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Investigating the significance of segmental aneuploidy findings in preimplantation embryos

--Manuscript Draft--

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We would like to thank the reviewers for their thoughtful comments and efforts towards improving our review. In response to their comments, we have incorporated a number of changes that are listed below.

**Reviewer comments are in black. Authors responses are in blue.*

Major:

-A section early in the manuscript specifically focused on the underlying biology of how 'true' segmental ANP may occur, including a brief review of meiosis and mitosis in the oocyte/zygote/embryo may make the review more accessible to a wider readership. The authors allude to how SAs are likely to be predominantly mitotic in origin throughout the manu, and a basic review of these processes and the biologic plausibility for why mitosis (as opposed to meiosis) lends itself to segmental anp will aid the reader.

Thank you for pointing this out. A new section has been added to briefly introduce this concept, named "Segmental Aneuploidies arise during cell division" before the "Etiology..." section where the molecular mechanisms are explained.

-In the abstract as well as under the prenatal dx header in the body of the paper, the authors describe having conducted a useful review of the literature, which found that the 'incidence' of SA in preimplantation embryos is higher that in prenatal diagnosis. Recommend that the authors provide more detail about how this systematic review/meta-analysis was conducted and to discuss potential confounding variables that could account for this disparity.

Please see response to next comment.

-Overall, recommend that the authors elaborate on their methodology for this review.

Thank you for sharing this with us. We agree that the methodology behind the literature review had to be described. In order to do so we have added a section named "Methods for search strategy and study selection", right after the introduction.

-At its core, this paper is an expert review. While SA remains an area of active research and much remains unknown (as the authors note), it would be helpful to the reader if, in the conclusion section, the authors offer their interpretation of how blasts deemed SA be handled at present, given the available evidence.

We appreciate your feedback. As a matter of fact, we have expressed an opinion but as you mentioned we have never specified it was what we, as authors, conclude to be the best "course of treatment" (see line 49 of the 1st submission).

We previously proposed that rebiopsies can be used to rule out the presence of a meiotic/uniform SA in the embryo. However, there is still a lack of clinical experience/trials to back up any of the potential practices. As a result, while avoiding any specific recommendations, in this work we have provided readers with a comprehensive overview of

all potential scenarios when dealing with a SA finding. Please see 1st submission line 44-49 for reference.

-Lastly, the authors should comment on what is known re neonatal outcomes of children born following dx of SA at the preimplantation and/or prenatal diagnosis stage. Even if nothing is known/reported in the available literature, this should be clearly stated.

While outcomes following a segmental finding during PGT or PND had already been discussed in the text, it was indeed only a brief discussion due to a limited amount of reliable data. Accordingly, we have reconsidered being more specific in response to your request. Therefore, we have decided to include a paragraph in the "Conclusion" section to discuss the implications of such findings on gestational and neonatal outcomes, as well as the limitations we continue to face when dealing with SA due to technical limitations and a scarcity of unbiased but powered studies on the subject. Thank you very much. Please see below 1st submission line 52 for reference.

Minor

-If using SA as abbreviation should define at first instance then use throughout. This is inconsistent in abstract/bullets.
-Bullet points line ~45. The authors likely mean 'predominantly' not 'prevalentaly'
Intro:
-Line 1, consider 'a leading cause' rather than 'the leading cause'
-Line 10, consider adding SNP-array here.
Body of paper:
Incidence of SA in PGT section
-In the first paragraph: in the context of PGTA the authors should clarify that they do not mean PGTA can determine that all the cells in the embryo have the SA, but rather only that all the cells in the biopsy do.
-In the 3rd paragraph, line 40 recommend to state that the data show SA is detected/reported in (since we don't actually know what percentage this phenomenon 'occurs in.'
-Similarly in the next paragraph, line ~57, would 'this finding' (or similar) rather than 'their occurrence'
-Will not comment further on this line of edits; however, the author should consider minor revisions throughout to avoid suggesting that PGTA findings entail true biologic incidence. I think is done
-same section, last paragraph - consider use 'along its length' rather than 'his'
Parental origin section:
-This sentence is unclear: "In fact, SA seems to be more frequently affecting those chromosomes inherited by the father instead of the mother (17,38)." Do the authors mean 'from the father'?
Concordance section:
-For this statement, the authors the authors should clarify whether they are recommending re-biopsy be done clinically or for research purposes: Nonetheless, findings from numerous studies, including non-selection prospective transfer of mosaic embryos, indicate that this is the best approach for determining the mitotic origin of chromosomal abnormalities.
WCA section
-for the following sentence 'More specifically, given that they are not found to be present in more than one biopsy, they seem to be prevalently mitotic in origin (15,51)'—suggest

"predominantly" or ":mostly" rather than "prevalently"
-consider renaming this section, since the paper is on SA not WCA
-This sentence is unclear. Recommend stating differently. Also, the authors may consider clarifying whether they are recommending re-biopsy be done clinically or for research purposes: "Given what is stated above, the best case scenario to obtain
a more reliable diagnosis if a SA is detected seems to be to proceed with a re-biopsy of the embryo to enhance the predictivity of the ICM chromosomal constitution"

Prenatal dx section

-Line 10 second page comma is misplaced should be after 'document'
Line 29 second page 'CNV' needs to be defined

Conclusion

-Line 41 recommend 'attributed to' or similar rather than 'accompanied by'

Thank you once more for bringing this to our attention. All minor revisions have been modified according to the reviewer's suggestions. Instead of simply changing the verb, the sentence in line 41 was rewritten to better convey the meaning.

Running Title: Segmental aneuploidies in preimplantation embryos

Title: Investigating the significance of segmental aneuploidy findings in preimplantation embryos

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Disclosure Summary:

Ludovica Picchetta and Antonio Capalbo are full time employee at Juno Genetics, Rome, Italy

Christian Ottolini has a fellowship at University college London and is an employee at The Evewell (Harley Street) Ltd and The Evewell (West London) Ltd.

Helen C. O'Neill is an employee at University College of London and Hertility health; has board membership and stock options for Hertility health and a grant with Innovative UK.

Abstract:

Segmental aneuploidies (SAs) are structural imbalances, namely gains or losses, involving a chromosomal segment. Most preimplantation genetic testing platforms can detect segmental imbalances greater than 5-10 Mb, either full or mosaic, however questions remain about clinical significance. An in-depth review was carried out to determine the accuracy, frequency, and types of SAs detected in preimplantation embryos. A comprehensive search of the literature revealed an incidence of around 8.15% in preimplantation embryos, compared to a prevalence of 3,55% in prenatal diagnosis samples. Several studies have used rebiopsy analysis to validate the accuracy and reproducibility of such findings in blastocyst stage embryos. A comparison of these studies yielded an average confirmation rate of SAs slightly above 30%. This result could be attributed to their mitotic origin as well as to the technical limitations of PGT. In addition, the few available studies in which embryos with a segmental finding were transferred in utero are analyzed to discuss the reproductive competence of such embryos. Except for one study, all outcomes were described for segmental embryos in a mosaic state. As a result, there is still insufficient evidence to provide accurate information about the effect of segmental imbalances on embryonic reproductive competence, as well as to determine gestational and newborn risks.

Key words: Embryo, PGT-A, segmental aneuploidies, incidence, rebiopsy

Bullet points:

- The detection of segmental aneuploidies by PGT-A is highly dependent on the resolution of the platform used for analysis.
- The prevalence of SA in blastocyst stage embryos ranges between 3.10% and 15.60%.
- Segmental imbalances occur as a result of incorrect chromosomal breakage correction, and the mechanisms involved are distinct from those linked with whole-chromosome aneuploidy.
- Segmental aneuploidies are rarely found in subsequent biopsies of the same embryo, suggesting they are prevalently mitotic in origin.
- Mosaic segmental embryos have a reduced but still significant reproductive potential.

Introduction:

Since its introduction at the turn of the century, the use of preimplantation genetic testing for aneuploidy (PGT-A) has significantly increased (1). PGT-A is used to screen preimplantation embryos for chromosomal changes, primarily numerical abnormalities like trisomy 21 or Down Syndrome, with current technologies enabling comprehensive screening of all 23 pairs of chromosomes. The goal of this test is to increase the live birth rate (LBR) and decrease the pregnancy loss rate per embryo transfer by selecting the most competent embryos (i.e., euploid embryos). As aneuploidy is recognized as a leading cause of preimplantation embryonic arrest, failed implantation, miscarriage, and congenital abnormalities of the newborn (2–4) and given that approximately half of the human preimplantation embryos produced using assisted reproduction are aneuploid (and that this rate increases proportionally with maternal age), advancements in PGT have facilitated improved IVF outcomes (5,6). Because of this, whole chromosome aneuploidy (WCA) detection following in vitro fertilization (IVF) has received a lot of attention in recent years, with a wide range of techniques, including FISH, qPCR, SNP/CGH-array and most recently Next Generation Sequencing (NGS) being used in its identification (7).

The development of NGS, along with the routine introduction of trophectoderm biopsy that allows for more starting material for DNA extraction, has enabled the detection of a broader range of chromosomal abnormalities (both structural and numerical) in the preimplantation embryo, overcoming the limits of previously used technologies for PGT-A that were only capable of detecting whole chromosome aneuploidy (8–10).

Because NGS allows for improved resolution and sensitivity in PGT, it is now possible to increase the diagnostic capabilities of PGT-A by detecting more subtle chromosomal changes such as mosaicism and sub-chromosomal copy number aberrations (11,12).

Currently, mosaicism is the third most common chromosomal status seen on a PGT-A report, following euploidy and aneuploidy (13,14). It is defined as the presence of two or more distinct cell lines with divergent chromosomal makeup within the same

organism, in this particular instance the embryo. While chromosomal copy number values thresholds are clearly set for full monosomy (one copy), disomy (two copies), and trisomy (three copies), copy number values for mosaicism fall in the intermediate ranges between 1 and 2 or 2 and 3.

As mentioned above, subchromosomal copy number changes or segmental aneuploidy (SA) is now detectable in PGT-A by NGS (or high-density array-CGH and high resolution SNP-array) (15,16). SA occurs when only a portion of a chromosome is lost or gained from the genome. These structural aberrations can either be de novo changes as a result of a meiotic error during gametogenesis, or a mitotic error during embryo cell divisions, or they can be inherited from a parent carrier of a structural abnormality such as reciprocal translocations or inversions(17). When the error occurs during the meiotic divisions of oogenesis and spermatogenesis, every cell of the future embryo will have the same abnormality and therefore be uniformly aneuploid. If the error occurs during the early mitotic divisions of the embryo, it will have two or more distinct cell lines with only a percentage of the cells having the segmental deletion or duplication, resulting in a mosaic state.

Whole chromosomal mosaicism has been and continues to be, a contentious topic in clinical embryology. Recently a number of studies, including some non-selection trials, have been published, providing more insight into the reproductive competence of mosaic embryos (18,19). However, there is still a lot of debate about SA owing to the small number of studies on the subject. Furthermore, the true fate of embryos where a SA has been identified by PGT-A is unknown. In fact, to date there have been few reports on the clinical outcomes and reproductive competence of embryos with SA. Additionally, all of these investigations only account for SA in a mosaic state rather than those that appear to affect an embryo uniformly. As a result, SA currently poses a unique diagnostic challenge in PGT-A.

This review sought to investigate the currently available literature to shed a light on segmental imbalances, their origin, incidence, and clinical implications.

Methods for search strategy and study selection:

A systematic search of the literature was performed in the databases of PubMed/Medline and Google Scholar, limited to articles published in peer reviewed journals up to November 2022. The search strategy included the following keywords and their respective combinations: “In Vitro Fertilization”, “IVF,” “Segmental Aneuploidies”, “Segmental imbalances”, “Deletion and duplications”, “Copy number variations- CNV,” “Preimplantation Genetic Testing”, “PGT-A”, “Chromosome abnormality”, “Aneuploidies”, “Outcome”, “Transfer”, “Concordance Rate”, “Prenatal diagnosis”, “Amniocentesis”, “Blastocyst”, “Biopsy”, “Chorionic villus sampling”, “Concordance rate”, “Embryo transfer”, and “Blastocyst”. The articles were then chosen based on various criteria related to the topic of review for which they were required. Selected papers for the evaluation of the frequency of a segmental finding during preimplantation genetic testing had to meet the following criteria: the platform had to be either NGS or CGH/SNP array, at least 300 blastocysts had to be analyzed, and they

had to be published between 2017 and 2022. To reduce the risk of technical biases, only papers using blastocyst stage embryos or embryo outgrowth and NGS as a platform for PGT analysis were investigated for the concordance rate in PGT analysis. In terms of the prevalence of SA in prenatal diagnosis, only papers with a minimum sample size of 400 were considered.

Incidence of segmental aneuploidies in PGT:

When a small region of a chromosome is lost or gained during cell division, segmental abnormalities, also known as segmental or partial aneuploidies, occur. This aneuploidy subtype can be found in two states: full, when all of the biopsied cells have the same chromosomal alteration and the copy number (as defined by the PGT-A assay) equals 1 or 3 (i.e., loss or gain), and mosaic, when the copy number has an intermediate value as a result of at least two karyotypically distinct cell lines within the biopsy specimen.
SA detection by PGT-A is highly dependent on the resolution of the platform used for the analysis. Usually, the average detectable size is 5-10 Mb and above (15,20). However, Lin and colleagues' recent study reported the detection of segmental losses and gains as small as 1 Mb using a targeted NGS-based platform (21). The authors were able to investigate SA below the standard resolution limit of 1Mb of most PGT-A platforms using a prior validated 1 Mb resolution NGS-based PGT assay. The detected sizes differed between inherited and de novo mutations. If inherited, the average length of SA was 1.7 ± 0.9 Mb. If SA occurred de novo, the average detected size was 5.05 ± 2.9Mb. Nonetheless, to date a platform with an accurate resolution limit below 10 Mb is more of an exception than the rule and as a result, the incidence of SA in preimplantation embryos is extremely variable (20,22,23).

According to a thorough review of the most recent literature, the prevalence of SA in blastocyst stage embryos fluctuates from 3.10% to 15.60%. Taken together, data from the last 5 years show that SAs are detected in approximately 8.51% of biopsied blastocyst stage embryos (*Table 1*).
This analysis included all embryos with SA, regardless of mosaic/full status or the presence of additional aneuploidies. However, the percentage of embryos with SA but no other chromosomal abnormalities, according to Girardi et al., is supposed to be much lower particularly when looked at in the typical population of patients undergoing PGT-A for advanced maternal age (24). In fact, after excluding all embryos with other aneuploidies and accounting for uniform SA only, the final incidence of SA was as low as 2.4% (vs 8.03%) in their work, accounting for less than 1% of their embryonic cohort of advanced maternal age women.
Unlike whole-chromosome aneuploidies (WCA), SA has no positive correlation with increasing maternal age (25–27). Interestingly, neither maternal nor paternal age influences their finding. Furthermore, the prevalence of different SA subtypes findings, namely losses and gains, is roughly equal at the blastocyst developmental stage (24). The q arm of chromosome 9 is an unusual outlier, with a high incidence of segmental

gains. It should be noted, however, that both gains and losses generally appear more frequently on the q arm than on the p arm, specifically for medium sized metacentric and submetacentric chromosomes. (26,28). Several papers report varying amounts of segmental abnormalities depending on the chromosome region involved. The distal regions appear to be more frequently affected than the proximal regions (24,28). This could be due to a technical limitation of PGT-A resolution rather than a true reflection of the biology. Indeed, interstitial segmental imbalances (involving proximal regions) detected pre and postnatally are typically less than 10 Mb, falling below the detection limit of the majority of PGT-A platforms. (29). Every chromosome can be potentially affected by SA, but the rate of this phenomenon seems to be associated with the size of the chromosome. Chromosomes with bigger length, such as those belonging to group A and C (e.g., chromosome 1), are more frequently associated to this type of aneuploidies compared to small chromosomes like those of group F or G (e.g., chromosome 19) (26). A few studies have found a remarkably low rate of segmental errors detection on chromosome 19 (26,30). Despite its small size, this chromosome is also the most gene dense chromosome out of all 23. This has given rise to the theory that the low frequency of SAs along its length could be due to an evolutionary mechanism of selective pressure. The low incidence of SA on other chromosomes, such as 21,22, or the Y chromosome, could also be explained by their smaller size and the detection limits of PGT-A.

Segmental Aneuploidies originate during cell division:

Cells in the human body can undergo two types of cell division: mitosis and meiosis, with the latter being limited to the process of gametogenesis (31). During oogenesis and spermatogenesis, primordial cells go through two meiotic divisions, known as meiosis I and meiosis II. Following one DNA replication prior to meiosis I and two consecutive cell divisions, the genome content goes from 2N or diploid to N or haploid in the final oocyte/sperm. Each sperm and egg contain a haploid nucleus that fuses during fertilization. Once the egg and sperm have joined their membrane and fused their pronuclei, the rising zygote will have a 2N content of DNA and will start undergoing a series of mitotic divisions. During mitosis each DNA molecule is replicated and segregated into two daughter cells only once, implying that the DNA content remains unchanged throughout the process. Errors in both types of cell division can cause SA. The percentage of affected cells in the future individual will be the main outcome difference depending on cell division of origin. If a SA forms during meiotic cell division, the rising gamete, either an oocyte or a spermatozoon, will be impacted. Following fertilization, the zygote will inherit everything that is present in the parental genome, including the SA.

After the zygote formation, DNA is duplicated and segregated via mitotic cell divisions into new embryo cells or blastomeres, propagating the chromosomal abnormality in every cell of the embryo. Exceptionally, a meiotic error can be rescued during the mitotic cell division of the embryo via a so-called self-correction. A zygote with a chromosomal abnormality undergoes mitotic division, during which a second error occurs on the same chromosome or chromosomal position, restoring the normal euploid configuration in all

or in part of the embryonic cells (32–34). Nonetheless, a segmental imbalance can occur after a normal haploid oocyte is fertilized by a normal haploid sperm. Following pronuclei fusion, the rising zygote will be a normal euploid. However, if an error occurs during mitotic embryonic division, some cells of the embryo will have the SA while others will not. The percentage of affected cells is highly dependent on the timing of the error and is extremely difficult to predict. The latter situation is what scientists commonly referred to as mosaicism. Indeed, meiotic errors frequently lead to uniformly aneuploid embryos, whereas mitotic errors usually result in a mosaic embryo with two or more cytogenetically distinct cell lines. The main characteristic of true mosaic SA is the presence of reciprocal aneuploidies within the same embryo biopsy, which unequivocally suggest its mitotic origin (see below).

Etiology of segmental aneuploidies:

Whole-chromosome aneuploidies are the most frequent abnormality detected in PGT-A(35). They are frequently reported in a full state due to meiotic segregation errors during oogenesis. Non-disjunction, premature separation of homologous chromosomes or sister chromatids and reverse segregation are all examples of these errors(36–38).
On the other hand, SA arises as a result of erroneous chromosomal breakage correction, and the mechanisms involved are distinct from those associated with aneuploidy of an entire chromosome. They often happen as a consequence of a fault in the repair mechanisms of double strand breaks (DSB) of DNA which are one of the most toxic lesions and must be repaired to preserve chromosomal integrity. The cell has a number of mechanisms for repairing such breaks, including homologous recombination, gene conversion, and break-induced replication(28,39). Although these processes are necessary for cell survival, they are not error-free. As a result, if a cell repairs the DSB incorrectly, the segment containing the break can be duplicated or deleted, culminating in SA. Furthermore, DSBs can also form as a result of exposure to a variety of endogenous and exogenous factors, including replication fork stalling, oxidative stress, and mutagens (39,40). So far, no research has investigated a possible link between exogenous sources and the occurrence of segmental alterations in IVF-derived preimplantation embryos.

Parental origin of segmental aneuploidies:

Aneuploidies' origin can be investigated through the study of genotyping information. When DNA samples from both parents are available, their genetic variations (usually in the form of single nucleotide polymorphisms or SNPs) can be compared to those of a blastocyst stage embryo to identify the parent of origin of any aneuploidy.
Many studies have proven that aneuploidies involving an entire chromosome are mainly maternally in origin, around 90% (319/357) according to Kubicek and colleagues (17). Specifically, the vast majority of meiotic errors happen during the first meiotic division of female gametogenesis. Furthermore, when plotted against maternal age,

chromosomal aneuploidies appear to follow a U curve (41); meaning that the risk of producing aneuploid embryos slightly decreases from menarche through a woman's 20s, then rapidly increases in women of 35 years of age and older. Once again, in contrast to whole chromosomal aneuploidies, for SA no relationship was observed between maternal or paternal age and the incidence of these abnormalities (17,42). On the other hand, segmental imbalances seem to have somewhat of a gender correlation. In fact, SA seems to be more frequently affecting those chromosomes inherited from the father instead of the mother (17,37). That could be explained by the high incidence of topoisomerases mediated DSBs in spermatozoa DNA to promote the substitution of histones with protamine, making the male germline more vulnerable to erroneous repairs (43,44).

Concordance rates of segmental aneuploidy- re-biopsy studies and cell division of origin:

Re-biopsies and multifocal analysis of blastocysts can be extremely useful in distinguishing between cell divisions of origin of specific chromosomal alterations. Embryo re-biopsies can indeed explain these phenomena by determining how frequently the result of a single TE biopsy is corroborated by subsequent biopsies of other parts of the embryo. When the same abnormality is found in all biopsy samples, it is said to be uniform or meiotic in origin. Although being uniformly present in all cells of an embryo or in all trophectoderm biopsies collected from the same blastocyst is not an assured proof of meiotic origin, this is a common assumption employed in multifocal studies to assign cell of origin to a multifocal analysis layout. In contrast, aneuploidies with a mitotic origin will be found in a mosaic state due to their biology, which means that the same alteration will not be found in all subsequent biopsies. Identifying true mosaicism origin in multifocal analysis also comes with some drawbacks. While some patterns can be accurately assigned as mosaic, for instance, if reciprocal abnormalities are observed in two different biopsies of the same embryo, this is not always the case. As a result, distinguishing between artefacts and true mosaicism is not always straightforward. Nonetheless, findings from numerous studies, including non-selection prospective transfer of mosaic embryos, indicate that rebiopsy to be the best clinical approach for determining the mitotic origin of chromosomal abnormalities.

The ability of PGT-A to detect SA is not as remarkable as it is for whole-chromosomal aneuploidy.

Concordance rates for full whole chromosomal aneuploidy, both monosomies and trisomies, are extremely high and usually exceeding 98% confirmation rate, with a single biopsy having a high predictive value for the constitution of the rest of the embryo (11,45). Indeed, the high concordance rates for whole chromosomal aneuploidy not only confirm the high diagnostic power of testing platforms applied to PGT-A but also demonstrate the meiotic origin of the vast majority of these numerical alterations. Recent studies have investigated karyotype concordance rates in case of positivity for the presence of a SA among clinical TE biopsies and either a second biopsy of the same

cell line or from the ICM (14,24,45–50). However, when it comes to detecting SA, the concordance rates for different biopsies, such as inner cell mass and multiple trophectoderm biopsies, are not so strong (Table 2) as those reported for WCA (11,24,46,47). The findings of these studies suggest a different etiology for sub-chromosomal alterations. More specifically, given that they are not found to be present in more than one biopsy, they seem to be mostly mitotic in origin (15,51). A review of the last five years' literature, including studies using NGS as a technique for PGT-A, indicates a variable karyotype concordance rate for SA from multiple embryo samples with an average just slightly above 30%. This evidence supports the theory that segmental errors are more frequently mitotic in nature, arising during the first embryonic cell divisions. Corroborating this hypothesis, both different aneuploidies as well as reciprocal segmental abnormality are often found in multifocal biopsies of blastocyst with a SA. Biologically this is clear evidence of mitotic nondisjunction errors occurring during the first stages of the embryo development. In particular, cleavage stage embryos appear to be more often affected by these errors than blastocysts or oocytes (28). This may be due to the fact that the human embryonic genome is inactive during the first few mitotic divisions, and the early stage of embryo development may be more susceptible to these chromosomal alterations due to the speed of mitosis and the impairment of cell cycle checkpoint mechanisms. Furthermore, new research suggests that early embryonic bottlenecks exist (52). It is possible that when the zygote genome is activated it can prevent gross chromosomal abnormalities stimulating bottleneck mechanisms for a negative selection against aneuploid cells. In a study by Babariya and colleagues, segmental abnormalities were found to increase dramatically during the first three days of embryonic development compared to oocytes (28). The incidence of segmental errors would then decline as the embryos kept developing to the blastocyst stage. The decline in SA in the later developmental stage could be supported by a mechanism of apoptosis or cell death, by which affected cells are not incorporated into the embryo as well as by a total developmental arrest of the affected embryo. However, besides the decline at the blastocyst stage, the detection rate of SA was still higher than that found in the polar bodies. This finding further supports the hypothesis that segmental imbalances less often maternally derived, compared to WCA, and are often mitotic in origin.

For this reason, so as to have as little variability as possible, only studies where blastocyst-stage embryos were investigated were included in this review with the exception of one study by Popovic and colleagues where culture was extended further (47). The authors of this paper, instead of focusing on the blastocyst stage, used embryos that were donated for research purposes and cultured in vitro under hypoxic conditions (to resemble the maternal in vivo environment) up until 12 days post fertilization (dpf), as a so-called outgrowth. This is another method, in addition to re-biopsy and multifocal analysis, for studying the embryonic karyotype in as many cells as possible. In their study, they found a concordance rate of 55% for segmental imbalances detected at the initial PGT-A by TE biopsy at the blastocyst stage (5 outgrowths out of 9). Outgrowths of euploid or aneuploid for a whole chromosome were found to have a 100% concordance rate in the same study. Surprisingly, the viable 12 dpf outgrowths

were mostly derived by euploid blastocysts or from blastocysts with trisomies, duplications, or aberrations in a mosaic state (34 out of 44 attached, 77%). Blastocysts with monosomies, deletions, or multiple anomalies, on the other hand, had significantly impaired embryo development and fewer reached the 12 dpf stage (3 out of 29 attached, 10%; P 0.0001). Aside from the proven reliability of PGT-A in WCA, it is important to remember that technical artifacts for mosaicism and segmental imbalances are still possible. This is especially important when it comes to clinical applications of such a tool, as only one TE biopsy is typically available per embryo. Girardi and colleagues developed a prediction model for SA to investigate how a single TE biopsy can represent the entire embryo constitution (24). Segmental imbalances were divided into two groups based on whether confirmation in ICM was obtained or not. A logistic regression analysis revealed that there were two main variables associated with the ICM confirmation rate: the length of the region involved in the imbalance and the result of a second TE biopsy. When a second TE biopsy was available and confirmed the SA finding, the likelihood of a diagnostic concordance increased from 21.4% to 84%. Alternatively, when the segment involved was smaller than 80 Mb in the first TE biopsy, but was not detected in the subsequent TE biopsy, the concordance rate declined to 10,5% from the initial a priori rate of 50,9%. Given the foregoing, re-biopsying the embryo to improve predictability of the ICM chromosomal constitution appears to be the best option for obtaining a more reliable clinical diagnosis if a SA is detected.

Transfer outcomes of embryos positive for segmental aneuploidy:

PGT-A technology is regarded as an effective strategy for whole chromosome aneuploid embryo deselection (3,11). However, how mosaicism and segmental PGT-A results should be interpreted in a clinical setting is still being debated. Several variables must be considered when dealing with a report of mosaicism or segmental imbalance. Biological variables include the imbalanced fragment size, gene content, embryo developmental stage, and percentage of cells containing the abnormality. Technical artifacts can also occur. Genetic artifacts, analytical noise in the PGT-A plot, and sampling bias due to the small number of cells available for analysis are examples of the latter (53). In terms of SA, it has been reported that their detection in single cells is not optimal if the cells are in the S phase of their cell cycle (54). Even using trophectoderm biopsy increases the likelihood of obtaining G0/G1 phase cells, this phenomenon should be considered as a possible source of a technical artifact until proven otherwise.

Several types of clinical studies can be used to investigate the predictive value of PGT-A, each with its own set of strengths and limitations. Non-selection trials provide the most unbiased population selection process, allowing for a more consistent interpretation of the results' reliability(3). This type of investigation has previously been used to uncover hypothetical differences between euploid, aneuploid, and mosaic embryos (18,19). However, there is currently a lack of non-selection studies available to investigate the performances of embryos with segmental imbalances in a full state. In 2021 Tiegs and colleagues published the only multicenter, prospective, blinded non-selection study in which embryos with segmental imbalances were transferred (18). 186

of the 2110 biopsied blastocysts had segmental imbalances, and 39 of them were transferred. The sustained implantation rate of euploid blastocysts transferred was 64.7%. In contrast, none of the 102 full aneuploid embryos transferred survived to implantation. Interestingly, twelve of the 39 embryos with SA reached sustained implantation, for a final sustained implantation rate of 30.8%, highlighting a reduced but still substantial reproductive potential of these embryos.

However, there are more studies available about the outcomes of embryos with a segmental imbalance in a mosaic state (55–59). A retrospective cohort study looked at how segmental mosaicism affected pregnancy outcomes and the live birth rate (55). When compared to euploid controls, the 20 segmental mosaic embryo transfers had a statistically significantly lower LBR (30% vs 53,8 p=0,04) and a statistically significantly higher miscarriage rate (40% vs 18%, p=0,04). These embryos were analyzed both via NGS and via aCGH. 11 out of 20 embryos had discordant results with the two platforms. If only NGS results were to be considered, then the live birth rate of segmental mosaic embryos would change to 42,8%. This shows how impactful the platform used for PGT-A can be on predicting outcomes.

In addition to this, another recent study compared the outcomes of mosaic embryos for whole chromosome or segmental imbalances to those deemed as euploid (58). Their detected LBRs were significantly lower after whole chromosome mosaic embryo transfer than after euploid embryo transfer (43,5% vs 59,1%, p=0,026). However, the rate of live birth did not differ statistically between the segmental mosaic embryo group and the control group of euploid embryos (48,3% vs 59,1%, p=0,26).

Segmental aneuploidies in prenatal diagnosis:

During prenatal diagnosis (PND), chorionic villus sampling and amniocentesis are the main two diagnostic interventions used to collect fetal and/or embryonic annexes cells. The samples can be tested for chromosomal abnormalities, including SA, using many methodologies. The main method used in PND is high-resolution G banding karyotype, but it can only identify fetal abnormalities with a resolution limit of >10 Mb (60). More recently, the investigation of chromosomal imbalances has also started to be performed by chromosomal microarrays such as CGH Array, SNP array, and by NGS(61–63). The application of these modern techniques has allowed for an increased detection of cryptic copy number variations regardless of clinical indications for the analysis.

In general, the frequency of SA in the post-conception stage (prenatal and postnatal) is extremely low. A review of recent publications investigating the prevalence of segmental imbalances in prenatal samples (i.e. Chronic villus samples; amniotic fluid; products of conception) was performed entering keywords into PubMed and google scholar (16,64–71) (Table 3). The incidence of segmental duplications and losses was found to be far lower than that detected in preimplantation human embryos in all of the papers. The average incidence obtained from the publication is roughly over 3.5%. Nevertheless, as already mentioned earlier in this document, chromosomal instability (CIN) is a common event in human preimplantation embryogenesis, probably explaining the high detection frequency of such aberrations in PGT-A (51,72) but not in PND. Cell

lineages containing chromosomal abnormality have been speculated to survive in the trophectoderm, having little to no influence on the inner cell mass and therefore on the embryo/fetus (73). This preferential allocation is also supported by recently published works reporting the birth of euploid mice after the transfer of genetically produced mosaic embryos and of lineage specific fate in chimeric mouse embryos where aneuploid cells go through apoptotic depletion in fetal lineages whereas only slow down their rate of division in the placental lineage (74–77). It is noteworthy, that there is no compelling evidence for an increased rate of segmental imbalances in children born after in vitro fertilization treatments (78). A recent paper by Esteki investigated de novo DNA copy number variations in DNA samples extracted for cord blood cells and placental tissue, from both IVF and naturally conceived pregnancies (79). The authors detected no differences in the incidence of de novo copy number variations (CNVs) between the naturally conceived pregnancy and the IVF-derived one. Furthermore, also when comparing IVF pregnancies with frozen or fresh transfer the same proportion of segmental samples were found. This suggests that neither the IVF procedure nor the type of transfer influence the frequency of segmental imbalances.

Conclusion and future perspectives:

As things currently stand, PGT specialists are particularly interested in SA of the preimplantation embryo. As far as SA, there is still uncertainty because these finding is surrounded by technical limitations and biological peculiarities that necessitate additional research to accurately report and inform patients about the true implications. Given the possibility of a mixed meiotic and mitotic origin, technical artifacts such as whole genome amplification issues, sampling bias, analytical noise, and S-phase artifacts, to date, the best way to process an embryo with a segmental finding in a clinical setting would be to request a re-biopsy for analysis (24,54). Despite their marginal contribution to PGT-A findings and studies proving that the incidence of segmental imbalances is not enhanced by IVF procedures, additional investigations on these imbalances are highly demanded (79).

SA as in partial chromosomal deletions and duplication are also defined CNVs which contribute significantly to individual genome variability. There is a wide spectrum of clinical outcomes associated with a wide range of phenotypes, ranging from polymorphic traits with benign to no clinical consequences, to embryonic lethality, miscarriage, and clinically recognized genetic syndromes. (26,55,80). With over 200 recurrent syndromes identified and a prevalence ranging from 1:100 to 1:25000, both chromosomal gains and losses have been recognized as the genetic cause underlying syndromic diseases (81).

Deletions reportedly result in more significant clinical features, such as intellectual disability and dysmorphic traits. Cri du Chat syndrome, caused by a deletion of the short arm of chromosome 5, Wolf-Hirschhorn syndrome, caused by a deletion on chromosome 4, and Di George syndrome, caused by a deletion on chromosome 22 are instances of such well-characterized syndromes (82–84).

Prenatally, segmental imbalances are quite rarely detected, even though their prevalence is enhanced if the fetus has ultrasound markers (61). Our current understanding of the impact of a segmental finding on neonatal outcome suffers from a selection bias. Indeed, during invasive prenatal diagnosis, a genetic test aimed at detecting SA is frequently performed only when a positive family history or ultrasonographic markers are present. Furthermore, the availability of repository databases where these imbalances are classified according to neonatal/implantation outcomes and miscarriages would be an important resource for the clinical management of SA. There is currently a lack of this type of database, specifically for PGT, which compromises clinical interpretation of SA even more. Future non-selection studies where the embryo is biopsied prior to the transfer but the result is disclosed only afterward, are required to help to elucidate the clinical impact of segmental imbalances on embryonic reproductive potential and gestational consequences.

Time-lapse microscopy (TLM) has seen significant success in assisted reproduction laboratories over the last ten years. TLM is a non-invasive tool that allows for the dynamic and continuous evaluation of the development of preimplantation embryo development. Chromothripsis and the formation of micronuclei are emerging phenomenon, that have been proposed as possible causes of SA formation in preimplantation embryos (85–87). TLM can be used to analyze a wide range of cleavage and morphokinetic parameters, both qualitative and quantitative, ranging from the study of cytoplasmic movements to fertilization events or the modalities and timing of cell divisions up to blastocyst formation (88,89).

TLM and morphokinetic parameters could indeed aid in the identification of micronuclei formation or really any other possible difference between the development of embryos with segmental imbalances (likely mitotic) and euploid/uniformly aneuploid embryos (meiotic).

Furthermore, because the discrimination of meiotic/mitotic origin based solely on multifocal biopsies may not always be a perfect estimation of reality, genotyping data could be used to improve this prediction. By comparing SNPs along the genome and using bioinformatic tools, it is possible to distinguish not only the parent of origin, as previously described in this text, but also between the mitotic and meiotic origin(17,37,38).

More information about the pathways of a developing embryo with a segmental imbalance will undoubtedly be required in order to define better criteria for improving bioinformatic algorithms used to provide a definitive diagnosis. Organoids in preimplantation genetics could be very useful in this regard. Blastoids, which are blastocyst models formed by stem cell self-organization, specifically mimic the pre-implantation stage of embryo development (90,91). Blastoids are models that cannot be used for direct reproduction or transfer; however, they are an ethical alternative for research purposes and may guide scientists through the discovery of hypothetical self-deselection of karyotypically imbalanced cells from the embryo, among many other possibilities (76).

New findings in this area will surely contribute to a deeper understanding of preimplantation embryo development and will aid in assessing the reproductive potential of embryos with full SA in order to rule out any potential negative effects on gestation and newborn health.

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Tables:

INCIDENCE OF SA IN BLASTOCYST STAGE EMBRYOS				
Reference	**Total n. of Blastocysts**	**SA Positive Blastocysts**	**Incidence of SA**	**Platform**
McCarty et al., 2022(27)	89226	2766	***3,10%***	NGS
Babariya et al., 2017(28)	1327	207	***15,60%***	CGH-Array
Tiegs et al., 2021(18)	2110	186	***8,82%***	tNGS
Escriba et al., 2019(26)	3565	299	***8,39%***	NGS
Zore et al., 2019(31)	377	20	***5,31%***	CGH-Array
Kubicek et al., 2019(17)	967	54	***5,58%***	Karyomapping
Rechitsky et al., 2020	14992	2099	***14,00%***	NGS
Girardi et al., 2020(24)	8137	653	***8,03%***	NGS
Nair et al., 2022(32)	1501	79	***5,26%***	NGS
Walters-Sen et a., 2022(33)	182827	20557	***11,24%***	FAST-SeqS
Coll et al., 2021(34)	1708	97	***5,68%***	NGS
Zhou et al., 2018(25)	2095	206	***9,83%***	NGS
Xie et al., 2022(21)	15411	2273	***14,75%***	NGS
Dviri et al., 2020(35)	3118	104	***3,34%***	NGS
Insua et al., 2018(30)	3628	314	***8,65%***	NGS
Average Incidence of SA	-	-	***8,51%***	-

Table1*. Incidence of segmental aneuploidy in blastocyst stage preimplantation embryos.*

CONCORDANCE RATE OF SA IN PGT-A				
Paper	**Platform**	**Embryo stage**	**Concordance Rate**	**Absolute values**
Chuang et al., 2018(46)	NGS	Blastocyst	55,50%	5/9
Popovic et al., 2019(47)	NGS	Outgrowth 12 dpf	38,46%	5/21
Victor et al., 2019(48)	NGS	Blastocyst	44,40%	4/9
Lawrenz et al., 2019(49)	NGS	Blastocyst	16,70%	1/6
Navratil et al., 2020(45)	NGS	Blastocyst	36,80%	14/38
Girardi et a., 2020(24)	NGS	Blastocyst	32,10%	17/53
Sachdev et al., 2020(50)	NGS	Blastocyst	0,00%	0/12
Kim et al., 2021(11)	NGS	Blastocyst	21,30%	36/196
Average Concordance rate	-	-	**30,66%**	-

Table 2. *Concordance rate of Segmental aneuploidy (SA) findings in PGT-A.*

PRENATAL INCIDENCE OF SA				
Reference	**Analyzed samples**	**SA Positive samples**	**Incidence of SA**	**Platform**
Breman et al., 2012(63)	1115	43	***3,80%***	CMA
Farcaș et al., 2013(64)	528	12	***2,27%***	Karyotype/FISH
Levy et al., 2014(65)	1861	43	***2,30%***	SNP-Array
Shen et al., 2016(66)	436	23	***5,30%***	CGH-Array and NGS
Sahoo et al., 2017(67)	7396	181	***2,40%***	SNP-Array/CGH-Array
Wang et al., 2018(79)	3398	41	***1,20%***	CNV-Seq
Peng et al., 2019(68)	836	40	***4,80%***	CGH-Array
Lin et al., 2020(69)	10377	223	***2,10%***	SNP-Array
Kowalczyk et al., 2022(70)	7400	579	***7,80%***	CGH-Array
Average Incidence of SA	-	-	***3,55%***	-

Table 3. *Incidence of segmental aneuploidy in prenatal diagnosis. Only studies with over 400 cases were investigated.*

Declaration of interest

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Supplementary Material

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