HEALTH RISKS OF BIRTH BY ASSISTED FERTILIZATION TECHNIQUES. THE TIP OF THE ICEBERG.

Nº7 HEALTH RISKS OF BIRTH BY ASSISTED FERTILIZATION TECHNIQUES. THE TIP OF THE ICEBERG

Content:

HEALTH RISKS FOR NEWBORNS BORN BY ASSISTED FERTILISATION TECHNIQUES. THE TIP OF AN ICEBERG.

When in 1978 the first girl produced by in vitro fertilisation (IVF) was born the question unavoidably arose: Will she have the same health risks as those conceived "naturally"?

In the case of Louise Brown there were no reasons to fear that she would suffer any problem as a consequence of her parents' sterility, whose gametes- ova and spermatozoa- were not defective. It was simply an obstruction of the mother's Fallopian tubes. It was not a multiple pregnancy, nor was she cultured or frozen in her embryonic stage, but was soon transferred into her mother's uterus.

Nonetheless, from that moment on the Medical Research Council began to compare the data of the first year of life of those born by IVF during 10 years with respect to those born in the same period of time and had been engendered naturally. Already then, a lack of health was found in those engendered by IVF.

The question of the lack of health in the children born by assisted reproduction techniques (ART) has been very controversial. The first comparative studies focused on the differences, in some type of alteration- prematurity, malformations, chromosomal alterations, etc.-, according to the mode that they had been conceived. The size of the samples was insufficient for a thorough statistical analysis, given the variety of characteristics with regard to the cause of sterility in the progenitors, as the type of technique used, or that they were subjected or not to the freezing of gametes of the embryo itself. It also varied according to the type of resulting pregnancy-multiple or single- mother's age, etc. The case study children, of the same age, geographic area, ethnic group etc., also had varied characteristics.

Around the years 2003 and 2005 meta-analysis was carried out, that gathered data from numerous studies and showed an increase in risk because of the growing implementation of the techniques, and a tenuous debate began on what could be the causes. The different studies gave an increase in risk of those born by the implementation of the techniques, which sometimes resulted as statistically significant and other times did not. Some do not show any difference [1]and in some cases apparently even have less risk for the health of those born[2]. The reality of that better health proved to be otherwise: when the embryos were frozen and afterwards thawed, they were only able to be implanted in the uterus, developed and be born those that were stronger, less damaged and therefore capable of resisting the process[3].

During many years it has been debated whether health problems, evidently greater in children generated by ART, are due to the condition of the gametes of the parents or to

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[1] Hansen, M., Bower, C., Milne, E., de Klerk, N., Kurinczuk, J.J. "Assisted reproductive technologies and the risk of birth defects—a systematic review". *Human Reproduction* 20, 2005, 328–338.

[2] De Neubourg, D., Gerris, J, Mangelschots, K, *et al.* "The obstetrical and neonatal outcome of babies born after single-embryo transfer in IVF/ICSI compares favorably to spontaneously conceived babies". *Human Reproduction* 21, 2006, 1041–1046.

[3] Mangelschots, K.J, *et al.* "The obstetrical and neonatal outcome of babies born after single-embryo transfer in IVF/ICSI compares favourably to spontaneously conceived babies". *Human Reproduction* 21, 2006, 1041–1046.

the techniques. The gathering of data is complicated with the donation of gametes when the cause of infertility is due to male factor or to the female's advanced age.

The growing propaganda of the use of gametes *donated* by others apart from the couple is made under a false supposition: the ART are very efficient and safe, the problem for the child will be the infertility subjacent of those that, precisely because of it, resort to assisted reproduction centres, to have a child.

It is a fallacy the disjunctive of posing if "the fault" is of the parents with problems in reproductive health. ART are born precisely to break down the barriers of sterility and, therefore, is based on the existence of a problem. The clinical practice of ART is designed to supple the natural inefficiency of those who suffer some form of infertility or sterility, which means the fertilization potential of the gametes has to be artificially forced, fertilized and the embryo maintained outside of its natural context, during a few days.

With time, the proliferation of studies with ample samples and adequate controls, allows for a good statistical treatment of the data, has dismissed doubts concerning the inherent negative effects of ART. At the same time, it is confirmed that so much the state of the gametes as the techniques used in artificial reproduction cause disorders in the offspring, with a different turnout according to the type of disorder.

A typical cycle of ART begins with ovary stimulation, usually hormonal, even when the fertilization is going to be natural or by insemination. In the case of IVF, after the stimulation the gathering in the ovarian follicles of the oocytes takes place and frequently they are induced to mature *in vitro*. The gametes are then co-incubated in a culture medium during some hours (IVF), or one sole sperm is injected directly in the oocyte in the culture to help the fertilization (ICSI). The resulting embryo is cultivated during two or three days until a 6-8 cell embryo is formed, or during several days more, until the blastocyst stage is reached (70-100 cells) before proceeding on to the implantation in the mother's uterus. This transfer takes place then or after a period of conservation in cold and prior thawing, that has to be accompanied by a reanimation of the embryo.

These "in vitro" events occur during the most critical period in the development of the nascent embryo, around its nesting period. The change in its natural medium-the mother's body-by the different mediums of the techniques, are not indifferent neither for the oocyte nor for the embryo[4]. For many years, the Biology of development has shown that different gene expression is dependent on the medium in which the cells are, with respect to its position in relation with the cells that they interact with, the molecular signals they receive and the environment the organism lives in, be it the maternal body or the external conditions where it lives after its birth.

It is important to have in mind this fact: the medium modifies the state of the genetic material, regulating in this way -epigenetically- the genetic expression. Logically, it is important that the techniques-with its changes in the environment and situation -are

[4] Thompson, J.R., Williams, C.J. "Genomic imprinting and assisted reproductive technology: connections and potential risks". *Seminars in Reproductive Medicine* 23, 2005, 285–295.

more invasive the greater the deficiencies of the gametes be, with the aim of forcing them to mutually fertilize.

The quality of the ovules depends on the mother's age as comparative studies show between women of the same age that conceive naturally or with the help of ART. A factor to have in mind is the infertility index that increases with age and since the age of the majority of women who resort to IVF has incremented in the last few years, makes the ART practice riskier. On the other hand, a series of environmental toxics, endocrine interruptors, have reduced masculine fertility by causing DNA mutations or in the same zones that regulate some specific gene expression known as *epigenetic mutations*.

The techniques play down the environment from the *in vitro* embryo: the "molecular dialogue" that is established since the beginning of fertilization between the gametes, that gives its origin and subsequently the "molecular dialogue" of the embryo with the mother while it travels through the tubes and between both prepare the nesting in the mother's uterus, inducing the immunological tolerance of the mother towards the foetus. The development of the conditions of the culture of the embryos in the laboratory, and in the case of freezing, by far that the experience has been perfected afterwards, does not substitute the specific natural environment, and the only one that avoids the vulnerability of the early embryo.

The reasonable hypothesis was raised that infertility treatments the more invasive they were the more health problems they would cause so much so in mothers as in their offspring.

a) Ovary stimulation is a habitual process, since it is necessary to provide for a greater number of ovules than those that mature in a menstrual cycle. Therefore, if the ovules are normal as if they have a defect-for example, by ageing -they are collected immaturely and in a greater number than normal, and they are forced to mature in an *in vitro* culture before being fertilized. The *in vitro* maturation of the ovules outside the ovaries, that is its niche, is a negative factor[5] by the change in the DNA state following the instructions of the medium.

b) Intrauterine insemination involves as many risks for the offspring as IVF/ICSI[6]. It requires ovarian stimulation of the woman and has a cause when the infertility is not explained, or when it is for diverse causes of male infertility. Both gametes can be engaged; in fact, the results in terms of pregnancy or of birth, are better when the semen of an external donor is used than when the semen of the partner is used. Insemination is not a substitute for IVF[7].

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[5] Buckett, W.M, Chian, R.C., Holzer, H, Dean, N, Usher, R, Tan, S.L. "Obstetric outcomes and congenital abnormalities after in vitro maturation, in vitro fertilization, and intracytoplasmic sperm injection". *Obstetrics and Gynecology* 110, 2007, 885–891.

[6] Sagot, P., Bechoua, S., Ferdynus, C., Facy, A., Flamm, X., Gouyon, J.B., Jimenez, C. "Similarly increased congenital anomaly rates after intrauterine insemination and IVF technologies: a retrospective cohort study". *Human Reproduction* 27, 2012, 902–909. Huang, C.T., Au, H.K., Chien, L.W., Chang, C.W., Chien, Y.Y., Tzeng, R. "Twin pregnancy outcome among cases of spontaneous conception, intrauterine insemination, and in vitro fertilization/intracytoplasmic sperm injection". *Fertility and Sterility* 86, 2006, 1017–1019.

[7] ESHRE Capri Workshop Group. "Intrauterina Insemination" *Human Reproduction Update* 1, 2009, 1–13.

The level of the prevalence of congenital abnormalities in Europe[8] show that the potential risk associated to insemination should not be ignored and this information should be transmitted not only to those responsible for carrying it out but also to the couples that wish to be treated.

c) ISCI is more prejudicial than the conventional IVF[9]. The forced fertilization of an ovary (IVF), more so if its by the direct injection of only one sperm into the cytoplasm (ICSI) has shown an elevated risk for the offspring, since the fertility incapability of sperm is usually due to genetic causes, associated to Y chromosome alterations or due to induced mutations by environmental toxics. This risk is then passed on to the following generations.

d) The tendency in the last few years has been an increase in ICSI use and of embryo transfer of those frozen or thawed[10]. Independently of the state of the ovule, in ISCI - especially indicated when the sterility of the couple is because of a male factor- the congenital malformations of those born. The freezing of the embryo acts as an embryonic selection, so much so because only the most fitted survive the thawing as because the uterus recuperates with time from the alterations due to ovarian stimulation and facilitates the nesting of the transferred embryo.

On the other hand, it is well-known that the proportion of those born alive has not increased, in the more than thirty years in spite of technology having been perfected, seeing the results that are obtained year after year in terms of an achieved pregnancy or of a born baby. Only approximately 15% to 30% of the embryos generated by these techniques[11] survive.

e) We lack reliable information about the added risks in the future of those born generated *in vitro* by ICSI and subject to a biopsy for the genetic diagnosis prior to implantation[12]. The current situation, in as much as it refers to the errors in analysis, as with perinatal deaths, should be looked from the perspective that this diagnosis does not have any therapeutic function for the analysed embryos but that it deals with a system of selection that chooses the embryos that are transferred by virtue of their characteristics.

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[8] Dolk, H., Loane, M., Garne, E. "The prevalence of congenital anomalies in Europe". *Advances in Experimental Medicine and Biology* 686, 2010, 349–364.

[9] Cfr. entre otros: Hansen, M., Kurinczuk, J.J., Bower, C., Webb, S. "The risk of major birth defects after intracytoplasmic sperm injection and in vitro fertilization". *New England Journal of Medicine* 346, 2002, 725-730. Bonduelle, M., Liebaers, I., Deketelaere, V., Derde, M.P., Camus, M., Devroey, P., *et al.* "Neonatal data on a cohort of 2889 infants born after ICSI (1991-1999) and of 2995 infants born after IVF (1983-1999)". *Human Reproduction* 17(3), 2002, 671-694. Lie, R.T., Lyngstadaas, A., Orstavik, K.H., Bakketeig, L.S., Jacobsen, G., Tanbo, T. "Birth defects in children conceived by ICSI compared with children conceived by other IVF-methods. a meta-analysis". *International Journal of Epidemiology* 34, 2005, 696–701.

[10] Källén, B., Finnstrom, O., Lindam, A., Nilsson, E., Nygren, K.G. Olausson, P.O "Trends in delivery and neonatal outcome after in vitro fertilization in Sweden: data for 25 years". *Human Reproduction* 25, 2010, 1026–1034.

[11] Gnoth, C., Maxrath, B., Skonieczny, T., Friol, K., Godehardt, E., Tigges, J. "Final ART success rates: a 10 years survey". *Human Reproduction* 26, 2011, 2239 –224.

[12] Liebaers, I, Desmyttere, S., Verpoest W., De Rycke, M.,Staessen, M.C., Sermon K., Devroey, P. Haentjens, P. Bonduelle, M. "Report on a consecutive series of 581 children born after blastomere biopsy for preimplantation genetic diagnosis". *Human Reproduction* 25, 2010, 275–282.

f) Some of the health problems of those born by ART are linked with the fact, showing in a consistent manner, multiple births, low birth weight and prematurity. These situations frequently involve lack of health in the long term.

On different occasions, pediatricians and obstetricians have offered to collaborate with Assisted Reproduction centres and to broaden the neurological development analysis of the children, growth, maturing process in puberty, fertility, etc., in the long term. Some illnesses appear later on and can not be detected until those born reach a certain age. They also try to be able to differentiate the health risk, according to the techniques used and the sterility diagnosis of the parents. Nevertheless, the repeated alarm on behalf of pediatricians and neonatologists, the key questions still have not been made or have rather been ignored.

Can the exposure to an *in vitro* environment, different from the natural one, of the gametes and/or embryo, alter the development of the organs and tissues?

Are we at the tip of an iceberg and can following generations be affected?

The diagnosis of the causes of sterility is essential but it has been investigated very little and is not known in many cases. It is indispensable to be able to move forward in that investigation to be able to determine a clear indication to use different procedures of ART, in order to minimize the risks related to its invasive character[13].

It has to be kept in mind that the implementation of these techniques has risks, and that this is not a process directed at curing sterility. Therefore, there exists from the start a scientific objection to the implementation of a technology without guarantees, and in some cases, possibly, unnecessary and inefficient.

We know with certainty that the risk is real. The paper published in *The New England Journal of Medicine* with a study of more than 300,000 children, of which 6,136 had been generated by ART, puts forth that the risk of being born with some type of defect is greater (8,3%) with whichever of the ART techniques than when they are engendered naturally (5.8%). Significant differences exist according to the ART used; with IVF it was 7.2% while 9.9% was reached with ICSI[14].

Also in 2012 it was clearly demonstrated that the ones born after the use of IVF/ICSI have a higher risk of suffering perinatal complications in comparison with those spontaneously engendered. And the urgency to determine what aspect of the techniques causes more risks and how they could be minimized[15] is posed.

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[13] Karpman, E., Williams, D.H., Lipshultz, L.I. "IVF and ICSI in male infertility: update on outcomes, risks, and costs". *The Scientific World Journal* 5, 2005, 922-932. Mandavilli, A. "As IVF becomes more common, some concerns remain". *Nature Medicine* 14, 2008, 1171.

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[15] Pandey, S., Shetty, A., Hamilton, M., Bhattacharya, S., Maheshwari3, A. "Obstetric and perinatal outcomes in singleton pregnancies resulting from IVF/ICSI: a systematic review and meta-analysis" Human Reproduction Update, 18,2012, 485–503.

The implementation of ART generates rare syndromes and recent cases have appeared that show alterations that have not yet been quantified[16].

We know that there are alterations that appear in the long term, such as the systemic pulmonary and cardiovascular disease, caused by the exposure of the embryo in the first few days- when it is especially vulnerable-, in an adverse environment and to ovarian stimulation[17].

All this tells us that even after more than 30 years of retrospective investigation ART is still not sufficiently controlled.

The reasons are very, very clear: since sterility can not be cured the child can suffer the consequences of the deficiencies of their parents' gametes. But even in the cases where the gametes are not defective as occurs with those that came from donors, the implementation of the techniques causes excessive deficiencies.

Obviously, we neither ignore nor take away importance from the problem of the great quantity of embryos that die in each cycle of IVF, nor of the certainty in the fact that an embryo is in *in vitro* and not in the mother's womb does not change the intrinsic nature of all human embryos. *In vitro* or *in vivo* are human. Also, what is not ignored is the indescribable joy that millions of born children have supposed in the world, during more than 30 years, of parents with sterility problems.

For many years, the majority of scientific publications on the risks for the children dwell on the obligation of informing those that resort to infertility aid, in a thorough and complete manner, of the diagnosis, possibilities and precise risks.

If how it begins to be thought of, and preoccupy, we can only be at the tip of a great iceberg, a rational public debate should take place that revises the ample legislative permissiveness and that perhaps puts forth the convenience of setting up adequate controls to the ART centres on the use of techniques.

We will describe here one of the grave problems that the use of ART brings about: the higher risk of suffering illnesses and malformations by the children generated by ART with respect to those engendered, that today is undeniable. Data that must be available to all who turn to them, available to society and that necessarily have to be taken into account by the National Commissions so much so of Bioethics as of ART control.

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HEALTH RISKS OF BIRTH BY ASSISTED FETRILIZATION TECHNIQUES. TIP OF THE ICEBERG.

Summary

Assisted Reproduction Techniques (ART)- Ovarian stimulation, intrauterine insemination (IUI) or In Vitro Fertilization (IVF)/Intracytoplasmic injection of sperm (ISCI)-have made possible the birth of hundreds of child, and many of them are entering adulthood. In the early 90's began a pediatric alert for defects and anomalies presented by these children, a higher proportion than those born naturally. Their health is unquestionably worse since 2002. Techniques used are extremely aggressive; the loss of embryos before implantation, spontaneous abortions and perinatal mortality is very high- Essential animal experimentation have not been carried out: and tests with fertile animals have shown that the offspring generated in vitro presented serious alterations.

However, damages observed were simply ascribed almost entirely to the advanced age of women using these techniques, and sterility to genetic alterations of the sperm, increasing by environmental contamination. Extensive direct human experimentation has been carried out, but without observing the minimum requirements of human experimentation; ignoring the role of the techniques themselves, and how they cause defects in a number of children thus generated, or what they might transmit to future generations. This is not a question of risk/benefit regarding a health problem. The ART are not interventions to solve a physical or physiological vital problem. The proportionality between the satisfaction of the desire for maternity/paternity and risks to the child's health should be a primary criterion, although not the only one.

Key words: assisted reproduction techniques, health of newborns, intergenerational problem, genome and epigenome, epimutations.

1. **Introduction**

The biology of the transmission of life has moved forward in a spectacular way in the last decades: hereditary alterations are not only due to defects in mutated genes in inherited genetic material. The forming and maturing of the ova or of the spermatozoa, the same fertilization, the first steps in embryonic life and in definite all of the life of each individual, modifies the state of the genetic material in a manner dependent of the medium. The regulation of what genetic information is expressed and which is silenced in each moment depends on the state of the genome in time and in corporal space.

The substitution, necessary when ART is used, of the environment by the means of *in vitro* can cause mutations at this level of regulation -*epimutations*- that can add up with the possible alterations, which on their own or by technical manipulation that the gametes of the progenitors can have.

A concatenation of causes brings about the fact that the health of those who are born by the use of ART will be worse than those born engendered. Moreover, some of the DNA epimutations can be passed on to the following generations.

The knowledge that the gene expression is epigenetically regulated that is to say that with the same process of development, and in a dependent form with the conditions of

the medium, it should not have been ignored. For many it has fortunately been a special preoccupation[18] although it has systematically been unheeded by the centres that carry out ART and is a call for thoroughness in research.

We will begin with a brief description in the close relationship genes-environment in the transmission of life and during the first stages of unborn life.

2. Genes and environment: epigenetic mutations as one of the causes of health alterations in those generated by ART

2.1 Epigenome and epigenetic mutations

Since the 60s it begins to appear that DNA is not the only deposit for information. On the contrary, there are two levels of information in the inherited chromosomes: the sequence of bases in DNA, the *genome*, and a second deposit that is known as the *epigenome* that carries out the regulated gene expression.

The term "epigenetic" was introduced for the first time in 1940 by Conrad Waddington[19] to describe the DNA modifications that permit regulating the gene expression without altering the base sequence and in function of the components of its interior or exterior medium. Therefore, as the genome- the sequence of the DNA base - does not change in none of the organism's cells nor throughout its life, the epigenome - that resides in chemical and structural changes of DNA- is in continuous change throughout life, to the beat of the interaction with the changing medium. It determines the state of development of the organism and of the diverse cells that constitute the organs, the tissues and systems of that organism. The most known changes are DNA methylation, the modification of the histone proteins and RNA expression in small quantity. Although in this paper we fundamentally refer to the changes in methylation, it is important to point out that the appearance and disappearance of RNA of small quantity plays an essential role as a regulator in the great epigenetic changes in the processes of life transmission and in the beginning of the development[20].

These changes permit DNA sequence to be accessible or to be blocked that signal the start of a new gene[21] by which it expresses or is silenced. Without this information, that is created step by step and controlling gene expression, there would not be embryonic development, maturation or natural ageing.

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Epigenetic mutations instead of genetic mutations are what alterations in the markings of DNA are called -methylation pattern and RNA union- that affect the regulation of gene expression. This regulation dependent on the medium fundamentally takes place during gamete formation, fertilization and in the first stages of embryonic development. So the changes in the medium generate alterations in embryonic development and these alterations can be transmitted onto the next generation. Epigenetic mutations are at the base of many diseases[22], especially in the processes of reproduction[23].

2.2 Influence of the medium in the processes of life transmission

The characteristic pattern of spermatozoa and of the ova that is known as parental imprinting, logically is inherited as a specific state of the genetic material of the gametes from those that are constituted in the fertilization of the new individual. The epigenome of the ova and of the spermatozoa has to rapidly change during the fertilization process so that the epigenome of the zygote is in situation to begin to develop. It is then, that each zygote begins to develop, *in vivo* or *in vitro*, with a genome and a brand new epigenome, and is renovated with fertilization[24].

This requires that the imprinting of the paternal and maternal chromosomes suffer an *epigenetic reprogramming* during the time that the fertilization process lasts.

Once the zygote is constituted and in the 5 or 6 days of embryonic development, before nesting begins, the majority of the marks are erased, with a rhythm and a different pattern, of the paternal and maternal genetic inheritance. At the same time other new ones are established that create the epigenome of a new individual, with a specific imprinting of each organ and tissue[25], therefore permitting the gene expression necessary for full development.

Only in one series of genes, denominated *genes with imprinting* does not change the regulatory markings during this wave of changes that take place during fertilization. Therefore, each copy stores the paternal or maternal parental imprinting, specifically regulates the expression of those genes: one copy is expressed and the other is silenced. These genes with imprinting[26] play a fundamental regulatory role in the first stages of embryonic development.

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2.3 The state of the gametes: epimutations

The genesis and maturity of the gametes is a process with an enormous precision that requires as a natural niche of the masculine or female body. Of its states depends a stable differentiation during the embryonic development after its mutual fertilization[27].

The epigenome of the sperm is established early at the beginning of the process of genesis and the maturation of the sperm, before it can be altered by the ART processes[28]. Because of this, the largest part of the causes of masculine sterility is due to existing defects in the genome or in the epigenome of the sperm and therefore it is transmitted to the offspring. However, it is alarming that diverse environmental toxins cause DNA alterations of the spermatozoa[29], and epimutations in specific genes that are implicated in spermatogenesis. The decline of human spermatogenesis is associated with defects of the imprinting of some genes[30]. The DNA alteration has very negative effects for the embryo generated by the ART[31], so it is not strange that it is raised to analyze the DNA of the sperm as a guarantee[32].

Little is known about the change of the imprinting in natural maturing or *in vitro,* of the ova but there are numerous data about how ovarian stimulation, a process common to all ART, affects imprinting[33]. The manipulation of the ova requires ovarian stimulation, the collecting of immature oocytes and their maturing in *in vitro* culture. If they suffer epimutations and come to be fertilized they have negative effects on the embryo. In animals, ovarian stimulation retards embryonic development, increases the abnormal

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[31] Borini, A., Tarozzi, N., Bizzaro, D., Bonu, M.A., Fava, L., Flamigni, C., Coticchio, G. "Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART". *Human Reproduction* 21, 2006, 2876–2881. Zini, A., Boman, J., Belzile, E., Ciampi, A. "Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: Systematic review and meta-analysis". *Human Reproduction* 23, 2008, 2663–2668.

[32] Barratt, C.L.R., Aitken, R.J., Bjorndahl, L., Carrell, D.T., de Boer, P., Kvist, U., Lewis, S.E.M., Perreault, S.D., Perry M.J., Ramos, L., Robaire, B., Ward, S., Zini, A. "Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications—a position report". *Human Reproduction* 25, 2010, 824–838.

[33] Borghol, N., Lornage, J., Blachere, T.S., Garret, A., Lefevre, A. "Epigenetic status of the H19 locus in human oocytes following in vitro maturation". *Genomics* 87, 2006, 417–426. Geuns, E., Hilven, P., Van Steirteghem, A., Liebaers, I., De Rycke, M. "Methylation analysis of KvDMR1 in human oocytes". *Journal of Medical Genetics* 44, 2007, 144–147. Sato, A., Otsu, E., Negishi, H., Utsunomiya, T., Arima, T. "Aberrant DNA methylation of imprinted loci in superovulated oocytes". *Human Reproduction* 22, 2007, 26–35.

formation of the blastocyst, delays the growth and increases foetal loss[34]. Immature human oocytes if they do not manage a correct reprogramming *in vitro*, can lead if they are forced to suffer fertilization to diseases even including the death of the embryo[35].

An epimutation of the gametes that affects genes with imprinting, essential for the normal development of the early embryo, leads to growth disorders of the placenta, causes reduced intrauterine growth and it is related with various syndromes and risks of some types of cancer as we will analyse further on.

2.4 Fertilisation

The requirement of ovarian stimulation, with its possible alterations of parental imprinting and the alterations that the spermatozoids might have, reinforces a combination of flawed parental genotype for the complex initial development of the embryo generated artificially. During the fertilization process, the imprinting of the paternal genome changes rapidly, fundamentally by the loss of methyl groups of cytokines of DNA[36]. The medium in which the fertilisation takes place is important as it is in all epigenetic processes.

2.5 Epimutations for the culture of the embryo

Various experiments with animals has shown that the embryo *in vitro* culture causes epigenetic mutations and alters the genes with imprinting[37] related with growth, in a dependent form of the conditions of the medium that is employed[38]. In mediums with poor conditions, mice embryos, coming from ovarian stimulation, have development faults and have more pathologies of the neuromotor system and some organs were of a bigger size than in those of control study[39]. These effects were also observed in sheep[40].

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[34] Van der Auwera, I., D'Hooghe, T. "Superovulation of female mice delays embryonic and fetal development". *Human Reproduction* 16 (6), 2001, 1237-1243. Stouder, C., Deutsch, S., Paoloni-Giacobino, A. "Superovulation in mice alters the methylation pattern of imprinted genes in the sperm of the offspring". *Reproductive Toxicology* 28, 2009, 536–541.

[35] Obata, Y., Hiura, H. "Epigenetically immature oocytes lead to loss of imprinting during embryogenesis" *Journal of Reproduction and Development* 57, 2011, 327-334.

[36] Mayer, W., Niveleau, A., Walter, J., Fundele, R., Haaf, T. "Demethylation of the zygotic paternal genome" *Nature* 403, 2000, 501–502.

[37] Rinaudo, P.F., Giritharan, G., Talbi, S., Dobson, A.T., Schultz, R.M. "Effects of oxygen tension on gene expression in preimplantation mouse embryos". *Fertility and Sterility* 86, 2006, 1252–1265.

[38] Giritharan, G., Talbi, S., Donjacour, A., Di Sebastiano, F., Dobson, A.T., Rinaudo, P.F. "Effect of in vitro fertilization on gene expression and development of mouse preimplantation embryos". *Reproduction* 134, 2007, 63–72. Fauque, P., Jouannet, P., Lesaffre, C., Ripoche, M.A., Dandolo, L., Vaiman, D., *et al.* "Assisted reproductive technology affects development kinetics, H19 imprinting control region methylation and H19 gene expression in individual mouse embryos". *BMC Developemental Biology* 18, 2007, 116. Rivera, R.M., Stein, P., Weaver, J.R., Mager, J., Schultz, R.M., Bartolomei, M.S. "Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development". *Human and Molecular Genetics* 17, 2008, 1–14. Rinaudo, P.F., Giritharan, G., Talbi, S., Dobson, A.T., Schultz, R.M. "Effects of oxygen tension on gene expression in preimplantation mouse embryos". *Fertility and Sterility* 86, 2006, 1252–1265.

[39] Fernandez-Gonzalez, R., Moreira, P., Bilbao, A., Jimenez, A., Perez-Crespo, M., Ramírez, M. A., Rodríguez De Fonseca, F., Pintado, B., Gutiérrez-Adán, A. "Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior". *Proceedings of the National Academy of Sciences of the United States of America* 101, 2004, 5880–5885.

[40] Gardner, D.K., Lane, M., Spitzer, A., Batt, P.A. "Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino

Generally the medium of culture contains foetal bovine serum that provides a rich environment for the development of the embryo. However, it contains active compounds, such as hormones, growth factors, that paradoxically reduce the early development potential of the embryo, causes metabolic abnormalities and abnormalities in embryonic structures. These erroneous signals provoke a gene dysregulation because of epigenetic modifications of the genome[41].

Development programming, growth and physiology are irreversibly affected during the period prior to implanting by an inadequate *in vitro* culture. It has been suggested that the possibility that the methionine contained in mediums of commercial cultures for ART can be critically involved in inducing epigenetic mutations and preoccupation has been expressed because the chemical contents of such mediums are not always clearly documented by the manufacturers[42].

A possible mechanism of the epimutations is that the culturing of the embryos gives way to an elevated production of reactive oxygen species on behalf of the mitochondrias. These molecules alter the DNA, the normal epigenetic pattern and the posterior genetic expression of the embryo[43]. In fact an altered expression of genes after *in vitro* fertilization has been described specifically in the placenta[44]; precisely genes that are involved in the energetic metabolism, in DNA repair and in stress response. The insufficient production of energy in the placenta and in genes in the dysfunction metabolism emphasizes the vulnerability of the placenta in relationship with its surroundings.

2.6 The freezing/thawing of the embryos and their posterior transfer

It is known that after being thawed some embryos present a loss in their capacity to develop. In publications in 2009 and 2011, which summarize the results of the European ART centres, describe that the amount of embryos, on an international level, fluctuates around 430,000 per year. More than half, -around 240,000- are generated by ICSI. The number of frozen embryos- after passing an exam based on basic morphological characteristics, usually in the blastocyst stage of some 5 or 6 days-, and thawed is of 108,000. 5%, even 10% of those embryos will not be able to be transferred due to developmental alterations. Only 19.37% of all thawed embryos will have succeeded to be implanted and of those 47.40 continued the gestation and were born alive. The

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acids, vitamins, and culturing embryos in groups stimulate development". *Biology of Reproduction* 50, 1994, 390-400.

41 Reik, W., Romer, I., Barton, S.C., Surani, M.A., Howlett, S.K. and Klose, J. "Adult phenotype in the mouse can be affected by epigenetic events in the early embryo". *Development*, 119, 1993, 933-942. Reik, W. "Genetic conflict in early development: parental imprinting in normal and abnormal growth". *Reviews of Reproduction* 1, 1996, 73-77. Reik, W., Walter, J. "Genomic imprinting: parental influence on the genome". *Nature Review Genetics* 2, 2001, 21-32.

42 Niemitz, E.L., Feinberg, A.P. "Epigenetics and assisted reproductive technology: a call for investigation". *American Journal of Human Genetics* 74, 2004, 599-609.

43 Chason, R.J., Csokmay, J., Segars, J.H., Decherney, A.H., Armant, D.R. "Environmental and epigenetic effects upon preimplantation embryo metabolism and development". *Trends in Endocrinology and Metabolism* 22 (10), 2011, 412-420.

44 Zhang, Y., Zhang, Y.L., Feng, C., Wu, Y.T., Liu, A.X., Sheng, J.Z., *et al.* "Comparative proteomic analysis of human placenta derived from assisted reproductive technology". *Proteomics* 8, 2008, 4344–4346.

percentage of success of babies born does not reach 15%. Also some are born prematurely and neonatal mortality reaches 2.54%.

Some malformations and cerebral paralysis are more frequent in embryos that have undergone ART with or without freezing than those conceived by natural form. Some risks- prematurity and low birth weight, neurological consequences and other diseases- are paradoxically less frequent in those embryos born having been frozen than in fresh embryo transfer[45]. However, it seems logical that it be so since freezing does not allow for defective embryos to resist thawing, be implanted in the uterus and to be born. Indeed, the microenvironment of the uterus a few days after a woman has undergone an ovarian stimulation process is toxic for embryo implantation. The mere distancing of time to preserve the embryo in the cold eliminates the adverse surroundings.

The filter of selecting embryos through its cryo-conservation is carried out in a special way in those fertilised by ICSI[46]. On the one hand, it has the effect of the injection in its membranes apart from the enormous fertilization force and since the indication of using ICSI is the masculine sterility factor, the sperms can be defective.

3. **Comparative systematic health studies according to how one was conceived**

Until the late eighties systematic studies on the health of children born by ART did not begin to be conducted. In 1990[47] the Medical Research Council published studies that gathered data on the health of the first year of life of those born between 1978 to 1987; when they were compared with those conceived naturally, it showed an increase- relatively low- of serious congenital malformations.

Throughout the nineties a clear relationship was observed between the implementation of IVF and prematurity and low birth weight of the children when born. Described are hypertension, heart diseases and osteoporosis and a higher incidence of malformations[48].

In the year 2002 the alarm is raised in the scientific community with the publication of Hansen´s[49] article, that shows that children conceived by IVF or by ICSI had almost more than half a prevalence (8.8% versus 4.2%) of chromosomal alterations, cardiac malformations, esophageal atresia and cranial malformations during the first year of

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[45] Pelkonen, S., Koivunen, R., Gissler, M., Nuojua-Huttunen, S., Suikkari, A.-M. Hyden-Granskog, C., Martikainen, H., Tiitinen, A., Hartikainen, A.-L. "Perinatal outcome of children born after frozen and fresh embryo transfer: the Finnish cohort study 1995–2006". *Human Reproduction* 25, 2010, 914–923. Pinborg, A., Loft, A., Aaris Henningsen, A-K, Rasmussen, S., Nyboe Andersen, A. "Infant outcome of 957 singletons born after frozen embryo replacement: The Danish National Cohort Study 1995–2006". *Fertility and Sterility* 94, 2010, 1320–1327.

[46] Källén, B., Finnstrom, O., Lindam, A., Nilsson, E., Nygren, K.G. Olausson, P.O "Trends in delivery and neonatal outcome after in vitro fertilization in Sweden: data for 25 years" *Human Reproduction* 25, 2010, 1026–1034.

[47] Beral, V., Doyle, P. "Births in Great Britain resulting from assisted conception 1978-1987. Report of the MRC Working Party on children conceived by in-vitro fertilization". *British Medical Journal* 300, 1990, 1229-123.

[48] Entre otros: Bergh, T., Ericson A., Hillensjö, T., Nygren, K.G., Wennerholm, U.B. "Deliveries and children born after in-vitro fertilisation in Sweden 1982-95: a retrospective cohort study". *Lancet* 354, 1999, 1579-1585.

[49] Hansen, M. and cols, *op. cit.*9.

life, than children engendered naturally. The preoccupation for the risk of damage in those born with the use of ART is not hidden[50] it begins to be significant and in fact has been confirmed with the passage of time.

Various revisions and meta-analysis gather numerous data, put forth the diverse types of risks and highlight the possible causes throughout the years until 2005[51].

As it reasonable to expect, the data is dependent on the type of damage that is examined, on the number of children, of the variables, that are taken into account or not-such as the mother's age, cause of infertility, etc.-, as well as those conceived by ART, as those naturally engendered control study groups. It is not surprisingly, therefore, that some articles do not find significant differences between those that are studies and the control study groups.

In Annex 1 there is a list of the scientific publications of the analysis conducted. Of some of the articles, commentaries are not made in the text. We will go on to continue the diverse types of risks and, in a manner that can be deduced by the information published, what is the cause or causes.

3.1 *Multiple births*

It is evident that ART techniques increase multiple births[52]. The reiterated international recommendation is to avoid these pregnancies, reducing ovarian stimulation and transferring only one embryo, but this has gone unheeded during a long time. Multiple births bring about higher rates of morbi-mortality during the perinatal period and disability in the long term[53]. In a follow up study during twenty years the negative effect

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[50] Winston, R.M., Hardy, K, "Are we ignoring potential dangers of in vitro fertilization and related treatments?" *Nature Cell Biology* 4, 2002, S14-S18 and *Nature Medicine* 8, 2002, S14-S18.

[51] Entre otros: Hansen, M., and cols *op .cit.*1. Bonduelle, M., Liebaers, I., Deketelaere, V., Derde, M.P., Camus, M., Devroey, P., *et al.* "Neonatal data on a cohort of 2889 infants born after ICSI (1991-1999) and of 2995 infants born after IVF (1983-1999)". *Human Reproduction* 17(3), 2002, 671-694. Bonduelle, M., Wennerholm, U.B., Loft, A., Tarlatzis, B.C., Peters, C., Henriet, S., Mau, C., Victorin-Cederquist, A., Van Steirteghem, A., Balaska, A., Emberson, J.R., Sutcliffe, A.G. "A multi-centre cohort study of the physical conception". *Human Reproduction* 20, 2005, 413-419. Schieve, L.A., Rasmussen, S.A., Buck, G.M., Schendel, D.E., Reynolds, M.A., Wright, V.C. "Are children born after assisted reproductive technology at increased risk for adverse health outcomes?" *Obstetrics and Gynecology* 103, 2004, 1154–1163. Schieve, L.A., Rasmussen, S.A., Reefhuis, J. "Risk of birth defects among children conceived with assisted reproductive technology: providing an epidemiologic context to the data". *Fertility and Sterility* 84, 2005, 1320–1324. Rimm, A.A., Katayama, A.C., Diaz, M., Katayama, K.P. "A Meta-Analysis of Controlled Studies Comparing Major Malformation Rates in IVF and ICSI Infants with Naturally Conceived Children". *Journal of Assisted Reproduction and Genetics* 21, 2004, 437-443.

[52] Reynolds, M.A., Schieve, L.A., Jeng, G., Peterson, H.B., Wilcox, L.S. "Risk of multiple birth associated with in vitro fertilization using donor eggs". *American Journal of Epidemiology* 154, 2001, 1043–1050. Vahratian, A., Schieve, L.A., Reynolds, M.A., Jeng, G. "Live-birth rates and multiple-birth risk of assisted reproductive technology pregnancies conceived using thawed embryos, USA, 1999–2000". *Human Reproduction* 18 (7), 2002, 1442–1448. Martin, J.A., Hamilton, B.E., Sutton, P.D., Ventura, S.J., Menacker, F., Kirmeyer, S., *et al.* "Births: final data for 2006". *National Vital Statistics Reports* 57, 2009, 1–101.

[53] Entre otros: Elster, N. "Less is more: the risks of multiple births". *Fertility and Sterility* 74, 2000, 617–623. Moise, J, Laor, A., Armon, Y., Gur, I., Gale, R. "The outcome of twin pregnancies after IVF". *Human Reproduction* 13(6), 1991, 702-1705. Sunderam, S., Chang, J., Flowers, L., Kulkarni, A., Sentelle, G., Jeng, G., Macaluso, M. "Assisted Reproductive Technology Surveillance--United States, 2006". *Morbidity and Mortality Weekly Report. Surveillance Summaries* 58(SS05), 2009, 1-25.

is confirmed[54]. Also ART twins require more medical care than those conceived naturally[55].

Multiple births have decreased considerably in the last years, since fewer embryos are transferred. Until 1992 the increase of twins approximately reached 30% of the births and reduced approximately around 5% in 2010 and is practically now null the birth of more than two[56]. It is important, in respect, that the transfer of only one fresh embryo has shown the same percentages of success than the simultaneous transfer of two embryos[57]. This is a question dependent on the quality of the centres, as women with a higher social-economic status show that since they have a more thorough follow-up and more perinatal care, generally they have less twins and better perinatal results[58].

Notwithstanding, there also exists risks for the health of singletons and, in fact, congenital malformations have not diminished[59]. And, in 1992, a clear alarm was raised when singleton children born through IVF/ICSI had a higher risk of perinatal complications than those conceived spontaneously[60].

3.2 *Low birth weight in relation to gestational age*

McDonald, S., Murphy, K., Beyene, J., Ohlsson, A. "Perinatal outcomes of in vitro fertilization twins: a systematic review and meta-analyses". *American Journal of Obstetrics and Gynecology* 193, 2005, 141–152. Yoon, G., Beischel, L.S., Johnson, J.P., Jones, M.C. "Dizygotic twin pregnancy conceived with assisted reproductive technology associated with chromosomal anomaly, imprinting disorder, and monochorionic placentation". *The Journal of Pediatrics* 146, 2005, 565-567. Li, S.J., Ford, N., Meister, K., Bodurtha, J. "Increased risk of birth defects among children from multiple births". *Birth Defects Research. Part A. Clinical and Molecular Teratology* 67, 2003, 879–885. Huang, C.T., Au, H.K., Chien, L.W., Chang, C.W., Chien, Y.Y., Tzeng, R. "Twin pregnancy outcome among cases of spontaneous conception, intrauterine insemination, and in vitro fertilization/intracytoplasmic sperm injection". *Fertility and Sterility* 86, 2006, 1017–1019.

[54] Scottland, G.S., McLernon, D., Kurinczuck, J.J., McNamee, P., Harrild, K, Lyall, H., Rajkhowa, M., Hamilton, M., Bhattacharya, S. "Minimising twins in *in vitro* fertilisation: a modelling study assessing the costs, consequences and cost-utility of elective single versus double embryo transfer over a 20-year time horizon". *British Journal of Obstetrics and Gynecology* 118(19), 2011, 1073-1083.

[55] Doornbos, M.E., Maas, S.M., McDonnell, J., Vermeiden, J.P.W., Hennekam Hansen, M., Colvin, L., Petterson, B., Kurinczuk, J.J., de Klerk, N., Bower, C. "Twins born following assisted reproductive technology: perinatal outcome and admission to hospital" *Human Reproduction* 24, 2009, 2321–2331. Pinborg, A., Loft, A., Rasmussen, S., Nyboe Andersen, A. "Hospital care utilization of IVF/ICSI twins followed until 2–7 years of age: a controlled Danish national cohort study". *Human Reproduction* 19, 2004, 2529-2536; Bower, C. "Admission to hospital of singleton children born following assisted reproductive technology (ART)". *Human Reproduction* 23, 2008, 1297–1305.

[56] Källén, B., Finnstrom, O., Lindam, A., Nilsson, E., Nygren, K.G. Olausson, P.O "Trends in delivery and neonatal outcome after in vitro fertilization in Sweden: data for 25 years" *Human Reproduction* 25, 2010, 1026–1034.

[57] Thurin, A., Hausken, J., Hillensjo, T., Jablonowska, B., Pinborg, A., Strandell, A., *et al.* "Elective single embryo transfer versus double embryo transfer in in vitro fertilization". *New England Journal of Medicine*. 351, 2004, 2392–2402.

[58] Boulet SL, Schieve LA, Nannini A, *et al.* "Perinatal outcomes of twin births conceived using assisted reproduction technology: a population-based study". *Human Reproduction* 23, 2008, 1941–1948.

[59] Knoester, M., Vandenbroucke, J.P., Helmerhorst, F.M., van der Westerlaken, L.A., Walther, F.J., Veen, S. "Matched follow-up study of 5–8 year old ICSI-singletons: comparison of their neuromotor development to IVF and naturally conceived singletons". *Human Reproduction* 22, 2007, 1638–1646.

[60] Cit en 15.

Another negative effect on health is the low birth weight that happens with more frequency with the use of ART than with those engendered naturally[61], be it those born of single pregnancies as with multiple births and with diverse ART. Low birth weight generates hypertension that affects neurological development[62].

3.3 *Cerebral paralysis, epilepsy and febrile seizures*

Cerebral paralysis is a permanent disorder, and not progressive, that affects psychomotor development, occasioning important limitations in the activity as a consequence of the complications in the brain development of the foetus. Diverse studies since 2006 have asserted that the children born from IVF/ICSI have more of a risk of suffering from it[63]; apart from presenting a higher incidence in mental retardation and the severe ocular dysfunction associated with this disease, as well as autism spectrum disorders [64].

Children of sub-fertile couples, with hormonal treatment of a year, have a higher risk of epilepsy and febrile seizures than those that have had less than five months of treatment and those that have been conceived spontaneously[65].

3.4 Principal causes

These risks seem to be related principally to the treatment for ovarian activation and the maturing of the oocytes so much so in those that arise from multiple births as is the case

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[61] Schieve, L.A., Meikle. S.F., Ferre. C., Peterson. H.B., Jeng. G., Wilcox. L.S. "Low and very low birth weight in infants conceived with use of assisted reproductive technology". *New England Journal of Medicine* 346, 2002, 731–737. McDonald, S.D., Han, Z., Mulla, S., Murphy, K.E., Beyene, J., Ohlsson, A. "Preterm birth and low birth weight among in vitro fertilization singletons: a systematic review and meta-analysis". *European Journal of Obstetrics, Gynecology, and Reproductive Biology* 148, 2010, 105–113. Helmerhorst, F.M., Perquin, D.A., Donker, D., Keirse, M.J. "Perinatal outcome of singletons and twins after assisted conception: a systematic review of controlled studies". *BMJ* 328, 2004, 261-264. Fujii, M., Matsuoka, R., Bergel, E., van der Poel, S., Okai, T. "Perinatal risk in singleton pregnancies after in vitro fertilization". *Fertility and Sterility* 94, 2010, 2113–2117.

[62] Pellicer, A., Bravo, M.C., Madero, R., Salas, S., Quero, J., Cabañas, F. "Early Systemic Hypotension and Vasopressor Support in Low Birth Weight Infants: Impact on Neurodevelopment". *Pediatrics* 123, 2009, 1369-1376.

[63] Zhu, J. L., Basso, O., Obel, C., Bille, C., Olsen, J., Hvidtjørnb, D. "Infertility, infertility treatment and psychomotor development: the Danish National Birth Cohort". *Paediatric and Perinatal Epidemiology* 23, 2009, 98–106. Zhu J, Hvidtjrn D, Basso O, Obel C, Thorsen P, Uldall P, *et al.* "Parental infertility and cerebral palsy in children". *Human Reproduction* 25 (12), 2010, 3142-3145. Hvidtjørn, D., Grove, J., Schendel, D.E., Vaeth, M., Ernst, E., Nielsen, L., *et al.* "Cerebral palsy among children born after *in vitro* fertilization: the role of preterm delivery—a population-based, cohort study". *Pediatrics* 118, 2006, 475–482. Hvidtjørn, D., Grove, J., Schendel, D., Sværke, C. Schieve L.A, Uldall, P. Ernst E., Jacobsson B., Thorsen, P. "Multiplicity and early gestational age contribute to an increased risk of cerebral palsy from assisted conception: a population-based cohort study" *Human reproduction* 25, 2010, 2115-2123.

[64] Hvidtjørn, D., Schieve, L., Schendel, D., Jacobsson, B., Svaerke, C., Thorsen, P. "Cerebral palsy, autism spectrum disorders, and developmental delay in children born after assisted conception: a systematic review and meta-analysis". *Archives of Pediatrics & Adolescent Medicine* 163, 2009, 72–83.

[65] Sun, Y., Vestergaard, M., Christensen, J., Zhu, J.L., Bech,B.H., Olsen, J. "Epilepsy and febrile seizures in children of treated and untreated subfertile couples". *Human Reproduction* 22, 2007, 215–220.

of singletons[66]; epimutations of some genes with imprinting affect growth by action on the placenta[67].

Some gene epimutations with imprinting that require using only the paternal copy in the sperm, could also be related with the low birth weight of children conceived by ICSI.

The data on the effects of ovarian stimulation can be understated, since it is known there are births from mothers that use it without going to the centres. It is estimated[68] that 4.6% of the children born in the United States in 2005 were conceived after this stimulation and this is an important group that has had no follow-up.

4. Malformations, chromosomal alterations and sterility inheritance

It has been confirmed that the highest risk of birth defects, in singletons as in those that come from multiple births, are in those conceived by ART in comparison with those conceived without treatment.

4.1 Malformations

Numerous articles refer to an increase in congenital malformations in children generated by *in vitro* with those that have been engendered[69]. The risk of suffering a malformation

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[66] Gaudoin, M., Dobbie, R., Finlayson, A., Chalmers, J., ameron, I.T. "Ovulation induction/intrauterine insemination in infertile couples is associated with low-birth weight infants". *American Journal of Obstetrics and Gynecology* 188, 2003, 611–636. Klemetti, R., Sevon, T., Gissler, M., Hemminki, E. "Health of children born after ovulation induction". *Fertility and Sterility* 93, 2010, 1157–1168. D'Angelo, D.V., Whitehead, N., Helms, K.,W., Barfield, Ahluwalia, I.B. "Birth outcomes of intended pregnancies among women who used assisted reproductive technology, ovulation stimulation, or no treatment". *Fertility and Sterility* 96, 2011, 314-320.

[67] Guo, L., Choufani, S., Ferreira, J., Smith, A., Chitayat, D., Shuman, C., *et al.* "Altered gene expression and methylation of the human chromosome 11 imprinted region in small for gestational age (SGA) placentae". *Developmental Biology* 320, 2008, 79–91.

[68] Schieve, L., Devine, O., Boyle, C.A., Petrini, J.R., Warner, L. "Estimation of the contribution of non-assisted reproductive techonology ovulations stimulation fertility treatments to US singleton and multiple births". *American Journal of Epidemiology* 170(11), 2009, 1396–1407.

[69] Entre otras: Rimm, A.A., Katayama, A.C., Diaz, M., Katayama, K.P. "A Meta-Analysis of Controlled Studies Comparing Major Malformation Rates in IVF and ICSI Infants with Naturally Conceived Children". *Journal of Assisted Reproduction and Genetics* 21, 2004, 437-443. Klemetti, R., *et al.* "Children born after assisted fertilization have an increased rate of major congenital anomalies". *Fertility and Sterility* 84, 2005, 1300–1307. Merlob, P., Sapir, O., Sulkes, J., Fisch, B. "The prevalence of major congenital malformations during two periods of time, 1986–1994 and 1995–2002 in newborns conceived by assisted reproduction technology". *European Journal of Medical Genetics* 48, 2005, 5–11. Zhu, J.L., Basso, O., Obel, C., Bille, C., Olsen, J. "Infertility, infertility treatment, and congenital malformations: Danish national birth cohort". *BMJ* 333, 2006, 679; Alukal, J. P., Lipshultz, L.I. "Safety of assisted reproduction, assessed by risk of abnormalities in children born after use of *in vitro* fertilization techniques". *Nature Clinical Practice Urology* 5, 2008, 140-150. Buckett, W.M, Chian, R.C., Holzer, H, Dean, N, Usher, R, Tan, S.L. "Obstetric outcomes and congenital abnormalities after in vitro maturation, in vitro fertilization, and intracytoplasmic sperm injection". *Obstetrics and Gynecology* 110, 2007, 885–891. El-Chaar, D., Yang, Q., Gao, J., Bottomley, J., Leader, A., Wen, S.W., Walker, M. "Risk of birth defects increased in pregnancies conceived by assisted human reproduction" *Fertility and Sterility* 92, 2009, 1557–1561. Wood, H.M., Babineau, D., Gearhart, J.P. "In vitro fertilization and the cloacal/bladder exstrophyeepispadias complex: A continuing association". *Journal of Pediatric Urology* 3, 2007, 305-310. Delbaere, I., Goetgeluk, S., Derom, C., De Bacquer, D., De Sutter, P., Temmerman, M "Umbilical cord anomalies are more frequent in twins after assisted reproduction" *Human Reproduction* 22, 2007, 2763–2767. Midrio, P., Nogare, C.D., Di Gianantonio, E., Clementi, M. "Are congenital anorectal

is different according to the type of ART used: the risk factor is 9.85 for gastrointestinal ones, 2.30 for the cardiovascular and 1.54 for muscularskeletal defects[70].

Muscular genital tract malformations have been described that are associated to paternal masculine sterility and, that were generated by ICSI; the risk is greater than the 5% against less than 1% of those engendered[71]. The risk of this type can be related with low sperm quality.

Other alterations described are the abnormal vascularisation of the retina[72] and heart anomalies[73].

4.2 Chromosomal alterations

It is known that in up to 60% of spontaneous miscarriages the foetus presents chromosomal alterations incompatible with life, and frequently of trisomies or loss of chromosomes. Spontaneous miscarriages with chromosomal abnormalities are higher in those generated by ART than those conceived naturally[74]; it is of interest that the losses of sexual chromosomes are greater (11.69%) in the group of those generated by ICSI than in the control group (6.45%) and of those generated by conventional IVF (3.23%). Of those generated by ICSI, in 55.71% the cause of infertility was due to the male factor. Similar results were obtained when comparing a total of 277 spontaneous miscarriages where the foetuses were generated with the use of ART. They have 63,2% of chromosomal alterations coming from those of conventional IVF and 71,5% of those engendered after ovarian stimulation treatment, 80% when the ICSI was a direct extraction of the sperm from the testicles and 85.7% after uterine insemination[75].

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ICSI treatment carries three times more risk for abnormalities in chromosomes than those conceived naturally[76]. The data is congruent: when fertilization is forced with a direct injection to the ova of a spermatozoid as in cases of masculine sub-fertility, it normally comes with alterations of the genetic material.

It is known that apart from the alterations, fundamentally inherited from the paternal gametes, there exists risks of generating some new chromosomal abnormality, not present in the gametes; supposedly 1.6 % for those generated by ICSI against 0,5% of those naturally conceived[77]. These foetal alterations *de novo* fundamentally consist in an increase in the number of sexual chromosomes, can cause sterility and its phenotype manifestation is undetectable at birth and in the first few years of life. They can be originated by the culture of the embryo[78].

The chromosomal alterations of the child can also be due to the mother's advanced age that is obviously accompanied by oocyte alterations and is manifested in chromosomal aberrations. These alterations are greater after ISCI with respect to conventional IVF, even if the mother is young[79].

In the past few years, the *in vitro* maturation of the oocytes obtained from ovarian stimulation has begun to be a routine step of ART. It has been observed that this process does not increase the risk of congenital alterations with respect to those born by being conceived naturally[80], except when female sterility is due to a polycystic ovary. In this case, the maturing of the ova *in vitro* carries a high level of embryonic loss if it is either generated by IVF as with ICSI[81].

4.3 Inheritance of the alterations that cause paternal sterility, after the use of ICSI

The possibility of inheriting paternal sterility when the ICSI technique has been used seems to be related with the underlying infertility[82]; in fact there exists a strong

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correlation between the chromosomal constitution of the embryo and the paternal infertility due to chromosomal alterations.

Part of male infertility is associated to alterations in the Y chromosome that the male children obviously inherit[83]. Actually it is possible to dissect the genes of chromosome Y which allows for a better diagnosis of the cause of infertility and, with the diagnosis, thorough information could be given to those that resort to ICSI and they could then take into account the expected malformations in their children.

Another of the causes of sterility that refers to the no production of spermatazoids -azoospermia- low or moderate production -oligozoospermia- and that have required ICSI to conceive had some chromosomal reorganization. The frequency of the three mentioned types was of 18.71%, 14.55%, and 2.37% respectively[84].

Thirdly, 6% of sterile men have a karyotype with anomalies of the type of trisomies -three instead of two- of the sexual chromosomes, less frequent. For example, Klinefelter Syndrome, which occurs in 1 of 500 males, affects 14% of the men with non-obstructive azoospermia. In other cases, it deals with chromosomal reorganizations, changes of places of the genes, inversions in some regions of DNA, or loss of zones. The ART cause that the offspring be passed on with genetic diseases[85].

It has been confirmed that genetic mutations that cause cystic fibrosis and follow with the absence of vas deferens, is transmitted to children generated by ART[86].

Epigenetic alterations have also been described in sperm with low motility[87] and also intrinsic errors in the imprinting in the sperm of men with normal sperm production[88].

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In types of female infertility that affects genes with imprinting it can be transmitted to children when the ART exceeds the fertilization selection barrier[89].

On the other hand, alterations have been described of the sexual hormones in those generated by ICSI whose consequences in the present and for future generations do not stop from being alarming[90].

It is imperative to carry out a thorough analysis of the causes of male sterility for those that resort to ART implementation; and it is necessary to advise against it in the case of any type of alteration not only in the chromosomes but also in the DNA state, that must correspond to the maturing situation.

5. **Defects in the early foetus**

Congenital defects classified as "blastogenesis defects" take place in the first four weeks of embryo development. It happens at the beginning of the formation of the diverse organs and therefore, tends to affect the formation of specific zones of the foetus that is developing in those moments. It originates defects in the neural tube, in the stomach wall, esophageal atresia and anal atresia and is more frequent in embryos generated by ART, so much so in singletons as in twins[91]. They are serious and many of the spontaneous miscarriages have this type of malformation. They do not have a genetic cause and are more frequent in those born after the use of IVF and ICSI[92]. They are present in 1 of every 160 pregnancies of ART in comparison to 1 for every 400 in control case studies which pose that they are due to the changes in the natural surroundings by the environment in which the ART embryo is developing in.

So much so the exposures related with ovarian stimulation, oocyte collection and the culture of embryos has probabilities of influencing in the early development of the embryo[93] and in its implantation. Ovarian stimulation treatment involves having a lower level of endometrial protein in pregnancy, PAPP-A, that plays a key role in the formation of blood vessels and in placenta formation during the first weeks of

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pregnancy. It is also relevant in that this protein is used in the diagnosis of Down´s Syndrome in foetus giving way to false-positive results[94].

Another negative effect of the implementation of ART in the early development of the embryo is the fact that they make the control of imprinting gene expression be lost, which is essential in this stage. As it has been commented, the cause of the loss of control of these genes can be multiple[95].

6. Rare Syndromes and risk of cancer because of epigenetic mutations

Some syndromes exist, related with aberrant expression of imprinting genes, very infrequent in the general population and that, however, have frequently been found in children born using assisted reproductive techniques.

a) *Beckwith-Wiedemann Syndrome* is characterized by premature birth, an abnormally long tongue, umbilical hernia, neonatal hypoglucemia and a predisposition for tumours[96] and a higher frequency for Wilms' tumour, a rare tumour that affects one in every 36,000 births[97]. There exists a clear association between Beckwith-Wiedemann Syndrome and patients conceived by ART[98].

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90% of children with Beckwith-Wiedemann, born by the use of ART, have specific imprinting defects, consistent in the loss of epigenetic marks in the KvDMR region- inside the KCNQ1- of the maternal copy of chromosome 11[99]. This same change in the imprinting is shared with 40-50% of those who suffer the syndrome and had been engendered naturally[100]. In those conceived by ART there seems to be other epimutations one of them tied to ICSI [101]and any of them can affect the regulation of specific genes.

A known example would be the gene with the imprinting, Igf2r -the receptor of the growth factor derived from insulin-that is directly related with excessive growth and that in sheep generated by *in vitro* has been related with a modification consisting of methylation loss[102].

b) Patients with Angelman Syndrome have loss or alterations in the methylation pattern. In this case, in chromosome 15, in the region SNRPN, also in the maternal copy, what it suggests, as in the prior case, is that the epigenetic mutation is related with a greater vulnerability of the oocyte obtained from the stimulated ovary and matured *in vitro*. In fact, SNRPN imprinting of the maternal copy is established in fertilization or even later on[103].

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Angelman Syndrome is characterized by severe mental retardation, speech deterioration, equilibrium disorder and "happy and nervous disorder" behaviour; it also includes excessive prenatal growth and defects in the abdominal wall. The frequency is approximately one in every 10,000 to 30,000 of the population. It is linked with ART, especially with the implementation of ICSI in sub-fertile couples[104]; although, it is evident that ICSI *per se* is not the principle determinant of the association observed between ART and imprinting disorders but more so because of ovarian stimulation[105].

c) Other rare syndromes have been associated with ART especially in ICSI twins: Goldenhar, and Rubenstein-Taybi, and Meckel-Gruber and mental retardation[106].

d) A single article -that has not been confirmed- shows a greater risk of suffering the infantile tumour retinoblastoma in children born by IVF; a tumour not so frequent that appears in one child for each 17,000[107]. A meta-analysis[108] has detected an increase in the risk for cancer in children generated by ART.

e) An increase in neurological after-effects has been described, such as mental retardation and serious vision defects[109]. The preoccupation for consequences in the long term by the use of ART, such as neurological developmental problems, raises the issue that makes the investigators pay more attention[110].

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f) As what was to be expected, those gestated alone have less risks of imprinting alterations as well as other problems than twins or triplets[111].

7. Epimutations in the genome originated by the use of ART that is manifested long-term and/or are transmitted to the following generations

There are health problems that appear in the long term. Consequently they are not detectable by the systematic studies that analyse health at birth or at short term of the life of those born. Some early defects prevail throughout life such as asthma or allergic diseases[112]. And actually there is evidence that epigenetic mutations in those born by ART generate alterations in the long run[113].

It is important to point out that epimutations can be inherited from the gametes or can be generated *de novo* in the first stages of embryonic development. Epimutation is generated in the processes of life transmission, although the corresponding defect will appear in the short or long term. The association between environmental influences and disorders in the origins of each individual[114] is well established.

In fact, in the differentiated cells of the organism, epigenetic reprogramming is very reduced and generally stable: 80% of the places of methylation remain methylated[115]. The effect of the possible epimutation can appear later on and, thus, affect the function of the corresponding organ that is not transmitted to the following generation unlike epimutations of germlike cells that can be transmitted, although the individual in which it has been generated has normal characteristics or phenotype[116].

7.1 *Cardiovascular problems due to ART implementation.*

Studies on mice[117] have documented that the ART technologies, especially the culture medium, alter the methylation patterns and with it gene expression, associating it with vascular dysfunction and hypertension in offspring.

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Beforehand, it has been described that this alteration can be directly linked to several factors[118]. In 2010 it became known that there is three times more risk of congenital heart defects, in the long term of those generated by INF/ICSI with respect to those naturally conceived[119]; the type of controls and variables used in this paper suggest that this problem is not related with factors of the parents but with the techniques themselves. However, in 2012 not only the risk was confirmed, in the long term for cardiovascular disease but also that the cause is the exposure of the *in vitro* embryo to an adverse environment[120]. They prove the incidence in children between the ages of 8 to 18 years old conceived by IVF techniques with generalized vascular dysfunction by the structural and functional characteristics that are linked to a higher risk of cardiovascular occurrences at adult age. For this, they have taken as a case-control study 65 youths that were generated *in vitro*, their brothers that were naturally conceived and also others engendered after the mother had ovarian stimulation treatment, vital for *in vitro* fertilization.

7.2 *Other epimutations*

Recent studies suggest that changes, almost inestimable of the methylation pattern, can be produced in whatever place of the genome, in those generated by ART and be passed

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on to the offspring[121]; apart from imprinting gene epimutations[122] and selective loss of methylation[123].

Several studies show that, sometimes, adverse conditions in prenatal life can later on be linked with the development of chronic diseases in adulthood[124]. For example, it has been described that the offspring of parents exposed to famine in the conception stage, show altered genes with imprinting as the growth factor derived from insulin[125].

Gene epimutations, after ART, has been associated to cardiovascular and metabolic alterations and could have future implications: changes in blood pressure, increase in the triglycerides, in fasting high glucose, an increase in the fat tissue and the increase in the incidence of the primary sub-clinical hypothyroidism. Adolescent girls conceived by IVF have hormonal imbalances[126]. These changes can result in a predisposition for illnesses such as diabetes type II, obesity and cardiovascular disease[127]

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8. Concluding

The information dealing with the frequency of malformations, chromosomal alterations, rare syndromes and a large etc., short and long term, in those born with the intervention of some of the processes included in the so-called Assisted Reproduction Techniques, are a call for attention, that demands a responsibility on behalf of the professionals and of the damage control systems. It requires an urgent and careful investigation of the health of those already born and detailed information to those that turn to ART as well as to society.

The implementation of whatever assisted reproduction technique first demands a thorough diagnosis on the sterility or infertility of the couple. The health risks on the offspring depend only partially on the quality of the gametes. In other cases the techniques are the sole causes of the alterations.

The masculine factor is mainly of genetic origin both by gene mutations as by epimutations. These alterations pass on to the offspring when the fertilization is forced by the injection of a spermatazoid into the cytoplasm of an ovum (ICSI). This technique is more invasive and the one that greater disorder originates so much so in the short term as in the long term and causes a grave intergenerational problem. ICSI should be advised against, even denied, if the diagnosis of masculine infertility is genetic. However, it is the most used not only in the case of infertility but also by indication of the protocol when a genetic diagnosis will be carried out before embryo implantation.

The risk of one of the techniques is greater the more invasive they are. Elevated is the number of epigenetic alterations (regulatory of the natural expression of the embryo's genome) that result from the exposure to an artificial environment so much so from the oocytes obtained by ovarian stimulation, as the culture medium of the fertilized ova, or the freezing-thawing and resuscitation of the embryo prior to implantation in the maternal uterus.

All this as has been reviewed beforehand has direct consequences in the short, medium and long term in the life of an individual generated by the techniques. Even when they are hereditary they can cause an intergenerational problem.

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