The mitochondrial component of human infertility- an increased mtDNA content and an excess of ultra-rare mtDNA variants in aneuploid versus euploid embryos.

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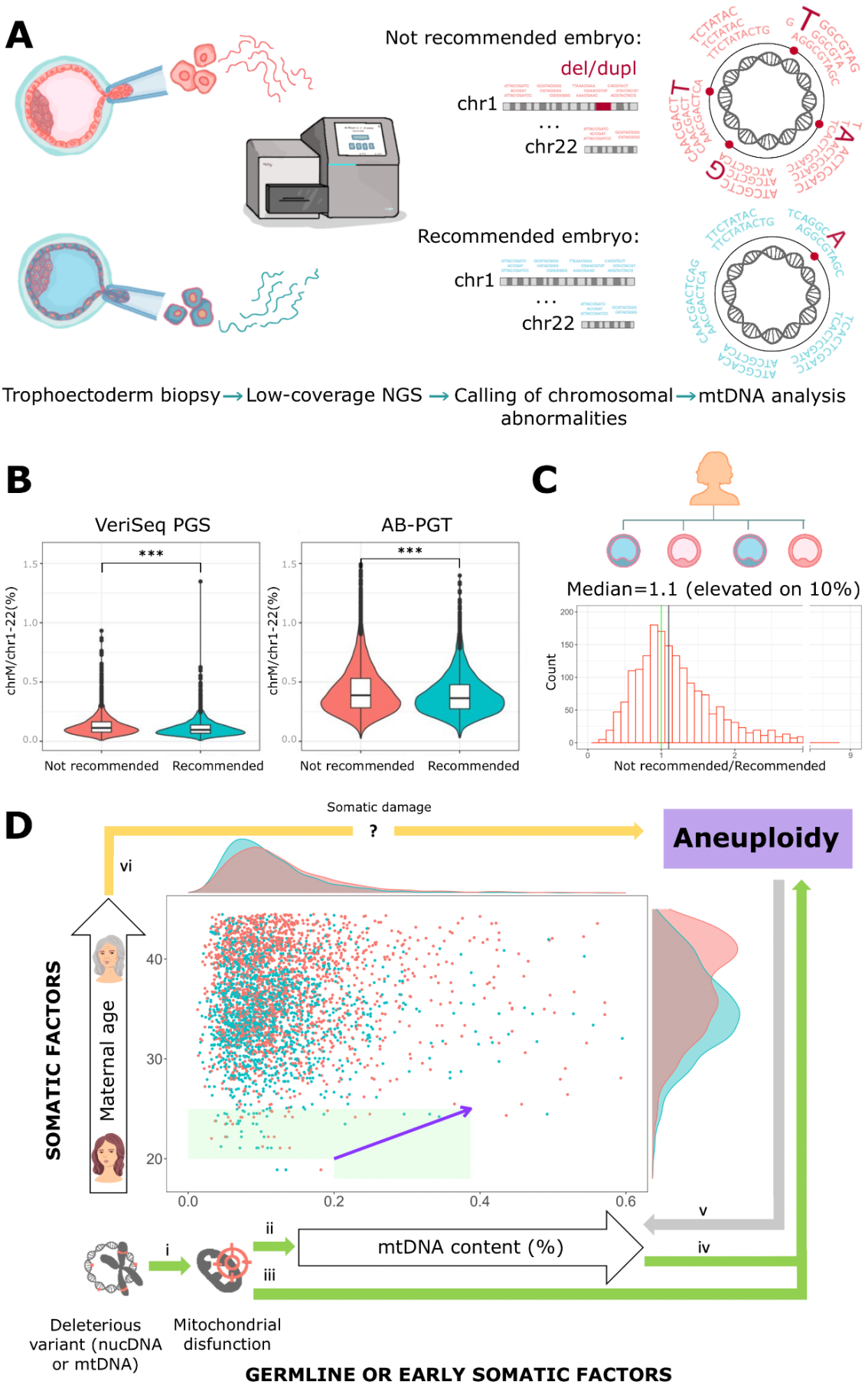
A
Not recommended embryo:
del/dupl
chr1
...
chr22
Recommended embryo:
chr1
...
chr22
Trophoectoderm biopsy → Low-coverage NGS → Calling of chromosomal abnormalities → mtDNA analysis
B
VeriSeq PGS
AB-PGT
***
chrM/chr1-22(%)
Not recommended
Recommended
C
Median=1.1 (elevated on 10%)
Count
Not recommended/Recommended
D
Somatic damage
?
Aneuploidy
vi
SOMATIC FACTORS
Maternal age
mtDNA content (%)
i
ii
iii
iv
v
Deleterious variant (nucDNA or mtDNA)
Mitochondrial disfunction
GERMLINE OR EARLY SOMATIC FACTORS

Figure 1

A. Graphical abstract of the study. We performed low-coverage whole genome sequence (WGS) of biopsied trophectoderm of 11610 morphologically good quality embryos as a preimplantation diagnostic for aneuploidy. Chromosomal status has been annotated for each embryo as euploid (recommended to transfer) or aneuploid (not recommended to transfer). Mitochondrial analyses revealed two additional characteristics of not recommended embryos: (i) an elevated level of mtDNA content (expressed as a percentage of reads mapped to mitochondrial as compared to the nuclear genome) and (ii) an excess of ultra-rare potentially deleterious mtDNA mutations (marked by red dots on mtDNA).

B. An increased mtDNA content in not recommended versus recommended embryos on the populational level. mtDNA content was higher in not recommended to transfer embryos versus the recommended ones. For VeriSeq PGS median for not recommended was 0.112 and for recommended 0.096, (p-value=1.92E-15, Mann-Whitney U test); for AB-PGT median for not recommended was 0.388 and for recommended 0.364 (p-value=8.56E-12, Mann-Whitney U test). Additional analyses confirmed the robustness of this trend and its independence of the maternal age (see SM1-SM12).

C. An increased mtDNA content in not recommended versus recommended siblings. The pairwise comparison of the mtDNA content between not recommended and recommended embryos within the 1827 families (VeriSeq PGS and AB-PGT) confirmed 10% increased mtDNA content in not recommended ones (p-value = 1.46E-36, paired Mann-Whitney U test). For more details, see SM13-SM14.

D. A scheme of potential causative interrelationships between mtDNA content, aneuploidy and maternal age. We interpret the X-axis "mtDNA content" due to its age-independency (see SM3-7) as germline or early somatic risk factors of aneuploidies. The potential chain of events can be the following: (arrow i) germline deleterious variant in nuclear or mitochondrial genomes leads to mitochondrial dysfunction; (arrow ii) cells attempt to compensate for the mitochondrial dysfunctionality by over-replicating mtDNA; (arrow iii) ongoing mitochondrial dysfunctionality is commonly associated with ATP deficiency which can lead to aneuploidy; (arrow iv) an increased mtDNA content is a marker of aneuploidy linking the causative deleterious germline variant with aneuploidy. The association between mtDNA content and aneuploidy can be also caused by aneuploidy (arrow v), where mtDNA content increases as a cellular response to aneuploidy. We interpret the Y-axis "maternal age" as a combination of all somatic factors associated with age (accumulation of somatic mutations, epigenetic changes, misfolded proteins with time) and causing aneuploidy (arrow vi). The scatterplot used for this scheme is from SM6. Not recommended embryos are marked by red points and recommended embryos - by blue points. The purple arrow indicates an increase in aneuploidy risk: every extra 5 years of the maternal age is equivalent to the increase in the mtDNA content by 0.187% (SM8C).

ABSTRACT

Using low-coverage whole genome sequences of trophectoderm biopsy samples from 11610 morphologically good quality embryos we analyzed associations between chromosomal abnormalities and mitochondrial genome. Comparing 6208 aneuploid and 5402 euploid embryos we observed an increased mtDNA content and an excess of ultra-rare potentially deleterious mtDNA variants in aneuploid embryos. The increased mtDNA content was associated with aneuploidy irrespectively of the maternal age, suggesting new age-independent causative mechanisms, based on deleterious germline (or early somatic) variants.

INTRODUCTION

Mitochondria maintain cellular energetics and its dysfunction can affect numerous ATP-demanding processes including the developmental ones leading to human infertility, embryo implantation failure and other disorders in the early neonatal period (Podolak, Woclawek-Potocka, and Lukaszuk 2022). Particularly, the increased mitochondrial DNA (mtDNA) content in trophectoderm biopsy samples at the blastocyst stage has been suggested to be a putative biomarker of implantation potential (Casper 2017). However, due to the technical differences and rather low sample sizes in the majority of the previous studies, there was a controversy about (i) the robustness of this association and (ii) the causative explanations - whether mitochondrial ploidy (directly or indirectly) causes aneuploidies or just marks them. Here we address both of these topics.

RESULTS

11610 human embryos with a sufficient number of trophectoderm cells and no signs of cell degradation were used for the preimplantation genetic testing for aneuploidies (PGT-A) using two different platforms: VeriSeq PGS (Illumina), N=3657, and AB-PGT (AB Vector), N=7953 (**Suppl Mat Methods**). Low coverage whole genome data obtained from trophectoderm biopsy samples at the blastocyst stage were used to derive medical recommendations, based on which (**Suppl Mat Methods**) all blastocysts were classified as recommended for embryo transfer (5402 euploid embryos or embryos with a low level of mosaicism: 1648 from VeriSeq PGS and 3754 from AB-PGT) or not recommended (6208 aneuploid or embryos with a high level of mosaicism: 2009 from VeriSeq PGS and 4199 from AB-PGT). For each blastocyst, we derived mtDNA content as a number of reads mapped to the mitochondrial genome normalized to the number of reads mapped to the nuclear genome (see methods). We observed that mtDNA content was 6-15% higher in a group of not recommended versus a group of recommended for transfer embryos within both platforms (VeriSeq PGS: p-value = 1.92E-15; AB-PGT: p-value = 8.56E-12, Mann-Whitney U test; **Figure 1B, SM1**). Splitting the entire mitochondrial genome into three separate regions: major arc, minor arc, and origins of replication, we demonstrated the uniformity of this trend - an increased mtDNA content in a group of not recommended versus recommended embryos for all these regions (**SM2**).

Maternal age is the strongest risk factor for aneuploidies (Hook, Cross, and Schreinemachers 1983) **Figure 1D**) (Hook, Cross, and Schreinemachers 1983; Thomas, Cavazza, and Schuh 2021; Mikwar, MacFarlane, and Marchetti 2020). The multiple logistic regression model has been performed to test the interrelationships between maternal age, mtDNA content and aneuploidy. We observed that the advanced maternal age and increased mtDNA content were significantly and independently associated with a high risk of aneuploidies (**SM3-7**). Additional analyses confirmed an increase in mtDNA content in not recommended embryos irrespectively of: total coverage of the genome (**SM8**), XX or XY embryo molecular karyotypes (**SM9**), types of chromosomal abnormality (**SM10**), embryo development day (**SM11**), and morphology of the blastocysts (**SM12**). Altogether we conclude that mtDNA content is higher in a group of not recommended versus a group of recommended for transfer embryos, other factors being equal. To control additionally for parental age and all other family-related factors such as nuclear genetics of both parents, maternal mtDNA haplogroups, social-economical status, diet, etc., we compared sibling embryos. One thousand eight hundred twenty-seven families in our dataset had at least one recommended and one not recommended embryo obtained after one controlled ovarian stimulation and *in vitro* fertilization. The paired test, based on the comparison of the median mtDNA content of siblings, confirmed significantly increased mtDNA content in not recommended versus recommended embryos (7.2% for AB-PGT, 17.9% for VeriSeq PGS and 10% for both platforms, **Figure 1C**, **SM13-14**). Thus, the main result obtained at the population level (**Figure 1B**) was unambiguously confirmed at the family level (**Figure 1C**).

An increased mtDNA content can be either a consequence of aneuploidies or a cause of aneuploidies (**Figure 1D**). One plausible causative scenario (see the germline factors in Figure 1D) can be the following: (i) a deleterious mitochondrially or nuclearly encoded germ-line, or early somatic, variant affects mitochondrial functionality; (ii) this mitochondrial dysfunctionality triggers the cellular compensatory mechanism of the over-replication of mtDNA, leading to the increased mtDNA content; (iii) the mtDNA over-replication often doesn't eliminate the mitochondrial dysfunctionality which leads to chromosomal abnormalities, for example, due to the energy deficiency, which can affect ATP-demanding processes such as the formation of the synaptonemal complex, chromosomal segregation, and division spindle recombination. According to the causative germline scenario, not recommended embryos can have an excess of deleterious variants. To test this expectation we used mtDNA of the VeriSeq PGS platform since it is the only platform, providing some regions with moderately high coverage, enough to call SNPs (similar regions are absent in AB-PGT mtDNA as well as in the nuclear genome sequenced on both platforms). Using all alternative mtDNA variants, supported by at least one forward and one backward read, we tested whether there is an excess of deleterious variants in not recommended embryos. As a proxy of the deleterious effect of each altered mtDNA position, we used corresponding frequencies of human population polymorphisms, based on 195,983 completely sequenced mitochondrial genomes around the globe (Bolze et al. 2020). We observed that the often-polymorphic (with three or more homoplasmic carriers of an alternative allele in a given position) and the rarely-polymorphic (with at least one carrier of a heteroplasmic alternative variant and less than three carriers of homoplasmic variants) positions were uniformly distributed among recommended and not recommended embryos. In contrast, the

never-polymorphic positions (positions without alternative variants) were enriched among not recommended embryos (**SM16**). Additional tests considering the nucleotide coverage of each alternative variant and recurrence of observed variants among not recommended and recommended for transfer embryos confirmed our results (**SM16**).

Another causative scenario (see somatic factors, **Figure 1D**) (Schon et al. 2000) assumes that mitochondrial somatic defects can be accumulated within the aged oocytes, similarly to a process well-known in somatic postmitotic tissues (Kraytsberg et al. 2006), and finally leads to aneuploidies. However, as was shown above, mtDNA content is an age-independent risk factor and thus, can unlikely be a result of accumulated somatic damages. Instead, we argue that mtDNA content, associated with aneuploidies, marks deleterious variants which are either present or absent from the very early stages of embryogenesis. The binary logic, above or below the phenotypically important heteroplasmic threshold, is commonly used for somatic mtDNA variants, and here we would like to use it for germline variants. There are several mechanisms, explaining how *de novo* or very rare deleterious mtDNA variants can affect the mitochondrial phenotype in the same generation: (i) a narrow stochastic mtDNA bottle-neck (about 20 inherited mtDNA copies) leads to a minimal heteroplasmy level of a deleterious variant of at least 5% or more; (ii) deterministic positive selection of deleterious variants in the germline (Franco et al. 2022); (iii) some mutations with a dominant effect may affect a phenotype even at very low heteroplasmy level (Fan et al. 2008); (iv) damage of oocytes in early life due to chemical or hormonal stresses can affect mtDNA nucleotide modifications, such as N-Deoxyadenosine methylation, having a pleiotropic effect on the whole mitochondria (Hao et al. 2020). Altogether, we propose that the effect of the germ-line or early somatic deleterious variants, mediated by the increased mtDNA content, can be a common age-independent mechanism of some aneuploidies. It has been proposed that deleterious mtDNA variants can be selected out during the apoptosis of follicles (Krakauer and Mira 1999; Perez et al. 2000) and here we suggest additionally that some mitochondrial deleterious variants can be selected out due to aneuploidies. For example, a potential role of mtDNA in the etiology of trisomy 21 has been proposed in several pedigrees with Familial Down syndrome cases (Arbuzova et al. 2001).

The high sample size combined with the family-based design allowed us to unambiguously confirm the importance of mtDNA content as a marker of embryo aneuploidy and demonstrate the first lines of evidence for ultra-rare potentially deleterious mtDNA variants, which can cause both increased mtDNA content and aneuploidy. It is important to emphasize that in our project we don't have data on the clinical outcomes of the consequent transfers and pregnancies. However, analysis of mtDNA content among euploid human embryos demonstrated that decreased mtDNA content is associated with high viability score (Diez-Juan et al. 2015; Casper 2017), suggesting that the clinical applications of the mtDNA content can be rather broad. Altogether, we recommend medical clinics use mtDNA content as an additional parameter for embryo selection. First, mtDNA content alone has a predictive power to annotate aneuploidies. For example, among the top 5% embryos with the highest mtDNA content, we observed 72% (for VeriSeq PGS) and 67% (for AB-PGT) of aneuploid embryos which is significantly higher than the average in our datasets (54.9% for VeriSeq PGS and 52.8% for AB-PGT) (**SM15**). Second, taking into account the age-independent

association of mtDNA content with aneuploidies, this parameter can be of special interest to young mothers with familial cases of aneuploidies or mitochondrial diseases.

ETHICS STATEMENT

The present study was approved by the Ethics Committee of Fomin Clinic, Moscow, Russia (ID No: 02/2021). Informed consent was obtained from all participants included in the study.

FUNDINGS

M.T., A.K. and I.M. were supported by the Russian Science Foundation [No. 21-75-10081].

SUPPLEMENTARY MATERIALS

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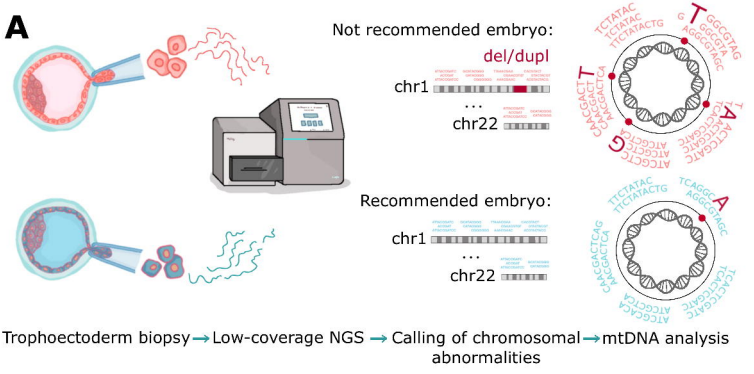
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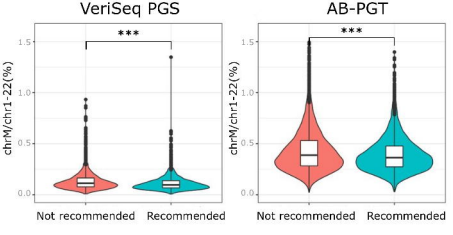
Trophoectoderm biopsy → Low-coverage NGS → Calling of chromosomal abnormalities → mtDNA analysis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AB-PGT
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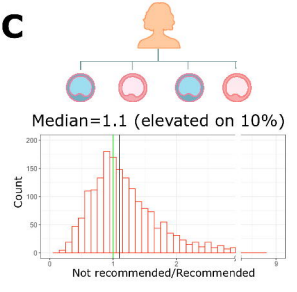
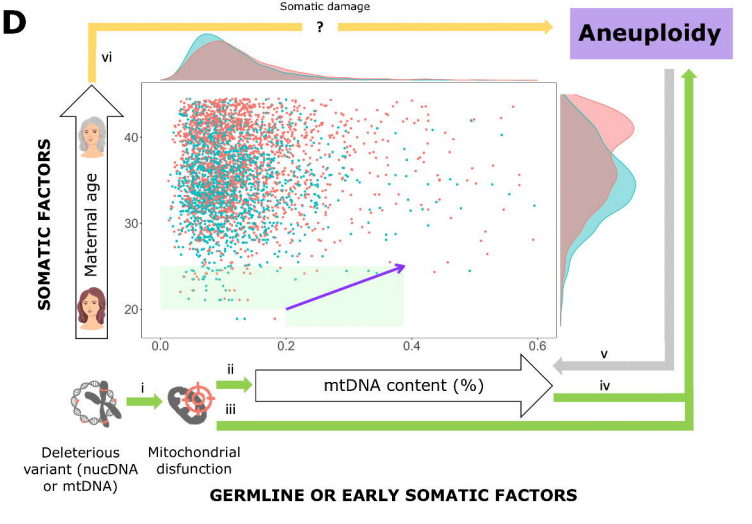
chrM/chr1-22(%)
Not recommended
Recommended


C
Median=1.1 (elevated on 10%)
Count
Not recommended/Recommended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?
Aneuploidy
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mtDNA content (%)
Deleterious variant (nucDNA or mtDNA)
Mitochondrial disfunction
GERMLINE OR EARLY SOMATIC FACTORS