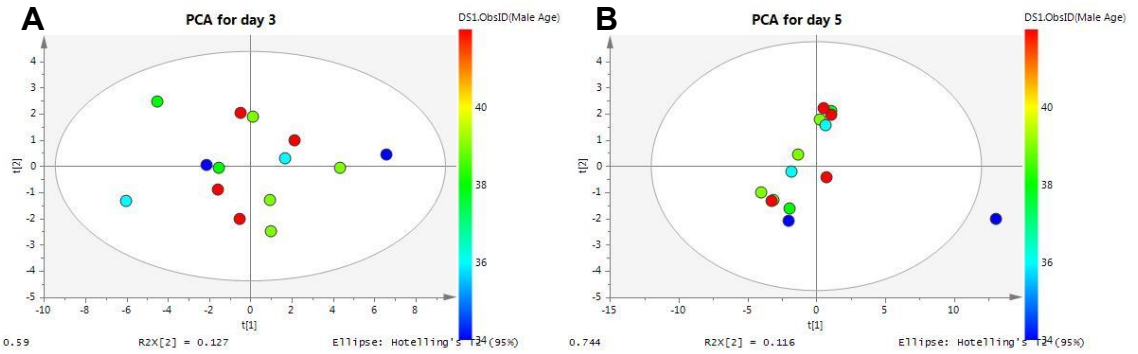

Figure 8.10. PCA score plots of SECM sample clustering from aneuploid and euploid embryos in (A) day 3 and (B) day 5 based on male age (years). Lower values are represented in the graphic in blue and higher levels are red. Data were analyzed using Simca. n=7 paired SECM from aneuploid and euploid embryos of 5 couples.

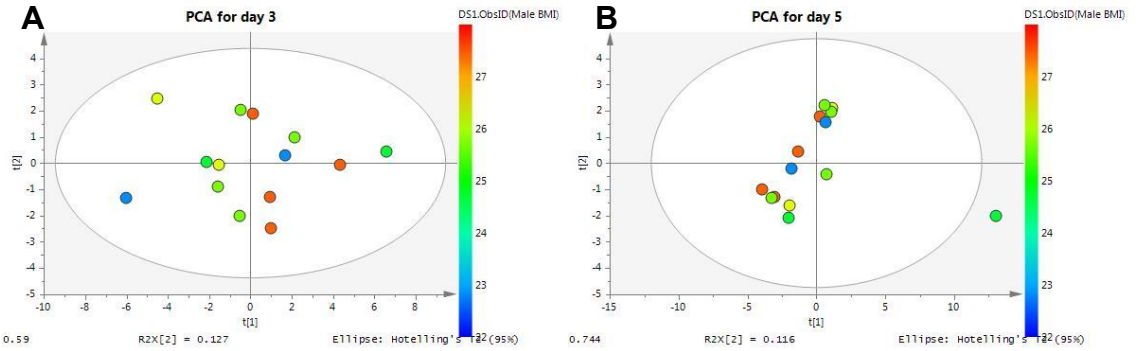

Figure 8.11. PCA score plots of SECM sample clustering from aneuploid and euploid embryos in (A) day 3 and (B) day 5 based on male body mass index (BMI) (kg/m^2). Lower values are represented in the graphic in blue and higher levels are red. Data were analyzed using Simca. n=7 paired SECM from aneuploid and euploid embryos of 5 couples.

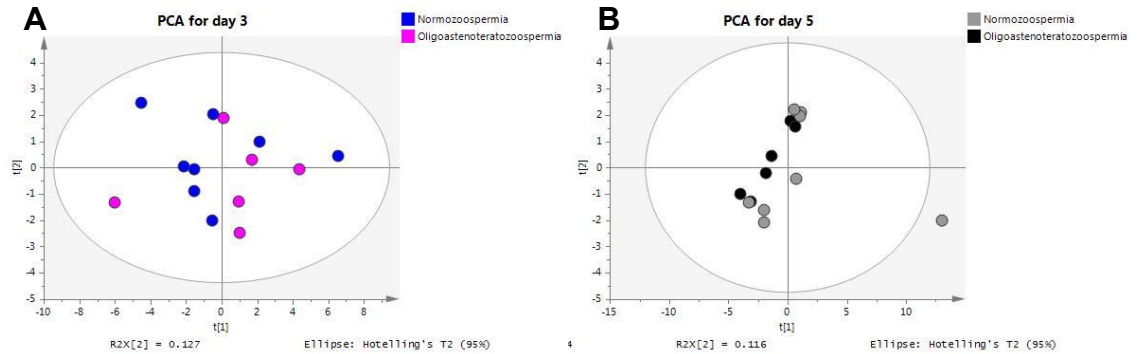

Figure 8.12. PCA score plots of SECM sample clustering from aneuploid and euploid embryos in (A) day 3 and (B) day 5 based on sperm quality. Blue and grey dots represent normozoospermia in day 3 and 5, respectively; pink and black dots represent oligoastenoteratozoospermia. Data were analyzed using Simca. n=7 paired SECM from aneuploid and euploid embryos of 5 couples.

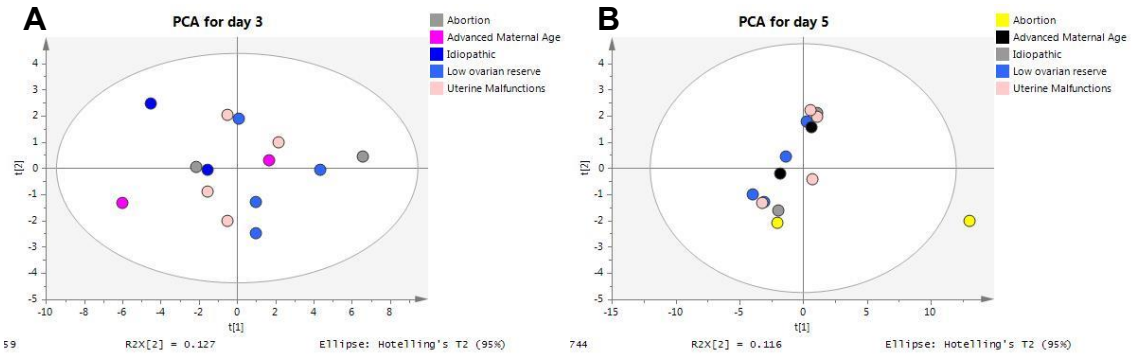

Figure 8.13. PCA score plots of SECM sample clustering from aneuploid and euploid embryos in (A) day 3 and (B) day 5 based on the medical conditions. Grey and yellow dots represent abortions in day 3 and 5, respectively; pink and black dots represent advanced maternal age; metallic blue and grey dots represent low ovarian reserve; and light pink dots represent uterine malfunctions. Data were analyzed using Simca. n=7 paired SECM from aneuploid and euploid embryos of 5 couples.

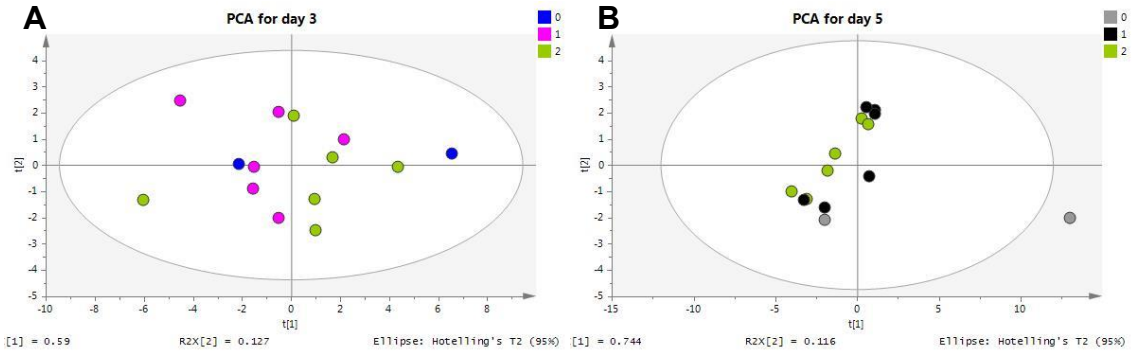

Figure 8.14. PCA score plots of SECM sample clustering from aneuploid and euploid embryos in (A) day 3 and (B) day 5 based on the number of previous fertilization in vitro (FIV) cycles. Blue and grey dots represent 0 previous cycles in day 3 and 5, respectively; pink and black dots represent 1 previous cycle; green dots represent 2 previous cycles. Data were analyzed using Simca. n=7 paired SECM from aneuploid and euploid embryos of 5 couples.

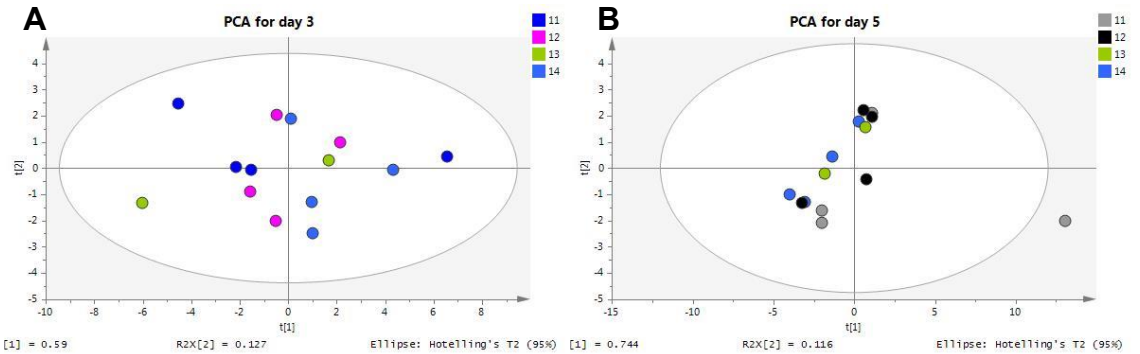

Figure 8.15. PCA score plots of SECM sample clustering from aneuploid and euploid embryos in (A) day 3 and (B) day 5 based on the duration of ovarian stimulation (days). Metallic blue and grey dots represent 11 days in day 3 and 5, respectively; pink and black dots represent 12 days; green dots represent 13 days; and blue represent 14 days. Data were analyzed using Simca. n=7 paired SECM from aneuploid and euploid embryos of 5 couples.

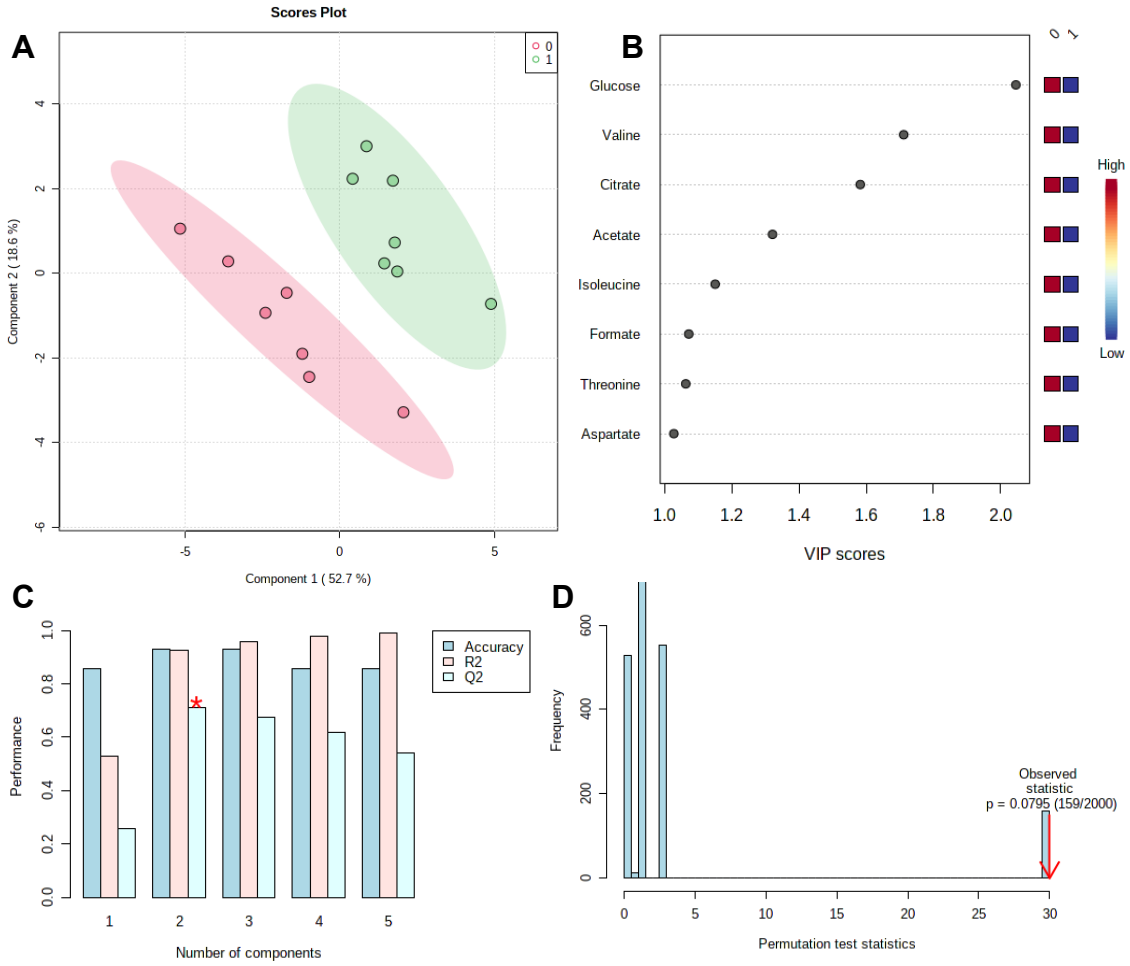

Figure 8.16 Summary of PLS-DA of day 3 data set. (A) PLS-DA score plot depicts the clustering patterns from the metabolite concentration data of SECM on day 3. Component 1 is responsible for 52.7% of the clustering, when component 2 only represents 18.6% of it. Shaded ellipses represent the 95% confidence interval region with no overlap. **(B)** The PLS-DA Variable Importance in Projection (VIP) scores indicates that 8 metabolites are significantly responsible for the group clustering. **(C)** The PLS-DA score plot discriminate both groups with a model including 18 metabolites with a R^2 of 0.925, a Q^2 of 0.709 at VIP threshold of 1.0. Both Q^2 and R^2 are above 0.7 with 2 components in the analysis which indicates a significative and robust predictive clustering model. **(D)** Also, the permutation test suggests the clustering formed initially is one of the best approaches, however not the best one (p=0.0795). Data were analyzed using Metaboanalyst. n =7 paired SECM from aneuploid and euploid embryos.

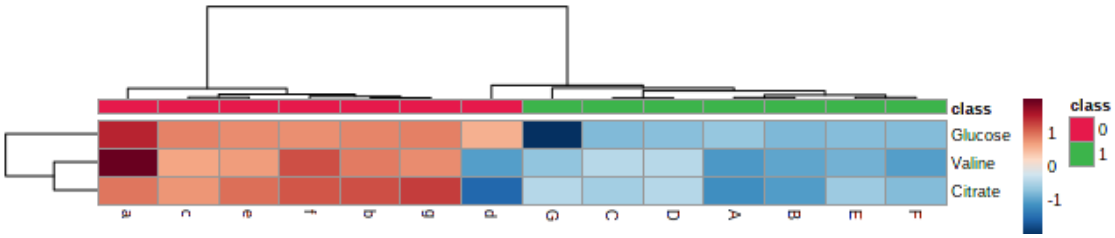

Figure 8.17 Heat map of 3 altered metabolites in day 3 SECM from aneuploid compared to euploid embryos. Red, increased metabolites levels on SECM; blue, decreased metabolites levels on SECM. The SECM from aneuploid and euploid embryos are colored in green and red, and represented by 1 and 0, respectively. Data were analyzed using Metaboanalyst. n =7 paired SECM from aneuploid and euploid embryos.

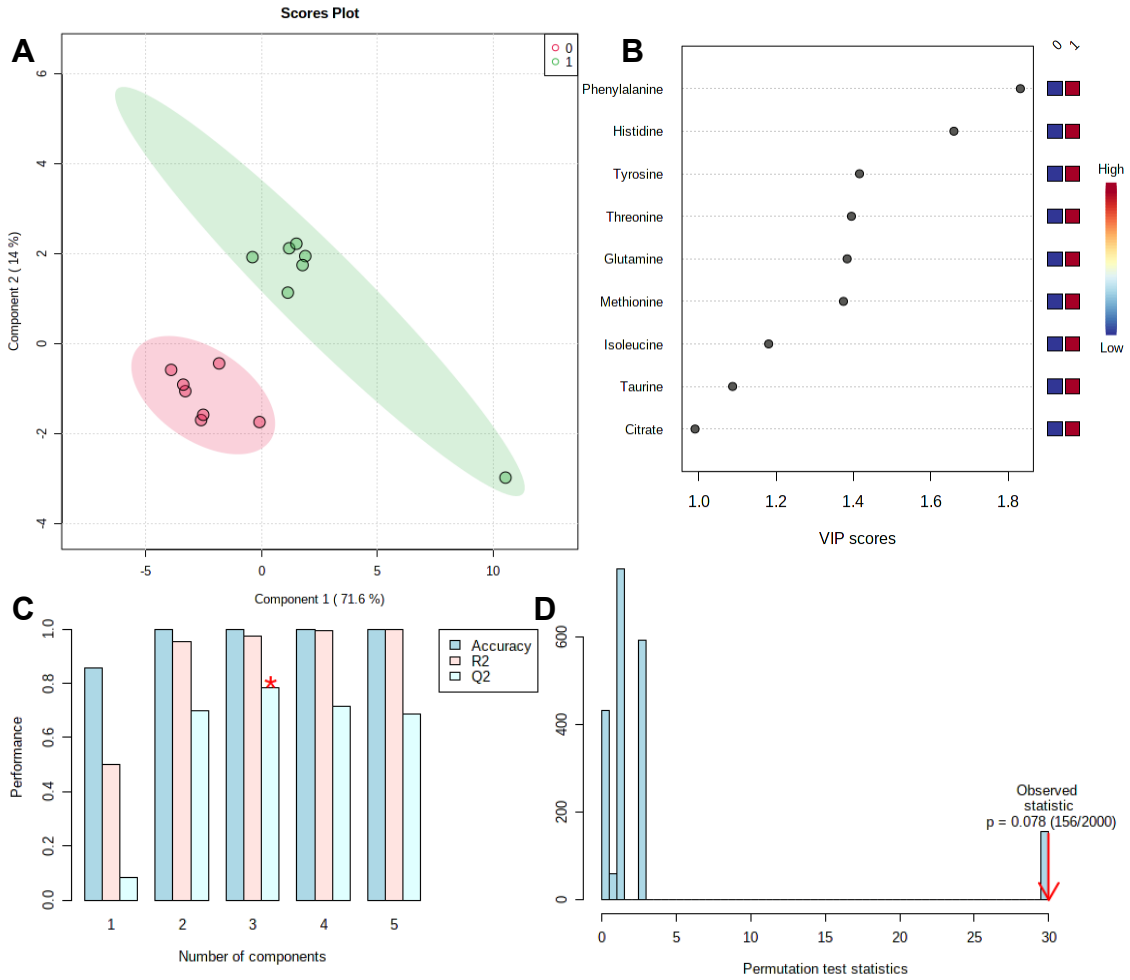

Figure 8.18 Summary of PLS-DA of day 5 data set. (A) PLS-DA score plot depicts the clustering patterns from the metabolite concentration data of SECM on day 5. Component 1 is responsible for 71.6% of the clustering, when component 2 only represents 14% of it. Shaded ellipses represent the 95% confidence interval region with no overlap. **(B)** The PLS-DA VIP scores indicates that 9 metabolites are significantly responsible for the group clustering. **(C)** The PLS-DA score plot discriminate both groups with a model including 23 metabolites with a R^2 of 0.975, a Q^2 of 0.783 at VIP threshold of 1.0. Both Q^2 and R^2 are above 0.7 with 2 components in the analysis which indicates a significative and robust predictive clustering model. **(D)** Also, the permutation test suggests the clustering formed initially is one of the best approaches, however not the best one (p=0.078). Data were analyzed using Metaboanalyst. n =7 paired SECM from aneuploid and euploid embryos.

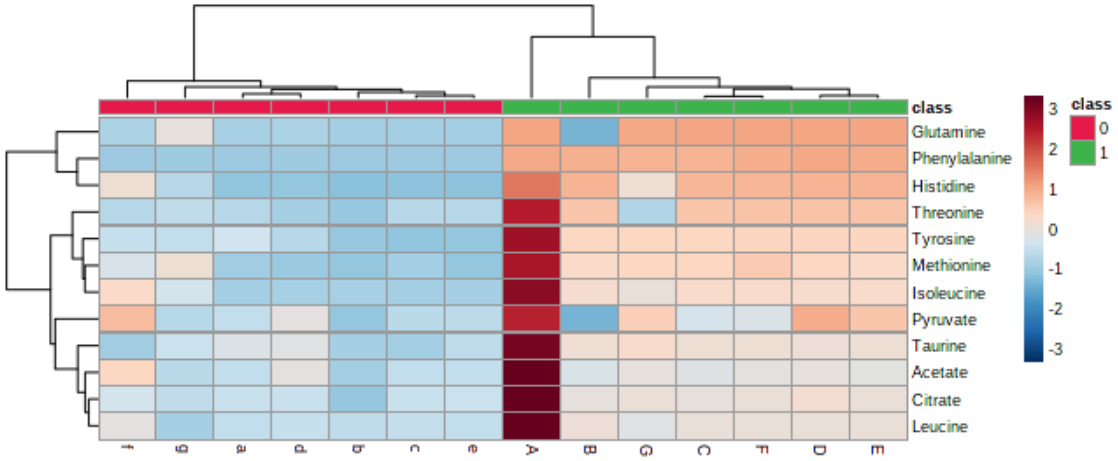

Figure 8.39 Heat map of 12 altered metabolites in day 5 SECM from aneuploid compared to euploid embryos. Red, increased metabolites levels on SECM; blue, decreased metabolites levels on SECM. The SECM from aneuploid and euploid embryos are colored in green and red, and represented by 1 and 0, respectively. Data were analyzed using Metaboanalyst. n =7 paired SECM from aneuploid and euploid embryos.