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MITOCHONDRIAL DNA COPY NUMBER, TELOMERE LENGTH AND DNA METHYLATION IN PERIPHERAL BLOOD OF WOMEN UNDERGOING IN VITRO FERTILIZATION CYCLES AS NEW PREDICTORS OF LIVE BIRTH

Settore Scientifico Disciplinare MED/40

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ABSTRACT

Background: The peripheral biomarker of female reproductive biological age proposed so far (*i.e*., mitochondrial DNA copy number (mt-DNAcn), telomere length (TL) and DNA methylation (DNAm) in peripheral blood are promising. Unfortunately, the results of studies aimed at investigating their predictive capacity are conflicting. The conduction of a prospective study including women at the beginning of their natural pregnancy seeking, at the present state of knowledge, goes beyond the possibilities offered by even the most favorable settings. In this context, assisted reproductive technology (ART) has emerged as the most reliable study model. Importantly, considering the absence of non-invasive predictors of ART success, the identification of a reliable biomarker would also have positive implications for the determination of the ART risk-benefit ratio and, in a public health perspective, for the rational allocation of economic resources.

Objective: To evaluate whether mt-DNAcn, TL or epigenetic age estimators based on DNAm pattern (biological age, epigenetic age acceleration, LINE-1 methylation rate), could be considered reliable predictors of in vitro fertilization (IVF) success in terms of live birth rate (LBR).

Design: Prospective cohort study

Setting: Infertility Unit of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, University of Milan, Italy.

Patients: 181 women aged 37-39 years who underwent IVF at a single center between January 2017 and December 2018.

Interventions: On the day of recruitment, blood samples were collected, and genomic DNA was isolated from white blood cells. TL, mt-DNAcn and DNAm assessment was performed using quantitative real-time polymerase chain reaction (qPCR). Biological age (DNAm age) was computed as the algorithm based on methylation pattern of five genes. Epigenetic age acceleration was estimated from the residuals of the linear model of epigenetic age regressed on chronological age. Long Interspersed Nuclear Elements-1 (LINE-1) methylation pattern was used as a surrogate for global DNA methylation.

Main outcome Measures: This study investigated whether peripheral TL, mt-DNAcn and DNAm could predict live birth in IVF cycles.

Results: TL, mt-DNAcn and LINE-1 methylation were not associated with IVF success. Conversely, DNAm age resulted significantly lower in women who had a live birth compared to women who did not (36.1 ± 4.2 and 37.3 ± 3.3 years, respectively, p=0.04). For DNAm age, odds ratio (OR) for live birth per year of age was 0.90 (95%CI: 0.82-0.99, p=0.036) after adjusting for FSH and antral follicle count (AFC) and 0.90 (95%CI: 0.82-0.99, p=0.028) after adjusting also for number of oocytes retrieved. A significant association also emerged for epigenetic age acceleration after adjustments (OR=0.91, 95%CI: 0.83-1.00, p=0.048).

Conclusion: DNAm age is associated with IVF success but the magnitude of this association is insufficient to claim a clinical use. However, our findings are promising and warrant further investigation. Assessment of biological age using different epigenetic clocks or focusing on different tissues may reveal new predictors of IVF success.

1. INTRODUCTION

Pivotal demographic and epidemiological studies demonstrated that female fertility declines with aging (1, 2). In particular, it decreases gradually but significantly beginning approximately at 32 years of age and more rapidly after 37 years of age (3). Approximately 75% of women starting pregnancy seeking at 30 years of age will have a conception ending in a live birth within 1 year, 66% at 35 years and 44% at 40 years. Within 4 years the success rates will be about 91, 84 and 64%, respectively (2). Using both historical and more recent data, one could estimate the proportion of women who remain childless according to the age at marriage: 5.7%, 9.3%, 15.5%, 29.6%, 63.5% of women who marry between 20 and 24 years, 25 and 29 years, 30-34 years, 35-39 years and 40-44 years, respectively (1, 2). Since the 70s of the last century, in high-income countries, the age of women starting to seek their first pregnancy is progressively rising (2, 4, 5). Not surprisingly, in the European Union, the mean age of women on giving birth to their first child stood at 29.4 years in 2019 (above 31 years in three member States: Italy (31.3 years), Spain and Luxemburg (both 31.1 years)) (4). Worryingly, in the future, this trend is expected to worsen: the progressive increase in female education attainment and in access to contraception will hasten decline in fertility and slow population growth (6). According to a forecasting model developed by Vollset *et al*., the global total fertility rate (TFR) is expected to decrease until reaching 1.66 (95% confidence interval (CI), 1.33 – 2.08) in 2100 and, as a consequence, the global population is projected to peak in 2064 at 9.73 billion (95% CI, 8.84 – 10.9) people and decline to 8.79 billion (95% CI, 6.83 – 11.8) in 2100 (6). The causal relationship between female aging and decrease in fertility has been repeatedly questioned due to the possible interference of confounding factors, the main one being the sexual activity which also declines with advancing age (3). In order to untangle this issue, Schwartz *et al*. studied healthy women with azoospermic husbands who underwent donor insemination and found that the cumulative pregnancy rate (CPR) observed in 12 intrauterine insemination (IUI) cycles progressively decreased with increasing age of the recipient female patient from 74% from women younger than 31 years to 54% for women older than 35 years (3, 7). The analysis of outcomes of in vitro fertilization (IVF) and embryo transfer (ET) programs reported by national and supranational registries seem to confirm the independent effect of age on female fertility (3). For instance, the 2018 Assisted

Reproductive Technology (ART) Fertility Clinic Success Rates Report published by the Centers for Disease Control and Prevention (CDC) show that, at a national level, the percentage of intended retrievals resulting in live births was 52% in women younger than 35 years, 38.1% in women aged 35-37 years, 23.5% in women aged 38-40 years, 11.2% in women aged 41-42 years and 3.2% in women older than 42 years (8). Finally, the fact that the success rates of ART cycles with donor eggs remain high and almost constant regardless of the age of the recipient strongly suggests that the decrease in female fertility with aging may be due to a deterioration in oocyte quality over time (3, 8).

Importantly, advancing maternal age is also associated with a significant and progressive increase of miscarriage rate from 20% at the age of 20 years to a high of more than 90% among women 45 years of age or older (9).

1.A. REASONS BEHIND THE DECLINE OF FEMALE FERTILITY WITH AGING

Ovarian senescence is a process characterized not only by a reduction in the number of oocytes but also by a progressive compromise of their competence because of defective physiological pathways including energy production and balance, metabolism, epigenetic regulation, cell cycle checkpoints and meiotic missegregation (10-12).

Multiple molecular and cellular alterations may be responsible for the age-associated oocyte quality decline: mitochondrial dysfunction, shortening of telomeres, cohesins dysfunction, and meiotic spindle abnormalities due to spindle-assembly checkpoint (SAC) impairment. A reduction in the blastulation rate and/or a significant increase in the incidence of chromosomal abnormalities are their putative consequences (12). Finally, a compromised ovarian reserve conditiong anovulatory cycles is an undeniable cause of infertility (12).

Mitochondrial DNA (mt-DNA) alterations

Oocytes need a lot of energy to undergo essential processes, including fertilization, translation during maturation, intracellular signaling, spindle formation, chromosome segregation, and polar body

extrusion (13). Not surprisingly, the mature human oocyte is the human cell with the highest number of mitochondria and largest mitochondrial DNA (mt-DNA) content. Mitochondria produce energy in the form of adenosine triphosphate (ATP) via oxidative phosphorylation to phosphorylate adenosine diphosphate (14). Noteworthy, proper oocyte maturation, fertilization, and embryo development require an adequate amount of mt-DNA molecules, membrane potential, and ATP production (15, 16).

Oocyte mitochondrial replication begins during early stages of fetal life: oogonia initially contain approximately 200 mitochondria. This number progressively increases as oocytes advance through their process of replication and maturation. Notably, an oocyte at metaphase II (MII) stage contains approximately 100,000 mitochondria and between 50,000 and 550,000 mt-DNA copies depending on the species and on the assessment technique used (17, 18). The mt-DNA replication capacity is not reactivated until the blastocyst stage. The embryo expansion is thus characterized by a dilution in the number of mitochondria. As a consequence, one may observe a reduced mt-DNA content in blastocyst stage embryonic cells when compared to that in oocytes. It follows that, in the period elapsing from fertilization to implantation, the embryo depends on the number and function of existing mitochondria (17, 19, 20).

The mitochondrial genome alterations involved in the ovarian aging process are both qualitative and quantitative (21). The former include mt-DNA damage caused by oxidative stress, the accumulation of acquired mt-DNA mutations, the effects of inherited mt-DNA mutations, and alterations in the mitochondrial stress response mechanism (21, 22). The latter refer to alterations in the oocyte mt-DNAcn (17).

Mt-DNAcn is considered a quantitative measure of the mt-DNA cell content. Considering its peculiarities in oocyte physiology, this biomarker attracted the attention of reproductive medicine researchers. In particular, the association between its quantification in oocytes, granulosa cells (GCs), embryonic cells, and peripheral blood cells (PBCs) with surrogate fertility outcomes such as the embryo implantation and pregnancy rates has been extensively investigated. In this scenario, IVF was adopted as study model (17). The initial number of mitochondria and the oocyte mt-DNA content emerged as key factors in the process of fertilization and embryonic development in different

mammalian species (23). In a pioneering study, Reyner *et al*. observed that the average mt-DNAcn in human oocytes was significantly lower in cohorts of patients suffering from fertilization failure compared to those with a normal fertilization rate (24). They concluded that low mt-DNA oocyte content might be the consequence of inadequate mitochondrial biogenesis or cytoplasmic maturation and might adversely affect the oocyte fertilizability (24). Some years later, Santos *et al*. confirmed these findings. In particular, the authors compared the mt-DNA content of 35 fertilized oocytes with that of 65 unfertilized oocytes and observed a significant higher mt-DNAcn in the first group (25). Mt-DNA oocyte content should thus be considered as critical to fertilization outcome and that it might be a useful marker of oocyte quality (25). Not surprisingly, low mt-DNA content is also associated with the known impaired oocyte quality of women with ovarian insufficiency (24). Furthermore, polar bodies from oocytes of women at an advanced reproductive age have been shown to contain less mt-DNA when compared to those of younger women (26).

Within the ovarian follicle, oocyte competence is determined by a network between the oocyte and the surrounding GCs (27). Boucret *et al.* showed that cumulus granulosa cells (CGCs), through the expression of factors involved in the replication and maintenance of mt-DNA, play a pivotal role in the creation of a sufficiently large mt-DNA pool during oogenesis (28). It follows that the mt-DNA content within the CGCs may contribute to the oocyte competence and supporting normal embryo development (17). In order to confirm this hypothesis, Desquiret-Dumas *et al*. deemed of relevance to establish if mt-DNA content of CGCs is related to the oocyte competence (29). Noteworthy, they did not find an association between CGCs mt-DNA content and oocyte maturity or fertilizability. On the other hand, a significant link between the CGCs mt-DNA content and the embryo quality was observed. In particular, a significantly higher mt-DNA content was associated with the development of a good quality embryo (28). Taugourdeau *et al*. observed a significantly higher mt-DNAcn in CGCs of embryos that successfully implanted when compared with that of non-implanted embryos. Importantly, their multivariate analysis, which took into account the age of included women, the quality of the embryos, and the Anti-Müllerian hormone (AMH) serum concentration, showed an independent relationship between the mt-DNAcn in CGCs and the embryo implantation capacity (17, 30). In summary, aging can compromise both oocyte mt-DNA integrity and/or mitochondria

morphology or alter the microenvironment within the follicle and impair the mutual crosstalk between the oocyte and its GCs (12).

Telomere length

Telomeres are repeated DNA-sequences (TTAGGG)n at the ends of chromosomes that play a pivotal role in protecting chromosome ends against degradation and fusion and, therefore, in preserving genome stability and integrity (31, 32). Their function is essential for early meiosis since they anchor chromosomes to the inner nuclear membrane to facilitate homologous pairing and initiate synapsis to form chiasmata, the physical sites of recombination responsible for normal segregation, thereby preventing non-disjunction (12, 33).

Telomere shortening and loss of telomere functionality have been shown to be associated with chromosome end-to-end fusions, resulting in chromosomal instability and genomic complexity (32, 33, 34). The progressive shortening of telomeres within GCs and ovarian stromal cells over time, (which can be due to inefficient DNA repair, chronic damage from oxidative stress, the late exit of the female gametes from their cell cycle arrest or to a reduced activity of the telomerase), has been suggested as one of the principal mechanisms underlying ovarian aging (12, 36-39). Furthermore, it has been demonstrated that the telomeres are shorter in oocytes from women who experienced IVF failure or recurrent pregnancy loss (40), as well as in oocytes resulting in fragmented (41) or aneuploid embryos (12, 42, 43).

Cohesin dysfunctions

Sister chromatid cohesion (*i.e.*, a process that holds sister chromatidis together until their separation) is achieved by a huge ring-like structure generated by the cohesin complex and named cohesin (16, 44).

Sister chromatid cohesion is required principally for two reasons. First of all, to maintain the proper alignment of sister chromatids on the spindle during metaphase and, together with chiasmata, contribute to physical attachment of homologous chromosomes (16, 45). Secondly, to generate tension around the centromere during the bipolar attachment of chromosomes (2, 46, 47). During

the meiotic process, the loss of sister chromatid cohesion from chromosome arms at anaphase I and from centromeres at anaphase II is required to achieve faithful chromosome segregation (16).

Two major defects affecting chromosome structure are associated with female aging: 1) reduction in cohesion subunit levels; 2) increase in the inter-kinetochore (iKT) distance between sister chromatids. Gathering evidence is outlining an age-related disruption of cohesin function leading to missegregation within the oocyte, especially in the presence of low recombination rate (16, 48, 49). As proof of this, cytogenetic studies of human oocytes and embryos showed that premature separation of sister chromatids (PSSC) is often associated with the reduction of cohesins and cohesion function associated with aging (16, 50, 51). Furthermore, also the activity of the regulatory proteins preventing a precocious removal of the cohesins seems to decline over time, exposing them to the detrimental effects of mechanical stress and/or reactive-oxygen-species (ROS) (12, 52, 53). It follows that in late reproductive age women, the above-mentioned mechanisms that affect the telomeres may trigger similar dysfunctions in the cohesins' activity (12, 49).

Spindle instability

The spindle assembly checkpoint (SAC) is a cell cycle surveillance mechanism that monitors the cell cycle and maintains genome stability by controlling both the time and order of cell cycle events in both somatic and germ cells (16, 54, 55). SAC prevents chromosome missegregation by delaying anaphase until accurate kinetochore attachment to the spindle apparatus occurs (16, 56). Alterations in the SAC mechanism are potentially contributing factors to oocyte aneuploidy. Noteworthy, recent evidence suggests that female aging contributes to its dysfunction (54).

The spindle of young oocytes is usually compact, orthogonally oriented with respect to the oolemma with every pole is associated with a ring of centrosome proteins. On the contrary, nearly 80% of the oocytes of women in their late reproductive age may exhibit abnormal spindles with an elongated and/or smaller profile and few microtubular foci at the cortex (12, 57). To this regard, also the SAC shows a reduced stability in this population (58-60). In fact, as already mentioned, different protein components of SAC have been observed in lower concentrations in oocytes from older women (2, 61). Evidence, emerged from both animal and human study models, suggests that female aging may

impact the ability of centromeres to recruit or retain core SAC components, which, in turn, affects the proper attachment kinetochore-microtubule attachment (16, 62). To summarize, available data demonstrate that the function of several SAC proteins in oocytes is affected by maternal aging, which could contribute to the increased incidence of aneuploidy (16).

Meiotic recombination failure

Meiotic recombination implies the exchange of genetic material between homologous chromosomes during prophase I, initiated by programmed DNA strand breaks (63, 64). Studies of human trisomies suggest that the number and location of recombination events may affect chromosome segregation and can lead to aneuploidy (16, 65). A reduction in recombination events has been observed in different types of maternally derived trisomies (*i.e.,* trisomy 15, 16, 18, 21, and sex chromosome) (65, 66). Examination of meiotic recombination patterns by genotyping of families and infants with different human trisomies tends to demonstrate an association between recombination failure and advanced maternal age (16, 67, 68). Notably, analysis of 400 trisomy 21 cases of maternal origin showed that approximately 50% of maternal MI errors in both younger and older women were due to failed recombination (67). The susceptible recombination occurs more frequently in advanced age women because older oocytes have a limited ability to resolve recombination errors (16, 67, 68). Studies on animal models support the important role of meiotic recombination in assuring normal chromosome segregation. Mice deficient for the synaptonemal complex protein SCP3 were at higher risk of recombination and of oocyte aneuploidy (69, 70). Evidence emerged from both animal and human studies suggest that the absence of recombination, reduced recombination and altered recombination patterns are risk factors for meiotic nondisjunction (NDJ) that increase with female aging (16).

1.B. *LOOKING FOR A PERIPHERAL BIOMARKER FOR FEMALE INFERTILITY*

Aging is not a uniform process and may affect each one differently. In other words, chronological and biological reproductive age may not completely overlap. Furthermore, chronological age has been recognized as an imperfect surrogate measure of the aging process. Since the beginning of the 1980s, the efforts of many researchers have been focused on the identification of valid and reliable markers of biological aging that allow to accurately predict the functional capability of a person or organ and how it changes over time (71, 72). Since their discovery, DNA methylation (DNAm) based biomarkers have provided answers to these long-standing questions in different areas of biology, such as medicine (73, 74), biodemography (75), endocrinology (76), dietary studies (77) and cell study (78).

Considering that the decline of fertility with aging affects all women but with a (often modest) inter-individual variability, the identification of a non-invasive peripheral marker capable of estimating the female biological reproductive age is gradually emerging as a research priority (79). The advantages of a reliable female fertility biomarker would in fact be extremely relevant especially considering the progressive trend toward postponement of childbearing (6). This concerns, in particular, public health and family planning. For example, in the eventuality of a young woman at high risk of rapid biological aging, it would be probably advisable to invite her to fulfil her reproductive desire as soon as possible or, if this is not feasible, to evaluate the option of cryopreserving the oocytes. On the contrary, an infertile woman with a proved advanced biological age selected for IVF should be advised not to persevere excessively with homologous treatments. At present, in fact, poor oocyte quality cannot be treated successfully with ART (80). Furthermore, a rapid access to egg donation cycles would have a beneficial effect on the whole gestation considering the known harmful impact of maternal aging on the risk of obstetric and perinatal complications (81). Finally, investigating how the estimated epigenetic age differs across a group of individuals of the same chronological age could help in determining the impact of endogenous or exogenous stress factors on biological aging. In this regard, one of the most exciting feature of DNAm based biomarkers is that epigenetic changes are reversible, raising the prospect that DNAm age estimates might thus be useful for identifying or validating anti-aging interventions (72).

In order to evaluate a hypothetical biomarker of female reproductive age, the ideal study design would be prospective. Participants should be young nulligravid women (under the age of 35/37 years) who wish to get pregnant naturally. Women should be excluded if they have known fertility problems (*i.e.,* polycystic ovarian syndrome (PCOs), tubal factor infertility, history of pelvic infections, endometriosis, previous or current use of fertility treatments, etc.) or a partner with a history of infertility (82). The primary outcome should be the association between the basal level of the biomarker and the chances of natural conception and/or the time to pregnancy (TTP). Unfortunately, conducting such a study presents many difficulties. A first problem is the recruitment of an adequate number of participants, particularly if the probability of natural conception is considered as the primary outcome. In fact, the culture of preconception counselling is still not widespread, especially among healthy young women (83).

In order to estimate the prevalence of young women who are infertile due to an advanced biological age, we focused those selected for IVF. The success rates were extracted from one of the very few studies on the subject and, to date, still considered the most reliable (84).

We made the following assumptions: i) the cumulative live birth rate (LBR) does not vary substantially with the indication for IVF (84); ii) couples diagnosed with repeated implantation failure (RIF) after 3 treatment cycles are successfully treated for known causes or drop out from treatment; iii) couples that fail to obtain a live birth after 6 complete IVF cycles are considered unable to obtain a live birth trough homologous IVF; iv) in a population of women with unexplained infertility, reasons of failure after 6 cycles of IVF can be: 1) unexplained (genetic causes excluded) and RIF, 2) a depletion of oocyte quality that causes repeated fertilization failures, failure to achieve viable embryos or production of only aneuploidy embryos. Considering that the incidence of unexplained and untreatable RIF is about 5% (85), one could hypothesize that, in a population of women with unexplained infertility, the prevalence of depleted oocyte quality that prevent the achievement of live birth is about 9%. It follows that, since the prevalence of infertility is between 7.3% and 9.1% in women younger than 35 years (86) and between 10 and 30% of cases its etiology is unknown (87), one can hypothesize that in the general population of young women (<35 years old) at least between 1 out 410 and 1 out of 1520 women are infertile due to a depleted oocyte quality. The real prevalence

of this condition (*i.e.,* faster depletion of oocyte quality that prevent live birth achievement) in general population is probably slightly higher considering that it can also affect a small proportion of women with other causes of infertility or with an infertile partner. Furthermore, one can also speculate that this condition (*i.e.,* faster depletion of oocyte quality that prevent live birth achievement) is more common in older women. However, in this scenario, determining its exact prevalence is more difficult due to the overlap of chronological aging.

As often in biology, there are probably conditions of intermediate severity. It can be hypothesized that, in some women, biological age is advancing rapidly but their oocyte quality is not yet depleted to such an extent to totally compromise their fertility. Nonetheless, the recruitment of a number of women to conduct a statistically convincing study probably goes beyond the possibilities offered by even the most favorable settings. Other study models have thus been proposed. Designs involving an infertile population (*e.g.,* women selected for IVF) are probably the most reliable. Furthermore, in this context, a female fertility biomarker would be of significant importance for other reasons as well. First of all, it would enrich the prognostic armamentarium. In fact, to date, the only variable which strongly correlates with IVF success rate (*i.e*., LBR) is the chronological female age (88). A precise prognostic evaluation before IVF is of utmost importance not only to counsel women but also to reliably estimate the treatment risk-benefit ratio (7-9, 84). According to the Ethics Committee of the American Society for Reproductive Medicine, in the field of ART, ‘‘futility’’ refers to treatment that has a <1% chance of achieving a live birth; ‘‘very poor prognosis’’ refers to treatment for which the odds of achieving a live birth are very low but not nonexistent (1% to <5% per cycle). Clinicians may refuse to initiate a treatment option they regard as futile or having a very poor prognosis (89). Unfortunately, to date, there are no pre-treatment prognostic scores capable of identifying with certainty patients who have a probability of success of less than 5% (90, 91). Consequently, many avoidable ART treatments are currently still performed with the exposure of patients to unnecessary risks and a substantial waste of (public) economic resources (90, 91). Possible candidates for the role of non-invasive biomarker of female infertility are described below.

Telomere length

Telomere length (TL) has been recognized as one of the most suitable biomarkers of biological age (92). However, findings from available epidemiological studies on the links between TL and age-related diseases and mortality are rather inconsistent and contradictory (92). This is probably also because telomere attrition does not have marked effects on cell physiology until a critical TL is reached, at which point the cell becomes senescent (72, 93, 94).

There is a growing body of literature looking into a possible role of TL in reproductive aging (32). Thus, several studies have investigated possible correlations between TL in spermatozoa and male fertility (95), embryo development and quality (96) during assisted reproductive treatment (97), and female pathologies, such as PCOs (98), premature ovarian failure (POF) (99), and endometriosis (100).

Several studies with different designs have been conducted in order to elucidate the association between TL in leucocytes and GCs and surrogate biomarkers of female infertility (101). Five studies focused on women with POF. Two of them investigated TL and/or telomerase activity in granulosa cells and 4 in leukocytes (99, 102-105). Butts *et al*. and of Xu *et al.* demonstrated that telomeres are shorter in GCs of women with POF than in those of healthy controls. Moreover, these two studies observed also decreased telomerase activity in GCs from POF patients (101). These results suggest that short telomeres in GCs are associated with a limited proliferation cell capacity and, consequently, with a fertility decrease (106). As other stem cells, GCs present a constitutionally high proliferative activity which could be partially linked to telomerase activity (107-111). A decrease in this activity may participate in POF (101). The 4 studies measuring leukocytes TL reported conflicting findings. In 2 of them, leukocytes TL was shorter in POF patients than in controls whereas the 2 other studies reported longer leukocytes TL in POF women (101).

The hypothesized link between leukocytes TL and female fertility is based on the premise that leukocytes TL and GCs TL should be correlated. It is known that all TL are synchronized in somatic tissues at birth (112) and that, despite the difference in TL observed between tissues due to different proliferative indexes, strong correlations in TL across somatic tissues subsist later in life (113). However, this synchrony may potentially not apply to granulosa cells since TL dynamics of these

cells are influenced by telomerase activity. Unlike Hanna *et al*. and Sayban *et al*., Xu *et al*. and Miranda-Furtado *et al*., reported shorter telomeres in leukocytes of women with POF than in controls (99, 103). These results are in line with the "synchrony hypothesis" (112). However, these two pilot studies were conducted with a limited number of subjects and must be confirmed with larger number of participants especially considering the contradictory results with the two other studies. If these results were confirmed, this would make it possible to avoid the invasive approach of taking GCs to measure TL. However, a recent study conducted in 35 fertile egg donors did not find any association between TL in GCs and TL in leukocytes based on quantitative real-time polymerase chain reaction (qPCR) measurements (114-117). These interesting but seemingly contradictory findings highlight the fact that a clearer understanding of the role of telomere length in human reproductive function is necessary (32, 37).

More recently, Hason *et al.* investigated this issue in a population of infertile women of reproductive age undergoing IVF (37). Specifically, the study seeks to determine whether relationships exist between the leukocytes and cumulus cells (CC) TL and patient age, AMH level, peak estradiol (E2) level, number of oocytes retrieved, number of mature (MII) oocytes retrieved, blastulation rate, and aneuploidy rate (37). There was a statistically significant relationship between shorter leukocytes TL and increasing patient age as well as embryonic aneuploidy rate emerged. No significant associations were noted between CC TL and any outcomes assessed (37). Prior human studies evaluating the relationship between TL and embryonic aneuploidy have primarily analyzed tissue samples obtained from CC, polar bodies, or blastomeres (41, 118-121). Apart from some exceptions, published studies have generally determined that shorter TL within these tissues is associated with higher rates of aneuploidy and meiotic dysfunction (118-122). If leukocytes TL can be used as an accurate biomarker of aneuploidy rates and female reproductive performance, this would greatly simplify the process of sample collection for subsequent TL assessment and improve the acceptability of testing among patients (118-123). Although leukocytes TL may potentially constitute a future biomarker of reproductive outcomes in infertile women, the clinical use of TL as a predictive marker currently remains limited.

M'kacher *et al.*, tested the hypothesis that telomere shortening and/or loss are risk factors for infertility. Results show that the mean lymphocytes TL of patients consulting for infertility was significantly lower than that of healthy donors of similar age. Moreover, patients with infertility showed significantly more extreme telomere loss and telomere doublet formation than healthy controls. Noteworthy, telomere shortening and/or telomere aberrations were more pronounced in patients with structural chromosomal aberrations (32).

Strengths and weaknesses of ovarian reserve tests (ORTs)

Since the late 1980s several tests (*i.e.*, blood biomarkers and ovarian imaging), have been proposed to more accurately assess the ovarian reserve (88, 124-126). Among all proposed ovarian reserve tests (ORTs), serum AMH concentration and antral follicle count (AFC), defined as the sum of antral follicles in both ovaries as measured by transvaginal ultrasonography during early follicular phase, seem to have the best predictive value for ovarian reserve (124-126). Experimental models further suggest that AFC and AMH accurately predict antral follicle pool size, which is also an indirect reflection of remaining primordial follicles (127). ORTs have been proposed as a possible solution to non-invasively determine oocyte quality (82). Unfortunately, overlapping age effects on both the residual ovarian reserve and oocyte quality hamper definitive conclusions. As expected, disentangling the independent impact of ovarian reserve remains challenging. To clarify this impact, authors have generally relied on several outcomes including age at menopause, IVF success rate, cumulative probability of conception after 6 and 12 cycles, and TTP. Depmann *et al*., in an individual patient data meta-analysis, demonstrated the capacity of AMH in predicting age at menopause (*i.e.,* the end of natural fertility) (128). However, individual age at menopause predictions showed poor accuracy, particularly when predicting early menopause (i*.e.,* ≤45 years). Clinical application of these findings is thus problematic (128). In IVF, AMH and AFC are used to predict reproductive success measures, including importantly the ovarian response to gonadotrophins. AMH and AFC measurements during IVF are beneficial for individualizing stimulation protocols during controlled ovarian hyperstimulation (COH). However, few published studies have demonstrated a convincing association between AMH and AFC and LBR (129, 130). Prospective studies designed to determine

the extent to which ovarian reserve biomarkers can accurately reflect the probability of conceiving naturally have so far failed to demonstrate an association (82, 128, 131, 132). Nested case control studies derived from cohorts of pregnant women found identical serum AMH concentrations among subfertile and fertile women and a comparable proportion of subjects with low serum AMH levels between the two groups (133, 134).

Fecundity, however, is defined by the capacity to reproduce, which includes not only the ability conceive but also to carry a fetus to viability (82). Within this context, miscarriage rate has been proposed as a possible measure of reproductive capacity (135). Reduced oocyte quality is thought to be the result of meiotic errors, which is considered the leading cause of embryo aneuploidy and, as a consequence, miscarriage (136). In a recent systematic review and meta-analysis, we aimed at elucidating the association between diminished ovarian reserve (DOR), as defined by serum AMH level and/or AFC, and miscarriage risk (88).

Synthesis of results showed that women with low serum AMH concentrations have an increased risk of miscarriage as compared to those with a medium or high AMH level. Pooling of data from retrospective cohorts also showed a significantly higher miscarriage rate in patients with low AFC. Sub-analyses suggested that the age of included subjects does not influence the association between AMH level and miscarriage risk. On the contrary, after splitting studies that reported outcomes according to AFC level, we observed an increase in miscarriage incidence only in young women (< 35 years old). Importantly, sub-analyses showed a slightly higher miscarriage risk in women with a serum AMH concentration < 0.7 ng/ml. An association between a severely reduced serum AMH concentration and miscarriage risk is an intriguing hypothesis that, if confirmed by specifically designed studies, would further strengthen the evidence (88). Unfortunately, due to study limitations, we were not able to make inferences regarding a causal relation between DOR and increased miscarriage risk. Also in a clinical perspective, the relevance of our findings was deemed modest (88).

Peripheral blood cells mitochondrial DNA copy number (mt-DNAcn)

Of utmost interest is the recent evidence demonstrating an association between the mt-DNA copy number (mt-DNAcn) in CGCs and that assessed in PBCSs (137-139). Measuring blood mt-DNA content is simple, rapid, and minimally invasive. Its use in the development of a diagnostic tool for ovarian aging thus emerged as an intriguing issue (138). In order to shed light on this issue, our group conducted a nested case control study. We recruited women at the time of screening for aneuploidies in the first trimester of pregnancy. The mt-DNA PBCS content of women that were seeking pregnancy for more than 12 months (defined as subfertile women) was compared with that of controls of the same age who achieved pregnancy in less than 12 months (defined as fertile women) (139). Interestingly, we found a significantly decreased mt-DNA content in blood cells of subfertile subjects. From the ROC curve analysis emerged that participants with a mt-DNAcn below 105 had a more than fivefold higher risk of being subfertile. Interestingly, the discriminatory ability of this biomarker was shown to be increased in younger women (139). In a previous contribution, Bonomi *et al.* found a diminished mt-DNAcn in PBCSs of women with DOR. A biological grading showing the lowest levels in subjects with POF and the highest in those with a normal ovarian reserve. Noteworthy, an intermediate level of mt-DNAcn was reported in patients with a poor response to COH for IVF (137). The interpretation of these findings is difficult. The low mt-DNA content assessed in blood cells could be a peripheral indicator of a low mt-DNAcn in the oocytes which would cause a fertilization impairment and, consequently, a lower reproductive capacity. This hypothesis is fascinating. However, the evidence demonstrating an association between mt-DNAcn in oocytes or follicular cells and mt-DNA content in PBCS is still weak (139). Alternative explanations may also be valid. One may hypothesize that oxidative stress, *per sè*, acts as the real mediator of fertility damage and that the reduction in peripheral blood mt-DNAcn is only the reflection of a high ROS level (140, 142).

If these preliminary results were confirmed, this biomarker could constitute a particularly important and useful tool in everyday clinical practice. In fact, it has all the features to become the much-sought noninvasive biomarker of female infertility (17, 141).

DNA methylation based (DNAm) biomarkers

Recent evidence from human and mouse studies demonstrates that DNA methylation based (DNAm) biomarkers seem to satisfy the formerly elusive criteria of a molecular biomarker of ageing: they apply to all sources of DNA and to the entire age spectrum (142-147).

A recent review of six types of potential biological age markers concluded that the epigenetic clock is the most promising one (148-149). The highly accurate age estimation based on DNAm levels as well as the concept of an innate process in the body that continues inexorably resulting in aging are commonly referred to as 'epigenetic clock' (72, 73).

The methylation states of all CpG dinucleotides in the human genome were seen to change with age (150). The advent of DNAm array technology enabled the identification of the specific genomic locations of these CpGs (151, 152). Epigenetic 'age estimators' are sets of CpGs that are coupled with a mathematical algorithm to estimate the age of a DNA source, such as cells, tissues or organs (72, 73).

There is limited knowledge about the epigenetics of ovarian follicle cells and how they differ from other somatic cells in the body (153, 154). Interestingly, Olsen *et al.* investigated whether DNAm profiles of leukocytes and mural GCs differ among women with different ovarian reserves (154, 155). The participating women were primarily included during their first, second, or third controlled ovarian stimulation cycle. Authors observed a distinctive epigenetic profile in somatic cells of human ovarian follicles in women with DOR. In particular, a high frequency of epimutations (*i.e*., a heritable change in gene activity that is not associated with a DNA mutation but rather with gain or loss of DNA methylation or other heritable modifications of chromatin) resulted associated with premature aging. Importantly, ovarian reserve status was not reflected in the leukocyte epigenetic profile (154, 155).

2. STUDY AIMS

The candidate biomarkers to reflect the female reproductive biological age proposed so far (*i.e.,* mt-DNAcn, TL and DNAm) are promising. Unfortunately, the results of studies aimed at investigating their predictive capacity are conflicting. The conduction of a prospective study including women at

the beginning of their pregnancy seeking, in the present state of knowledge, goes beyond the possibilities offered by even the most favorable settings. In this context, IVF has emerged as the most reliable study model. Importantly, considering the absence of non-invasive predictors of ART success, the identification of a reliable biomarker would also have positive implications for the determination of the ART risk-benefit ratio and, in a public health perspective, for the allocation of economic resources.

Against this background, in this study, we aimed at disentangling the possible association of peripheral determinants of aging and IVF success. To this aim, we set-up a prospective cohort study in women in the late thirties undergoing IVF cycles and compare mt-DNAcn, TL and DNAm between those who did and did not achieve a live birth.

3. MATERIALS AND METHODS

Women referred to the Infertility Unit of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico and scheduled for IVF or Intracytoplasmic Sperm Injection (ICSI) between January 2017 and December 2018 were prospectively evaluated for study entry. Inclusion criteria were as follows: 1) indication to IVF, 2) age 37-39 years, 3) body mass index (BMI) 17-35 Kg/m^2, 4) serum FSH < 15 IU/mL, 5) less than 3 previous IVF cycles. Criteria for exclusion were as follows: 1) uterine abnormalities such as fibroids, adenomyosis, endometrial polyps and uterine septum, 2) hydrosalpinx at basal ultrasound, 3) severe male factor infertility (azoospermia requiring surgical recover of spermatozoa, cryptozoospermia, necrospermia, globozoospermia), 4) systemic diseases that could affect pregnancy outcome and cause miscarriage (such as diabetes, uncompensated thyroid disease, anti-phospholipid antibody syndrome). In addition, we excluded women who did not initiate the IVF cycle for any reason. Eligible subjects were invited to participate and signed an informed written consent prior to be recruited. The study was approved by the local Ethical Committee (*Comitato Etico Milano area B*, 2016/2176).

Recruited women provided a blood sample prior to initiate the IVF cycle. They were collected in EDTA-containing tubes and immediately stored at -80° C until assayed. Baseline clinical

characteristics and IVF outcome of the selected women were obtained from patients' charts. Missed information were obtained by direct contact. An active investigation of the IVF cycle outcome was performed by phone call or by consulting patients' charts of the obstetrical unit of our hospital.

Molecular analyses were performed simultaneously after completing patients' recruitment and follow-up. Blood samples were thawed, and DNA extracted using commercial kits as reported in detail elsewhere (137).

TL and mt-DNAcn were measured by using the real-time quantitative PCR method as described by Cawthon (155) and Hou *et al.* (156). These assays measure relative TL and relative mt-DNAcn in DNA by determining, respectively, the ratio of telomere repeat copy number (T) and mitochondrial (mt) copy number to a single nuclear copy gene (S), which was the human (beta) globin (*hbg*). The T/S ratio and mt/S ratio are calculated in a given sample relative to a reference pool DNA. The reference pool DNA was prepared from 10 participants randomly selected from this same study, using 6 *μg* for each sample. A fresh 7-points standard curve prepared from the pooled DNA, ranging from 40 ng/*μ*l to 0.62 ng/*μ*l (serial dilutions 1:2), was included in every "T," "mt," and "S" PCR runs. For each sample, 9 ng of DNA was used as a template, and the reaction was run in triplicate. A high-precision MICROLAB STARlet Robot (*Hamilton Life Science Robotics, Bonaduz AG, Switzerland*) was used for transferring a volume of 7 *μ*l reaction mix and 3 *μ*l DNA (3 ng/*μ*l) in a 384-well format plate. All PCRs were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Primers and thermal cycles were previously reported (156). At the end of each real-time PCR reaction, a melting curve was added to confirm the amplification specificity and the absence of primer dimers. The average of the three T and three mt measurements were divided by the average of the three S measurements to, respectively, calculate the T/S or the mt/S ratio for each sample. The coefficients of variation for mt-DNAcn and for TL were respectively 4.3% and 5.9%.

Biological age (DNAm age) was calculated considering the methylation pattern of 5 CpG sites at five genes (ELOVL2, C1orf132/MIR29B2C, FHL2, KLF14, TRIM59) as reported elsewhere (158). The DNA samples (500 ng) were plated at a concentration of 25 ng/μL in plates of 96 wells each and were treated with sodium bisulfite using the EZ-96 DNA Methylation-Gold™ Kit (*Zymo Research; Irvine, CA, USA*) following the manufacturer's instructions and eluted in 200 μL. 10 μL of bisulfite-

treated template DNA were added to 25 µL of GoTaq Hot Start Green Master mix (Promega), 1 µL of forward primer (10 µM), and 1 µL of 5′ end-biotinylated reverse primer (10 µM) to set up a 50 µL PCR reaction. PCR cycling conditions and primer sequences have been previously reported (158).

Biological age (Y) was calculated as follows:

$$Y = 8.052 + 55.673*ELOVL2 + 47.141* FHL2 + 62.870*KLF14 -$$

$$- 29.075*MIR2B29B2C + 41.281*TRIM59$$

Epigenetic age acceleration was defined as the residuals of DNAm age regressed on chronological age.

To assess DNA methylation of LINE-1, bisulfite-PCR was performed with the following primers: forward 5'-TTTTGAGTTAGGTGTGGGATATA-3', reverse 5'-biotin – AAATCAAAAA ATTCCCTTTC-3' and sequencing primer 5'-AGTTAGGTGTGGGATATAGT-3' as previously reported (159).

The sample size was calculated for mt-DNA. Based on previous studies of our group on this variable (79, 137), we estimated that at least 170 women (of whom one third achieving live birth) were necessary to demonstrate a mean difference of 15% with type I and II errors set at 0.05 and 0.20. Given the sub-optimal reproducibility of the assessment of mt-DNA in our experience, we were not stringent on the sample size and opted a priori for a slightly larger recruitment (up to 200). Baseline characteristics and ART outcomes of two groups were compared according to Student's *t* test, Mann-Whitney and Fisher's Exact test, as appropriate. P values below 0.05 were considered statistically significant. In case of significant differences in baseline characteristics, a multivariate logistic regression model was used to obtain adjusted measures of association between the chances of live birth and the studied peripheral biomarkers. More specifically, we first performed a multivariate analysis for the whole cohort that took into account only baseline variables found to differ between the study groups (i.e., serum FSH and AFC). Then, we performed a second multivariate analysis focusing only on women who retrieved oocytes and adjusting for the baseline variables included in the first model as well as variables of the cycles up to the number of available oocytes (including therefore information on ovarian hyperstimulation and oocytes retrieved). Specifically, the model included serum FSH, AFC and number of oocytes retrieved. The aim of this second analysis was to better extract pure biological effects, thus excluding women who could not provide oocytes.

4. RESULTS

One-hundred ninety-one women were initially recruited. Ten of them did not initiate the IVF cycle for personal reasons, leaving 181 women for data analyses. Overall, 58 women (32% of our cohort) obtained a live birth. An overview of the baseline clinical characteristics of the whole cohort as well as of the comparisons between women who did and did not deliver are shown in Table 1. Statistically significant differences emerged for AFC and serum FSH between the two groups.

Table 1. *Baseline clinical characteristics of the whole cohort and comparison between women who did and did not obtain a live birth*

Characteristics	All cohort n=181	Live birth n=58	NO live birth n=123	p
Age (years)	37.9 ± 0.8	37.8 ± 0.8	38.0 ± 0.9	0.22
BMI (Kg/m^2)	22.4 ± 3.6	22.3 ± 3.4	22.4 ± 3.7	0.99
Previous deliveries	34 (19%)	10 (17%)	24 (20%)	0.84
AMH (ng/ml)	2.8 ± 2.4	3.2 ± 2.7	2.6 ± 2.3	0.21
AFC	12 ± 7	15 ± 8	10 ± 6	<0.001
FSH (IU/mL)	7.4 ± 2.2	6.9 ± 2.1	7.7 ± 2.3	0.02
Duration of infertility (years)	3.7 ± 2.6	4.1 ± 3.2	3.5 ± 2.3	0.21
Smoke	33 (18%)	10 (17%)	23 (19%)	1.00
Previous IVF cycles				0.33
None	136 (75%)	42 (73%)	94 (76%)	
One	31 (17%)	13 (22%)	18 (15%)	
Two	14 (8%)	3 (5%)	11 (9%)	
Indication to IVF				0.90
Unexplained	55 (30%)	15 (26%)	40 (33%)	
Endometriosis	33 (18%)	10 (17%)	23 (19%)	
Anovulatory	10 (6%)	4 (7%)	6 (5%)	
Tubal factor	26 (14%)	8 (14%)	18 (15%)	
Male factor	48 (27%)	18 (31%)	30 (24%)	
Mixed	9 (5%)	3 (5%)	6 (5%)	

Table 1 legend: AFC: Antral Follicle Count. AMH: Anti-Müllerian hormone. Data are reported as mean ± SD or number (percentage).

Table 2 illustrates the IVF cycle outcomes, again for the whole cohort and separately for women who did and did not achieve a live birth. Statistically significant differences emerged for number of oocytes retrieved, number of suitable oocytes, fertilization rate and number of available embryos at cleavage stage.

Table 2. *IVF cycle outcome in the whole cohort and comparison between women who did and did not obtain a live birth*

Characteristics	All cohort n=181	Live birth n=58	NO live birth n=123	p
Protocol of hyperstimulation				0.11
GnRh Antagonist	148 (82%)	45 (78)	103 (84%)	
Long Protocol	22 (12%)	11 (19%)	11 (9%)	
Flare up	11 (6%)	2 (3%)	9 (7%)	
Cancelled cycles	7 (4%)	0 (0%)	7 (6%)	0.10
Total dose of gonadotropins (IU) [a]	2,001±671	1,864±626	2,070 ±684	0.06
Duration of stimulation (days) [a]	8.8 ± 1.8	9.0 ± 1.9	8.7 ± 1.7	0.34
Nr. of oocytes retrieved [a]	7.6 ± 5.5	9.5 ± 6.0	6.7 ± 5.1	0.001
No oocytes retrieved [b]	2 (1%)	0 (0%)	2 (2%)	0.55
Nr. of suitable oocytes [a]	6.3 ± 4.4	7.9 ± 4.6	5.5 ± 4.1	0.001
Nr. of women without suit. oocytes [a,c]	2 (1%)	0 (0%)	2 (2%)	0.55
Technique [d]				0.08
Conventional IVF	88 (52%)	36 (62%)	52 (46%)	
ICSI	82 (48%)	22 (38%)	60 (54%)	
Fertilization rate (%) [d]	71 [50 - 93]	75 [63 - 100]	67 [50 - 85]	0.009
No embryos available for transfer [d]	9 (5%)	0 (0%)	9 (8%)	0.03
Nr. cleavage stage embryos (72 h) [e]	3.6 ± 2.4	4.8 ± 2.4	2.9 ± 2.1	< 0.001

Table 2 legend: Data are ported as mean ± SD or median [interquartile range] or number (percentage). IU: International Units, IVF: In vitro fertilization. ICSI: Intracytoplasmic Sperm Injection. [a] Refers to women who were not cancelled (n=174). [b] Suitable oocytes refer to metaphase II oocytes and type 1 cumulus-oocyte complex according to the European Society for Human Reproduction and Embryology Istanbul Consensus Conference, 2011. [c] Data refer to subjects retrieving at least

one suitable oocyte (n=170). [d] *Percentages refer to the number of women with available embryos (n= 161).*

Mt-DNA, TL, LINE-1 methylation, biological age and epigenetic age acceleration in women who did and did not obtain a live birth are shown in Table 3. A statistically significant difference emerged only for DNAm age. Specifically, the mean ± SD biological age was 36.1 ± 4.2 and 37.3 ± 3.3 years respectively (p=0.04) for women who did and did not have a live birth. The ROC curve aimed at assessing the accuracy of DNAm age in predicting live birth rate showed an AUC of 0.57 (95%CI: 0.48-0.66). Women with a biological age ≤ 10th centile (corresponding to 32 years) had an Odds Ratio (OR) of 2.4 (95%CI: 1.0-5.9, p=0.05) for live birth.

Table 3. *Biomarkers of aging in women who did and did not achieve a live birth*

Variable	Live birth n=58	NO live birth n=123	p
Mitochondrial DNA (copy number)	1.01 ± 0.27	1.04 ± 0.30	0.47
Telomere length (TTAGGG repeats)	1.04 ± 0.23	1.00 ± 0.27	0.32
LINE-1 methylation (%5mC)	76.4 ± 1.4	76.5 ± 1.5	0.71
Biological Age (years)	36.1 ± 4.2	37.3 ± 3.3	0.04
Age acceleration (years)	0.66 ± 4.0	0.34 ± 3.3	0.08

Table 3 legend: Data are reported as mean ± SD. p: p-value; DNAm age: Biological age.

Multivariate analyses adjusting for the variables found to differ between women who did and did not achieve a live birth are shown in Table 4. Two different models were used for adjusting the data. Firstly, we used a clinical model that took into account only baseline variables found to differ between the study groups (*i.e.*, serum FSH and AFC). These analyses included the whole cohort. Using this

model, only DNAm age was found to differ between the study groups. The adjusted OR of live birth per year of age was 0.90 (95%CI: 0.82-0.99, p=0.036). Secondly, we used a model that took into consideration both baseline characteristics and IVF variables. Specifically, the model included serum FSH, AFC and number of oocytes retrieved. We exclusively included in this model women who underwent oocytes retrieval (n=170). A statistically significant difference persisted for DNAm age. The adjusted OR of live birth per year of age was 0.90 (95%CI: 0.82-0.99, p=0.028). In addition, a significant association emerged for epigenetic age acceleration. The adjusted OR of live birth per year was 0.91 (95%CI: 0.83-1.00, p=0.048).

Table 4. *Univariate and multivariate analyses on the relation between biomarkers of aging and live birth*

Variable	Univariate analysis			Model 1 *			Model 2 **		
	Crude OR	95% CI	p	Adj. OR	95% CI	p	Adj. OR	95% CI	p
Mt-DNAcn	0.67	0.22 - 2.00	0.47	0.94	0.29 - 3.03	0.91	0.95	0.29 - 3.07	0.93
Telomere length	1.81	0.55 - 5.96	0.33	1.44	0.41 - 5.01	0.57	1.54	0.44 - 5.43	0.50
LINE-1 methylation	0.96	0.78 - 1.18	0.71	0.97	0.78 - 1.22	0.80	0.94	0.75 - 1.19	0.61
Biological Age	0.91	0.84 - 1.00	0.048	0.90	0.82 - 0.99	0.036	0.90	0.82 - 0.99	0.028
Age acceleration	0.92	0.84 - 1.01	0.08	0.91	0.83 - 1.00	0.06	0.91	0.83 - 1.00	0.048

*Table 4 legend: *Data was adjusted for AFC and FSH using a multivariate logistic regression model. **Data was adjusted for AFC, FSH and oocytes retrieved using a multivariate logistic regression model. AFC: Antral Follicle Count. Mt-DNAcn: Mitochondrial DNA copy number. OR: Odds ratio. CI: Confidence interval. p: p value. ORs refer to the chance of live birth.*

Finally, we performed a subgroup analysis based on the leading cause of infertility. Only two groups (endometriosis and mixed factor) were characterized by a statistically significant difference of

biological age and age acceleration between women who delivered and who did not (p value: 0.03 and 0.04 respectively) (Table 5). Besides, the difference in biological age persisted even comparing endometriosis to the unexplained cause of infertility, that could be considered our internal control group (p value: 0.04) (data not shown).

Table 5. Biomarkers of aging in women who did and did not achieve a live birth according to the IVF indication

Unexplained infertility

Variable	Live birth n=15	NO live birth n=40	p
Mitochondrial DNA (copy number)	0.94 ± 0.19	1.03 ± 0.28	0.25
Telomere length (TTAGGG repeats)	1.00 ± 0.15	0.99 ± 0.22	0.76
LINE-1 methylation (%5mC)	76.55 ± 1.85	76.64 ± 1.47	0.86
DNAm Age (years)	37.47 ± 3.5	37.25 ± 3.64	0.84
Age acceleration (years)	0.36 ± 3.35	0.31 ± 3.56	0.96

Anovulatory infertility

Variable	Live birth n=4	NO live birth n=6	p
Mitochondrial DNA (copy number)	0.89 ± 0.18	1.32 ± 0.41	0.09
Telomere length (TTAGGG repeats)	1.22 ± 0.25	1.08 ± 0.29	0.45
LINE-1 methylation (%5mC)	76.98 ± 1.05	75.87 ± 1.03	0.14
DNAm Age (years)	36.75 ± 3.69	38.33 ± 2.34	0.43
Age acceleration (years)	0.48 ± 2.84	1.72 ± 2.16	0.20

Endometriosis

Variable	Live birth n=10	NO live birth n=23	p
Mitochondrial DNA (copy number)	0.99 ± 0.28	1.07 ± 0.27	0.41
Telomere length (TTAGGG repeats)	1.02 ± 0.23	0.98 ± 0.25	0.66
LINE-1 methylation (%5mC)	76.05 ± 1.26	76.28 ± 1.42	0.66
DNAm Age (years)	33.50 ± 5.52	36.57 ± 2.41	0.03
Age acceleration (years)	-3.35± 5.69	-0.31 ± 2.38	0.04

Tubal factor infertility

Variable	Live birth n=8	NO live birth n=18	p
Mitochondrial DNA (copy number)	1.12 ± 0.25	1.03 ± 0.28	0.46
Telomere length (TTAGGG repeats)	1.06 ± 0.34	0.95 ± 0.18	0.28
LINE-1 methylation (%5mC)	76.38 ± 1.12	76.84 ± 1.95	0.54
DNAm Age (years)	37.0 ± 3.30	36.56 ± 4.03	0.79
Age acceleration (years)	0.30 ± 2.79	-0.46 ± 4.14	0.64

Male factor infertility

Variable	Live birth n=18	NO live birth n=30	p
Mitochondrial DNA (copy number)	1.07 ± 0.35	1.04 ± 0.29	0.79
Telomere length (TTAGGG repeats)	1.04 ± 0.21	1.06 ± 0.37	0.84
LINE-1 methylation (%5mC)	76.59 ± 1.50	76.38 ± 1.61	0.66
DNAm Age (years)	36.22 ± 4.29	37.67 ± 3.26	0.19
Age acceleration (years)	-0.33 ± 4.16	0.63 ± 3.11	0.37

Mixed infertility

Variable	Live birth n=3	NO live birth n=6	p
Mitochondrial DNA (copy number)	0.97 ± 0.15	0.81 ± 0.40	0.51
Telomere length (TTAGGG repeats)	1.01 ± 0.32	0.96 ± 0.33	0.85
LINE-1 methylation (%5mC)	75.63 ± 0.65	77.1 ± 1.35	0.13
DNAm Age (years)	34.33 ± 2.08	39.67 ± 2.66	0.02
Age acceleration (years)	-1.57 ± 2.08	2.53 ± 2.05	0.03

Table 5 legend: Data are reported as mean ± SD; p: p-value; DNAm Age: biological age.

5. DISCUSSION

In this study, we failed to show a marked association between mt-DNA, TL, LINE-1 methylation, and the chances of live birth in IVF. In contrast, a statistically significant difference emerged for DNAm age. Given the modest difference, the clinical relevance is however doubtful. The area under the ROC curve indicates a low performance (0.57, 95%CI: 0.48-0.66). An exploratory analysis aimed at disentangling whether this biomarker could be more reliable to predict the success, or the failure tended to favor the former (the OR for a biological ≤ 10^{th} centile being 2.4, 95%CI: 1.0-5.9) but additional evidence is needed.

The absence of correlation between mt-DNA and IVF success stands in apparent contrast to the current state of research. Bonomi *et al*. noted a biological grading of mt-DNA copy number in peripheral blood along with ovarian reserve, highlighting the poorest mt-DNA content in case of premature ovarian aging (137). However, it must be underlined that oocytes quality and quantity are two sides of the same *coin*, that is female fertility (132, 160). They both decrease with age, but they have a different and independent trajectory. The contrast of our findings with those reported by Busnelli *et al*. is more difficult to reconcile (79). Indeed, in that study, mt-DNA correlated with time

to natural pregnancy, indirectly suggesting a benefit on oocytes quality rather than on ovarian reserve. However, again, it would be simplistic to assimilate natural and IVF pregnancies. The occurrence of natural pregnancies implies a plethora of other physiological mechanisms that are not involved in IVF pregnancies (including ovulation, tubal function, uterine motility).

Considering TL, the literature is ampler but also more conflicting. The implication of the telomere pathway in fertility is supported both by animal and human models (161). Keefe *et al*. hypothesized that progressive shortening of telomeres, from fetal oogenesis to the adult ovary, would be the cause of ovarian impairment related to age (39). Moreover, in case of ovarian insufficiency, shorter telomeres have been registered in leukocytes and granulosa cells (104). On the other hand, it has recently been supposed that the maintenance system of telomere length may be atypical in the ovary. Both Morin *et al*. (123) and Lara-Molina *et al*. (114) found out that telomere length is higher in cumulus cells rather than in peripheral blood leucocytes, suggesting that the follicular environment could possess peculiar mechanisms to cope against telomere shortening. Similarly, Hanson *et al*. and Olsen *et al* have confirmed a difference in telomere length between cumulus or granulosa cells and white blood cells (37,155). Based on their studies, Lara-Molina *et al*. stand against the use of telomere length of peripheral cells as a reliable indicator of follicular cell telomere length. These latest data fuel the idea that follicular telomere maintenance could not mirror other somatic tissue (114).

Despite the modest magnitude of the difference in DNAm age between women who did and did not deliver herein observed, evidence on this biomarker is the most promising and intriguing finding of our study. Based on a very small sample size, Monseur *et al*., 2020 observed a correlation between epigenetic clock and AMH and the number of oocytes yield, claiming the need to verify the parameter as an additional tool in ovarian reserve testing (162). To note, in the present study, the association of DNAm age and the success of IVF was found to be independent from ovarian reserve biomarkers. It remained statistically significant even after adjusting for baseline characteristics (AFC and FSH) that sorted out to be statistically different among the two groups. Not only, but we could prove that the strength of the association between DNAm age and IVF success even increased adopting a more composite model for multivariate analysis. When adjusting for both baseline characteristics

and the number of oocytes retrieved, we could appreciate that also epigenetic age acceleration turned out to be statistically significant. The possibility that epigenetic clock estimators could emerge as additional and independent predictors of ART success, assessing aspects different from ovarian reserve (oocyte retrieval, FSH and AFC), is particularly appealing. One could foresee a predictive algorithm taking into account ovarian reserve tests, chronological and DNAm age or epigenetic age acceleration.

There is a wide variety of models that can compute DNAm age and the most recent one relies on more than 30,000 genes (163). We presently relied on a validated but simple method of DNAm age calculation based on the methylation of five genes. More comprehensive and potentially more precise approaches could be used. In addition, there is a recent trend to develop and validate surrogate clocks for clinical outcomes, including life span or age-related diseases such as cancer and cardiovascular events rather than chronological age (163). One may foresee the advance of clocks specifically dedicated to female fertility.

Although multi-tissue age estimators, such as the first one called Horvath's clock (75) have already been validated, it is known that the different epigenetic pattern among cells underlies a specific epigenetic profile of tissues or cellular populations (164). Some of the genes proved to be tissue specific (FHL2 for saliva and blood, TRIM59 for blood and buccal swab) (165) while accuracy of others (such as ELOVL2) results independently from the type of biological sample (166). Up to now, the research has focused mainly on tissues that are easy to reach (blood, buccal swab, saliva, sperm). In fact, even if epigenetic clock revealed the synchronicity of DNAm age across several tissues, some exceptions have already been detected (female breast epigenetically older and cerebellum younger) (167). Of utmost relevance here is that Morin *et al*. observed a remarkably younger age in cumulus cells when compared to peripheral cells (123). Indeed, Hanson *et al*. confirmed this result in a larger cohort of infertile women undergoing ovarian stimulation (167). However, while Morin failed to prove the association of different epigenetic ages of leukocytes to ovarian response (123), in a subgroup analysis Hanson *et al*. observed that white blood cells were epigenetically older in poor ovarian responders (167). Even if these observations may be of scant interest in disentangling predictors of success (cumulus cells can be obtained only when performing

IVF), studying more in-depth aging of ovarian cells could reveal new avenues of research. In line, Olsen *et al*. have recently developed a “Granulosa Cell clock” (155) apparently more accurate for mural granulosa cells than *Horvath’s clo*ck and *Skin & Blood clock* (72). These results could be a promising confirm of the distinct methylation trajectory with age of follicle cells compared to other somatic cells. Moreover, epigenetic profile changes have been defined in granulosa cells in women with different ovarian reserve, suggesting the possibility of a methylation DNA variability in relation to granulosa cells functionality (167).

Some limitations of the study should be recognized. Firstly, other methodological approaches could be used to assess mt-DNA, TL and DNAm. As alluded above, this limitation could be particularly important for DNAm age. On the other hand, it should be underlined that the methods we adopted were already validated by others (168). In addition, one has to point out that available scientific evidence is insufficient to definitely indicate a referral method (169). Secondly, infertile patients were enrolled irrespective of infertility diagnosis. This could have diluted the results and precluded meaningful subgroup analyses. Thirdly, we decided to exclusively focus on women aged 37-39 years and conclusions should thus be considered valid only for this age group. This decision was taken because we believed that the evaluation of biological age could be particularly relevant in women in their late thirties, when natural fertility is rapidly declining. Evidence from less selected women is however required to better disentangle the possible clinical utility of assessing biological age.

In conclusion, peripheral assessment of mt-DNA, LINE-1 methylation, TL and DNAm age do not provide a potent mean to predict the success of IVF. However, our findings strengthen the interest of investigating more in-depth the possible role of DNAm age. Along with the state of research, our study highlights the urgency to shed light on the epigenetic mechanisms of female infertility. The use of alternative and more precise epigenetic clocks for different cellular populations (*i.e.*, granulosa, cumulus cells) could open new fruitful avenues of research, providing new potential markers not only to predict IVF success but also to streamline the selection of the “eligible woman” to ART treatment.

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