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Age-associated changes in cumulus cells and follicular fluid: the local oocyte microenvironment as a determinant of gamete quality

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Abstract

The ovary is the first organ to age in humans with functional decline evident already in women in their early 30s. Reproductive aging is characterized by a decrease in oocyte quantity and quality, which is associated with an increase in infertility, spontaneous abortions, and birth defects. Reproductive aging also has implications for overall health due to decreased endocrinological output. Understanding the mechanisms underlying reproductive aging has significant societal implications as women globally are delaying childbearing and medical interventions have greatly increased the interval between menopause and total lifespan. Age-related changes inherent to the female gamete are well-characterized and include defects in chromosome and mitochondria structure, function, and regulation. More recently, it has been appreciated that the extra-follicular ovarian environment may have important direct or indirect impacts on the developing gamete, and age-dependent changes include increased fibrosis, inflammation, stiffness, and oxidative damage. The cumulus cells and follicular fluid that directly surround the oocyte during its final growth phase within the antral follicle represent additional critical local microenvironments. Here we systematically review the literature and evaluate the studies that investigated the age-related changes in cumulus cells and follicular fluid. Our findings demonstrate unique genetic, epigenetic, transcriptomic, and proteomic changes with associated metabolomic alterations, redox status imbalance, and increased apoptosis in the local oocyte microenvironment. We propose a model of how these changes interact, which may explain the rapid decline in gamete quality with age. We also review the limitations of published studies and highlight future research frontiers.

Summary Sentence Aging is associated with genetic, epigenetic, transcriptomic, proteomic, metabolomic, and redox status changes in the local oocyte microenvironment: cumulus cells and follicular fluid.

Keywords: telomere, epigenome, mitochondrial DNA, transcriptome, proteome, metabolism, reactive oxygen species, apoptosis, angiogenesis, extracellular matrix

Introduction

Tissue function declines with age, and this also holds true for the reproductive system. However, there are major sex differences in the onset of reproductive aging; it occurs much earlier in females than males. In fact, the female reproductive system is the first to age, beginning when women reach their mid-30s. In female mammals, the dogma is that a finite number of oocytes are formed during early development, which dictate the ovarian reserve or reproductive lifespan. A main hallmark of reproductive aging is a decline in both gamete quantity and quality. Reproductive aging also has adverse general health outcomes because the gonadal hormone estrogen regulates numerous organs, such as the brain, heart, and bone [1–5]. The gap between menopause and lifespan is ever-widening, resulting in more women living longer in an altered endocrine milieu.

There are also tangible clinical ramifications of reproductive aging, including an increased incidence of infertility which necessitates high usage of Assisted Reproductive Technologies (ART) and an increased risk of spontaneous abortions and miscarriages [6, 7]. For those women of advanced reproductive age who are able to conceive, they have a higher chance of having dizygotic twins even independent of ART [8]. They also have an increased risk of obstetrical complications with increased risk of maternal and fetal mortality and severe maternal morbidity, including high rates of cesarean delivery, preeclampsia, postpartum hemorrhage, gestational diabetes, and deep vein thrombosis [7, 9–11]. Advanced reproductive age is also associated with a greater risk of having offspring with birth defects that are chromosomal in origin, such as Trisomies 13, 18, and 21, or non-chromosomal in origin such as heart defects, hypospadias, craniosynostosis, club foot, and diaphragmatic hernia [12–16]. These clinical ramifications are becoming more pronounced as women globally are delaying childbearing [6, 17].

Data from ART cycles clearly demonstrate that decreased egg quality underlies the reproductive age-associated decline in fertility. For example, the percentage of fresh embryo transfers that result in live births decreases with age such that the likelihood of a woman having a child with autologous eggs after her mid-40s drops to nearly zero [18]. However, this maternal age effect is essentially abrogated if a woman of advanced reproductive age uses eggs from reproductively young, healthy donors to conceive [19]. Thus, the biological age of the egg is critical in dictating reproductive outcomes. As a result, the majority of research in reproductive aging has focused on changes intrinsic to the gamete and include primary defects in both meiotic and cytoplasmic competence. For example, advanced reproductive age is associated with chromosome defects in terms of recombination, DNA

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repair pathways, micromechanical properties, cohesion, and kinetochore function, which may all contribute to increased incidences of age-dependent aneuploidy [20–22]. At the same time, age-associated alterations in mitochondria numbers, morphology, distribution, and function as well as mtDNA copy number and mutations result in an overall state of reduced energy production and increased reactive oxygen species and oxidative stress [23, 24].

The gamete, however, does not develop in isolation but rather as part of a broader and heterogeneous ovarian microenvironment of cells and extracellular matrix components that influence gamete quantity and quality [25]. In fact, it has recently been appreciated that robust changes occur in the ovarian microenvironment with age. For example, there is a difference in immune cell populations and a shift to a pro-inflammatory aging milieu consistent with the concept of inflammaging [26–28]. This inflammation often correlates with increased tissue fibrosis, which results in a quantifiably stiffer ovary with age [29–33]. The biophysical properties of the ovary are mediated by a combination of increased collagen and decreased hyaluronan levels [30]. Moreover, there are age-dependent changes in interstitial fiber thickness and orientation as well as the size and the number of pores between extracellular matrix fibers [31]. Importantly, many of these age-dependent changes both in the gamete and ovarian microenvironment have been documented in both mouse and human suggesting conserved mechanisms of aging [20, 30, 34].

The immediate local follicular environment also influences gamete development and quality in addition to the broader ovarian microenvironment. Oocytes within primordial follicles are enclosed by squamous granulosa cells. Following primordial follicle activation which is gonadotropin-independent, granulosa cells transform into a cuboidal shape and proliferate while the oocyte undergoes significant volumetric expansion during its active growth phase [35, 36]. Following the secondary follicle stage, subsequent follicle growth becomes gonadotropin dependent, and fluid then accumulates in between granulosa cells and eventually a single antral cavity is formed. Follicular fluid is formed from blood flowing through the thecal capillaries. Granulosa cell synthesis of large molecules, including hyaluronic acid (HA) is believed to create the osmotic gradient which facilitates fluid accumulation in the antral follicles [37, 38]. Antrum formation separates the preantral granulosa cells into mural granulosa cells that line the wall of the follicle and cumulus cells that surround the oocyte. Physical separation, influence of FSH from the pituitary, and oocyte secreted factors (GDF9, BMP15) promote the formation of these two physiologically distinct cell populations; mural granulosa cells are endocrinologically active and secrete estrogen whereas cumulus cells become intimately linked to the oocyte through specialized physical connections called transzonal projections [39, 40]. Cumulus cells are essential for supporting the metabolic needs of the oocyte during its final growth phase to ultimately generate a developmentally competent gamete and also play an important role in the regulation of meiotic arrest and resumption [40–43]. In addition, cumulus cells undergo expansion following the luteinizing hormone (LH) surge, and this is necessary for ovulation and fertilization [44–46].

Cumulus cells and follicular fluid represent the local microenvironment during the final maturation stages of the oocyte and likely influence gamete quality (Figure 1). Understanding the age-related changes in these compartments can provide new insight into the mechanisms of reproductive aging. Furthermore, their study across reproductive lifespan can provide us with invaluable information regarding the associated gamete and may lead to the discovery of new non-invasive biomarkers of oocyte quality. Our goal was to systematically evaluate the literature on the age-dependent changes in the immediate oocyte microenvironment: cumulus cells and follicular fluid. We searched MEDLINE (through August 2021) using various combination of terms: age, aging, follicular fluid, cumulus cell, oocyte environment, and oocyte microenvironment. A web application, Rayyan, was used to screen 1475 abstracts for studies that addressed the changes in the oocyte local environment with aging [47]. Of these, 286 relevant articles were identified and then evaluated at full-text level. Studies that did not directly address the changes in the oocyte microenvironment with advancing chronological age were excluded. Examples of excluded studies were those that focused on the mechanisms of "post-ovulatory aging" or ones that primarily investigated in vitro fertilization (IVF) outcomes. Only a handful of included studies were performed in animal models (3 in mice and 1 in bovine species), with a significant majority focusing on human subjects likely due to the accessibility of these materials at the time of oocyte retrieval during IVF. Therefore, the age-related cumulus cell, mural granulosa cell, and follicular fluid parameters reported in this review were primarily based on material obtained at the time of follicular puncture and aspiration and oocyte retrieval following ovarian stimulation during IVF unless otherwise specified. It is also important to note that oocyte retrieval during IVF is typically performed after human chorionic gonadotropin (hCG) injection to induce oocyte maturation and therefore, the majority of described age associated changes are after the exposure to the ovulatory cue. Therefore, earlier stages of follicle and oocyte development are not addressed in these studies, and this should be considered when interpreting the findings.

The local oocyte microenvironment undergoes unique nuclear and mitochondrial genomic alterations with advancing age

Age-related changes in the cumulus cell genomic DNA integrity, mitochondria copy number and mutation status, and the levels of cell-free mtDNA (cf-mtDNA) in follicular fluid were evaluated in several studies. Older ($\geq$37 yo) patients show higher rates of DNA double-strand breaks in cumulus cells compared to younger ($\leq$29 yo) counterparts [48]. Although not directly addressed, it would be interesting to determine the mechanism by which genomic instability impacts cumulus cell function and whether it contributes to the age associated decrease in gamete quality. The research on telomere length of follicle somatic cells also shows interesting findings. Telomeres are highly preserved nucleoprotein structures of (TTAGGG)*n* repeats located at the ends of chromosomes, which maintain genomic stability and prevent chromosome degradation [49]. Telomere shortening is associated with aging. The proposed mechanism is that short telomeres fail to protect the chromosomal ends from being recognized as DNA double strand breaks, cause genomic instability, and activate DNA repair pathways, which eventually lead to cellular senescence or apoptosis [50]. Reproductive aging is associated with shortened telomeres in human oocytes [51–53]. The degree of telomerase activity that regulates telomere length is believed to regulate ovarian aging [54]. Telomerase deficient mice demonstrate early

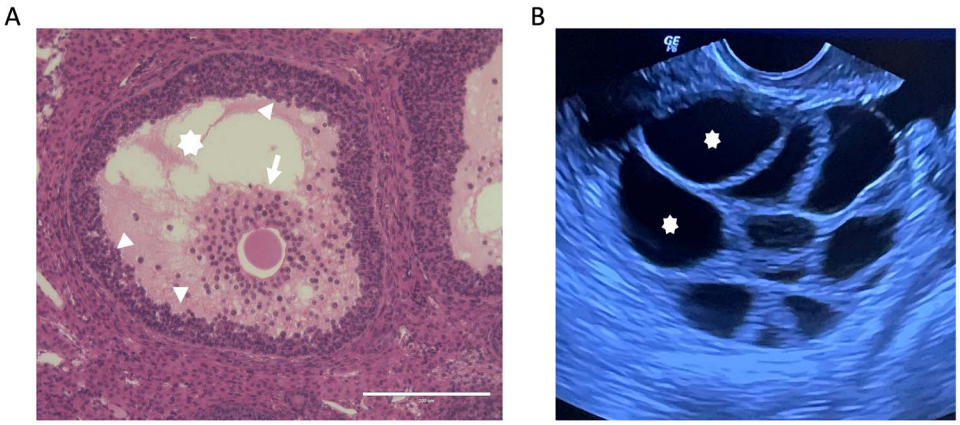

Figure 1. The local oocyte microenvironment. (A) Mouse antral follicle. Asterisk shows antral cavity filled with follicular fluid. Arrow points to the expanding cumulus cells 11 h after human chorionic gonadotropin injection, and arrowheads show mural granulosa cells. (B) The appearance of human antral follicles on ultrasound at the time of oocyte retrieval. Asterisks show fluid filled antral cavity of 18–20 mm follicles.

meiotic arrest, compromised chromosome synapsis, and recombination [55]. Several studies focused on the evaluation of cumulus cell telomere length as a function of age [56–58]. Interestingly, telomeres of cumulus cells are significantly longer compared to the telomere length of white blood cells (WBCs) in the same woman. However, telomere length did not correlate with age in CCs or WBCs in this study [58]. The finding of longer telomere length in CCs compared to WBC was independently replicated in a separate study of 35 oocyte donors with a mean age of 25 [57]. Another recent study analyzed cumulus and white blood cell telomere length in relation to IVF outcomes in 175 patients [56]. In this study, WBC relative telomere length was inversely associated with the patient age and embryo aneuploidy rate; however, the relative telomere length in cumulus cells was not associated with age or aneuploidy. These data show that the regulation of telomere length in cumulus cells is different from somatic tissues, and it appears that telomere shortening is not one of the hallmarks of aging in these cells. Selective experimental manipulation of telomere length in follicle somatic cells may help us understand the physiological importance of this differential regulation. The local oocyte microenvironment may have evolved to maintain longer telomeres with age to ensure the development of a competent gamete.

The mitochondrion is the only animal organelle containing DNA outside of the nucleus, and all proteins encoded by the mitochondrial genome are part of the electron transport chain. The number of mitochondria and mitochondrial DNA copy number is determined by tissue metabolic activity. Mitochondrial DNA is prone to mutations due to the lack of protective histones and its exposure to high levels of reactive oxygen species (ROS) because of the close proximity to the electron transport chain. [23]. mtDNA copy number and mitochondria function affect the developmental competence of the oocyte, with lower mtDNA numbers and/or reduced mitochondria function in the oocyte associated with age, decreased fertilization, and impaired embryo development [23, 59]. Accumulation of mtDNA mutations and deletions over time may reduce oocyte quality [23, 60, 61]. Similar to what occurs in the gamete, increasing age is associated with lower relative mtDNA copy number in cumulus cells [62]. Cumulus cell mtDNA from older women is also more likely to have large deletions [63]. The relative cf-mtDNA content in FF, measured as mitochondrial gene/nuclear gene transcript ratio using real-time quantitative PCR, appears to positively correlate with age and diminished ovarian reserve [64]. These studies demonstrate that aging is associated with abnormalities in nuclear and mitochondrial genomic integrity, a decrease in CC mtDNA numbers, an increase in mtDNA mutations, and an increase in FF cf-mtDNA levels. Future studies are needed to determine whether the cf-mtDNA levels in FF are due to active expulsion of defective mitochondria from the cumulus cells. Overall, the number of studies on this subject are limited, and more research is needed to corroborate these observations.

The "epigenetic clock" moves slower in cumulus cells compared to somatic tissues

The epigenome is a sum of chemical changes in DNA and histones which can be inherited [65]. Aging is associated with a predictable change in the epigenome including increased or decreased DNA methylation at specific loci. The analysis of large datasets across thousands of tissue specimens from multiple longitudinal studies demonstrated that age-associated DNA methylation changes are consistent and predictable between individuals and have led to the development of "DNA methylation or epigenetic clocks" that can accurately predict and measure the chronological age of humans [66, 67]. The risk for all-cause mortality increases when the age of an individual's "epigenetic clock" is more advanced than his or her chronologic age [68, 69].

There is considerable variation in the pace of ovarian aging across individuals as manifested in ovarian reserve parameters and response to gonadotropin stimulation, and this triggered investigations into the methylation profile of cumulus and mural granulosa cells in the follicles [58, 70, 71]. Interestingly, the epigenetic algorithm fails to accurately predict chronological age when applied to cumulus and mural granulosa cells [58, 71]. These cells consistently demonstrate "younger" methylation profiles when compared to the individual's chronological age or the "epigenetic age" of WBCs [58, 71]. Furthermore, the "epigenetic clock" of cumulus cells is not different across the spectrum of ovarian response to gonadotropin stimulation during IVF. However, the methylation profiles in WBCs of younger patients with poor ovarian response (i.e., $\leq$5 oocytes retrieved) are consistent with accelerated aging [70]. Mural granulosa cells also show overall younger DNA methylation with age compared to leukocytes

[71]. These data demonstrate that there is a distinct age-related regulatory mechanism of the epigenetic profile in somatic cells within antral follicles. These cells seem to maintain a younger epigenetic profile compared to other cell types, and it is tempting to speculate that this has likely evolved to support the development of a competent oocyte. Dissection of the molecular mechanisms of this differential regulation may elucidate signaling pathways important for the understanding of ovarian aging.

The transcriptomic landscape of the local oocyte microenvironment and how it changes with advancing age

Cumulus cells are transcriptionally active, and their transcriptome has been the target of myriad of investigations to identify non-invasive biomarkers of oocyte and embryo quality [72]. Transcriptomic analysis of the oocyte microenvironment may shed light on the mechanisms of the age-related decrease in gamete quality. High-throughput transcriptomic analysis of young (2 months), middle age (9 months), and old (14 months) BDF1 mice oocytes and surrounding cumulus cells after superovulation demonstrates that the cumulus cell transcriptome undergoes age-related changes before any alterations in the oocyte transcriptome can be detected. In this study, genes involved in transcription regulation were down-regulated and genes involved in DNA damage/repair and cell cycle regulation were up-regulated with aging in cumulus cells [73]. Although there are numerous investigations evaluating differential messenger RNA (mRNA) expression in human cumulus cells with advancing age, the majority of these studies performed either targeted microarrays or RT-PCR of a priori determined genes [74–81]. Only one study used unbiased RNA sequencing (RNASeq) and analyzed differential gene expression in cumulus cells from reproductively young (<35 yo) and older (>40 yo) women [82]. In this study, 45 out of 11 572 genes were differentially expressed between these age groups in cumulus cells. The analysis demonstrated that genes involved in hypoxia stress response (*Nos2*, *Rora,* and *Nr4a3*), vasculature development (*Nr2f2*, *Pthlh*), glycolysis (*Ralgapa2* and *Tbc1d4*), and cAMP turnover (*Pde4d*) are significantly overexpressed in cumulus cells of older women.

Targeted investigations of select genes revealed that creatine kinase B and peroxiredoxin 2 expression are upregulated in cumulus cells of women older than 38 years old compared to women less than 28 years old [74]. In a different study, CC from women older than 37 years old showed increased expression of angiogenic genes (*Angptl4*, *Lepr*, *Tgfbr3*, and *Fgf2*) and down-regulation of genes implicated in TGF-β signaling pathway (*Amh*, *Tgfb1*, inhibin, and activin receptor) compared to women 31–34 yo and < 30 yo [76]. Similarly, three TGF beta family receptor genes (*Bmpr2*, *Alk4*, and *Alk6*) were downregulated in CC of women ≥35 years old, whereas *Alk5* was upregulated when compared to women <35yo [79]. Lastly, the expression of genes encoding integral components of cumulus cell extracellular matrix and molecules important for cell-ECM interaction were up-regulated (*Vcan*, *Tnfaip6*, *Ptx3*, *Sdc4*) in cumulus cells of women ≥35 yo compared to <35 yo [80]. Although limited, collectively, this evidence shows that there are significant age-related changes in the cumulus cell transcriptome and that the transcript levels of hypoxia, angiogenesis, DNA damage/repair, glycolysis, and ECM-related genes are increased, whereas the expression of TGF-β related genes are down-regulated. These transcriptomic signatures may indicate a potential compensatory upregulation in response to follicular stress due to a potential suboptimal environment (e.g., hypoxia).

In addition to mRNA, several studies have investigated the differential expression of non-coding RNAs in the oocyte microenvironment with age. Targeted analysis of 752 human miRNAs in cumulus cells revealed miRNome profiles with enrichment of biological functions of oocyte growth and maturation, embryo development, steroidogenesis, ovarian hyperstimulation, apoptosis, cell survival, glucose and lipid metabolism, and cell trafficking [83]. Differential miRNA expression of 32 or 36 miRNAs, depending on the type of hormonal stimulation, was observed between women ≥36 yo and ≤35 yo [83]. In addition, overall higher concentrations and increased numbers of miRNAs were reported in younger than in older women [83]. The analysis of publicly available RNASeq databases identified 147 long non-coding RNAs (lncRNAs) that are differentially downregulated in CCs from older (>40 yo) compared to younger women (<35 yo) [84]. Interestingly, lncRNAs (*IL10RB-AS1, APOA1-AS, IGF2BP2-AS1, LINC00548, PSMB8-AS1*, and *LAMTOR5-AS1*) with a possible role in CC–oocyte communication because of their association with transcript networks involved in this process, are down-regulated in CCs of older women, which may point to the impaired CC–oocyte communication in women with advanced reproductive age. Collectively, these differentially expressed lncRNAs are enriched in carbohydrate and lipid metabolism, molecular transport, and cell cycle pathways. One small study with 20 women undergoing IVF, 10 in poor and 10 in normal ovarian response groups, showed that even among women of similar age, accelerated follicular loss and diminished ovarian reserve is associated with altered small non-coding RNA expression profile [85]. Circular RNAs (circRNAs), which are single-stranded RNAs with covalently closed continuous loops and unknown biological functions in most cases, are also differentially expressed in granulosa cells of women ≥38 yo compared to ≤30 yo [86]. Two circRNAs levels (circRNA_103827 and circRNA_104816), potentially involved in glucose metabolism, mitotic cell cycle, and ovarian steroidogenesis, were positively associated with age after adjustment for ovarian stimulation [84].

Besides cumulus and mural granulosa cells, the non-coding RNA profile in follicular fluid has also been investigated. miRNAs can localize to extracellular vesicles (EVs), which protect them from degradation, or can be found free floating in FF. EVs are membrane-bound nanovesicles, which facilitate intercellular communication via shuttling proteins, mRNAs, and miRNAs [87]. The oocytes and somatic cells within the follicle utilize EVs to communicate, which is important for follicular development and oocyte maturation [88, 89]. Three studies analyzed the age-related miRNA profile in follicular fluid [90–92]. Microarray analysis of 866 miRNAs revealed hsa-miR-424 as an age associated differentially expressed miRNA [91]. In a different study, the analysis of the miRNome in EVs in follicular fluid revealed four differentially expressed miRNAs between younger (<31 yo) and older (>38 yo) women. The predicted targets of these miRNAs are enriched in genes involved in heparan-sulfate biosynthesis, extracellular matrix-receptor interaction, carbohydrate digestion and absorption, p53 signaling, and cytokine-cytokine-receptor interaction [90]. In another recent study, authors identified twice as many EVs in follicular fluid of older women (>38 yo) compared to young (<35 yo) with 46 deregulated miRNAs both in the FF and inside

the EVs of FF from older women [92]. Protein targets of these miRNAs are involved in biological processes related to vesicle-mediated transport, mRNA processing, apoptotic signaling, nucleocytoplasmic transport, protein targeting, and chromatin organization.

Overall, the analysis of miRNA profiles in the oocyte microenvironment reveals overlapping age-related differences with mRNAs. Transcripts involved in carbohydrate metabolism, ECM synthesis, ECM-cell interaction, and cell cycle regulation show altered expression. Importantly, the available data points to the changes in post-transcriptional regulation of gene expression with advancing age. Specifically, many of the predicted targets of age-dependent differentially expressed miRNAs play important roles in oocyte metabolism and oocyte-somatic cell communication, highlighting the importance of these processes in reproductive aging.

Reproductive aging alters cytokine and protein composition of the oocyte milieu

Similar to transcriptomic studies, the majority of investigations into the proteome of the oocyte microenvironment primarily focused on a targeted analysis of a single or group of proteins. Only two studies used an unbiased approach to examine the aging associated changes in the cumulus cell [93] and follicular fluid [94] proteome utilizing liquid chromatography–tandem mass spectrometry (LC–MS/MS) and matrix-assisted laser desorption-ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF-TOF-MS), respectively. Proteomic analysis of cumulus cells identified 1423 proteins [93]; 110 (7.7%) of these proteins were differentially expressed between women of advanced maternal age (40–45 yo) and oocyte donors (20–33 yo). Proteins involved in cumulus cells fatty acid metabolism demonstrated increased expression (ACAT2, HSD17B4, ALDH9A1, MVK, CYP11A1, FDFT1), whereas proteins with a role in oxidative phosphorylation (NDUFA1, UQCRC1, MT-ATP6, ATP5I, MT-ATP8) and post-transcriptional RNA processing (KHSRP, SFPQ, DDX46, SNRPF, ADAR, NHPL1, U2AF2) showed downregulation [93]. Proteomic analysis of FF from younger (20–32 yo) and older (38–42 yo) women via two-dimensional gel electrophoresis followed by MALDI-TOF-TOF-MS revealed five proteins with decreased expression in the older cohort [94]. Identified proteins are involved in innate immunity (complement C3, C4) and may affect angiogenesis [95]; iron transport (serotransferrin, hemopexin precursor), which may act as anti-oxidants by chelating iron [96, 97]; and kininogen which is proangiogenic [98].

Similar to the aforementioned study which highlights the alteration of angiogenic factor levels in FF with aging [94], vascular endothelial growth factor (VEGF), a well-established angiogenic protein, may also have a potential role in reproductive aging [99–104]. VEGF stimulates angiogenesis and vascular permeability, and its expression is induced under hypoxic conditions [105, 106]. It plays an important role in the establishment of ovarian vascularity and is detectable in ovarian follicular fluid [107, 108]. Studies that measured VEGF levels in human follicular fluid during IVF [99–101, 103, 104] or in natural cycles [102] consistently demonstrate increased levels with aging. In two studies, follicular fluid VEGF levels in young patients (<35 yo and <37 yo) were positively correlated with the grade of perifollicular vascularity as measured by power Doppler ultrasonography [105, 109]. Importantly, severe reduction of dissolved oxygen in human follicular fluid in overall young patient populations (25–37 yo) is associated with an increased rate of chromosome abnormalities on meiotic spindles in metaphase II eggs. However, FF VEGF levels are not significantly different across groups with different dissolved FF oxygen content [109]. These studies provide potential mechanistic insight into how a hypoxic follicular environment may control ovarian angiogenesis and how abnormalities in these processes can lead to aneuploidy and decreased gamete quality. The age-related increase in VEGF levels may be compensatory in response to follicular hypoxia. However, the direct negative effects of its increased levels on follicle viability and function cannot be excluded and need to be investigated in future studies.

Reproductive aging is associated with increased levels of pro-inflammatory cytokines, and this sustained low-grade inflammation, "inflammaging," may contribute to fibrosis [27, 29, 30, 33]. Semiquantitative profiling of follicular fluid fibroinflammatory cytokines following controlled ovarian hyperstimulation in women 27–45 years old revealed that the levels of 6 out of 80 cytokines measured (IL-3, IL-7, IL-15, TGFβ1, TGFβ3 and MIP-1) positively correlate with aging and negatively correlate with AMH levels [110]. In the same study, an additional 16 cytokines, including VEGF, platelet-derived growth factor-BB (PDGF-BB), Leukemia inhibitory factor (LIF) levels increased with advancing age but did not correlate with AMH levels. In a separate study, researchers investigated FF cytokine profiles using a bead-based multiplex immunoassay of 23 cytokines in 10–40 yo patients undergoing ovarian tissue cryopreservation for nongynecological malignancies [111]. The comparison of cytokine levels between age groups (<35 yo vs. ≥35 yo) showed decreased levels of IL-1Ra and IL-5 and increased levels of IP-10 in the older group [111]. Although these ovaries were not stimulated with gonadotropins and therefore the potential confounding effect of hyperstimulation on cytokine expression is eliminated, the effect of a systemic illness, such as malignancy, on FF cytokine profile cannot be excluded. In a separate study, the measurement of 27 cytokines and growth factors in FF in patients with diminished ovarian reserve revealed decreased levels of PDGF-BB in this group compared to women with normal ovarian reserve independent of age [112]. This points to the potential role of PDGF-BB in reproductive aging and may be the underlying reason of the initial promising results obtained after IVF following platelet rich plasma injection to the ovaries with premature ovarian insufficiency (POI) [113].

Studies looking into FF levels of individual cytokines demonstrate that IL-6 is higher in FF of older women with poor response to stimulation compared to older women with normal response or younger women [114]; Granulocyte colony-stimulating factor (G-CSF) levels are lower in FF of older women (>36 yo vs. <30 yo) [115]; IL-8 levels in large follicles decrease with advancing age [116]; IL-1α levels are significantly upregulated in FF of patients with POI [117]; FF Nerve growth factor (NGF) levels positively correlate with age [118]; Transforming growth factor-β1 (TGF-β1) levels are lower in older patients (≥35yo vs <35yo) [119], which is consistent with cumulus cell transcriptomic data reported above [76] but in contrast to what was observed in FF with cytokine arrays [110]. Similarly, although the association between BMI and FF leptin levels is relatively well established [120, 121], the data on its association with aging demonstrate variable results with one study showing increasing levels with age [122] and another study showing

no significant change [110]. The inflammatory molecules in FF may potentially reflect the cytokine profile of intraovarian vessels with likely contribution from granulosa, cumulus, theca, and ovarian immune cells but this needs to be confirmed in future studies. The comparison of these cytokine levels in the local oocyte environment to their systemic levels is also an important area for future investigations. Furthermore, it needs to be established whether the changing inflammatory signature is the cause or the consequence of reproductive aging and whether there is a feedback loop whereby low-grade inflammation causes cellular damage which in turn exacerbates this inflammation. Altogether, these studies highlight that our understanding of the role of the changing inflammatory milieu in reproductive aging is still limited, and more studies are needed to investigate inflammaging in the ovary and elucidate the mechanisms of how these changes can lead to tissue damage and increased fibrosis.

Advanced maternal age is associated with an altered metabolomic profile of the local oocyte microenvironment

Bidirectional communication between the oocyte and surrounding cumulus cells is important for the transfer of signaling molecules and nutrients [42, 123]. There is an extensive network of carbohydrate, amino acid, and lipid metabolism transfer within the cumulus oocyte complex (COC), where the COC functions as a unit to support the development of a competent gamete [42, 123, 124]. Oocyte secreted factors regulate the metabolism of the surrounding cumulus cells to fine tune their metabolic activity to meet the metabolic needs of the oocyte [125, 126]. Therefore, the study of cumulus cells and surrounding FF metabolites and their byproducts may provide insights into the associated oocyte. In addition, this dynamic relationship between the oocyte and cumulus cell changes with follicle growth and in response to the various hormonal stimuli [42, 123, 127], and given significant transcriptomic and proteomic alterations with aging, metabolomic differences in the oocyte microenvironment are also expected.

In COCs, glycolysis is outsourced to cumulus cells [124, 125]. Cumulus cells metabolize glucose to pyruvate which is then taken up by the oocytes via gap junctions [128, 129]. Compared to younger women (<35 yo) with normal ovarian reserve, older women and patients with diminished ovarian reserve (DOR) show decreased glucose and increased lactate levels in follicular fluid. This is associated with increased glucose uptake, lactate production, and increased expression of phosphofructokinase platelet gene in cumulus and granulosa cells [130]. The levels of one of the glycolytic enzymes, lactate dehydrogenase (LDH), is also increased in FF with advancing age [131]. These findings are corroborated in a high-resolution 1H-NMR (nuclear magnetic resonance) spectroscopy study of FF of women >40 yo with control group of women 25–35 yo with normal ovarian reserve. The comparison of FF composition between these groups reveals decreased glucose and increased lactate levels in FF of older women [132]. These studies indicate increased glycolytic activity in the cumulus cells of women with advanced reproductive age perhaps due to compensatory upregulation to counteract follicular stress (i.e., hypoxia, increased ROS) or to increase ATP production to meet the changing demand in oocytes from older individuals. The regulatory role of the oocyte in this process and whether these metabolic changes in the oocyte microenvironment can be used as indicators of gamete quality in the clinical setting remains to be investigated.

Some amino acids, including L-alanine and L-histidine, are transported from cumulus cells to the oocyte via gap junctions in mice because the oocytes cannot take these up directly from the environment [133]. Only one study investigated the age-related levels of amino acid, D-aspartic acid, in the oocyte milieu. This amino acid is not incorporated into proteins but can induce hormone biosynthesis and release (e.g., injection of this molecule in rats increases the serum levels of LH, testosterone, progesterone, and prolactin) [134–136]. The levels of this amino acid in FF decrease with age (35–40 yo vs. 22–34 yo), and higher levels within FF are associated with morphologically better appearing MII oocytes as assessed by the appearance of the cytoplasm and zona pellucida and the size of perivitelline space as well as increased fertilization rates [137].

Cholesterol synthesized de novo in cumulus cells is the primary source of this molecule for oocytes with some additional possible contribution from lipoproteins taken up from the follicular fluid by CCs [124]. In addition, free fatty acids appear to be an important energy source in COCs during oocyte maturation in mice [42, 138]. Metabolomic analysis of follicular fluid from younger and older women (28–34 yo vs. 35–48 yo) using a combination of ultra-high-performance liquid chromatography and high-resolution mass spectrometry reveals that lipid metabolites are the primarily affected class with four downregulated (Arachidonate, LysoPC (16:1), LysoPC(20:4), LysoPC(20:3)) and two upregulated (LysoPC(18:3), LysoPC(18:1)) molecules [139]. In a separate study, mass spectrometric analysis of lipids in the follicular fluid of women over and under 35yo demonstrated that 11 out of 15 identified lipids are more abundant in the older group. Pathway analysis shows enhanced metabolism of glycosphingolipid, phosphatidylinositol phosphate, and glycerophospholipid in women >35 yo [140]. The levels of apolipoproteins are also altered with advancing age. Specifically, apolipoprotein A1 (Apo A1) and apolipoprotein CII (Apo CII) levels are decreased, and apolipoprotein E levels (Apo E) are increased in follicular fluid of older women. These proteins bind and transport lipids in the form of heterogenous complexes (i.e., HDL, LDL etc.), and their distribution within these complexes is also altered in women with advanced age [141]. Overall, the available evidence demonstrates age-related alterations in lipid levels, composition, and interaction with the apolipoproteins in the follicular fluid. Given that these are important energy sources and building blocks of cells, this disrupted microenvironment may affect oocyte quality. Future mechanistic studies may shed light on if/how altered lipid milieu contributes to changing gamete developmental competence with aging.

The age-associated changing metabolomic profile in the local oocyte environment affects the expression of sensor proteins, sirtuins, which modify downstream protein functions in response to metabolic cues. Sirtuins play a role in DNA repair and recombination, epigenetic modifications, and the regulation of mitochondrial function [142, 143]. Sirtuin 3 (SIRT3) is a mitochondrial protein, which alters the function of proteins in this organelle via deacetylation. SIRT3 transcript levels and protein activity are decreased in cumulus and granulosa cells in women with DOR and advanced maternal age (AMA) (≤35 yo vs. ≥40 yo) [144]. Similarly, another mitochondrial sirtuin, SIRT5, exhibits reduced transcript and protein levels and activity in somatic cells of antral follicles in women with reduced ovarian reserve and advanced age [145]. Consistent with this, mitochondrial metabolism, and the expression

of enzymes important for Coenzyme Q (CoQ) production appears to be reduced in cumulus cells of both mice and humans with age [146]. In contrast to mitochondrial sirtuins, the expression of nuclear sirtuin, SIRT1, which regulates the function of transcription factors, is increased in cumulus cells of older women (>38 yo vs <34 yo) [147]. These data point to the altered metabolic milieu in the oocyte microenvironment and provide insights into how these changes affect the regulation of downstream targets through regulation of sirtuin activity.

Hormonal composition of the oocyte microenvironment remains largely unchanged with advancing age

Follicles are endocrinologically active and the investigation of hormone composition of FF can help us assess the changes in this endocrine activity with age. Inhibin α and β levels are lower in FF of older women undergoing IVF [148–150]. Consistent with this, in vitro synthesis of inhibin molecules is compromised in granulosa cells of older women [149]. In contrast, some studies demonstrate that inhibin levels in FF do not change with age in spontaneous cycles [102, 151]. In another study, although FF inhibin levels did not change with age, serum inhibin B was lower in older women [152]. Estradiol levels appear to be lower in FF of older women undergoing IVF compared to their younger counterparts [148, 150], but are of similar value to younger patients in spontaneous cycles [102, 152]. The correlation of FF progesterone levels with age in IVF cycles show conflicting results which is likely due to differences in IVF stimulation protocols between studies [148, 153]. Growth hormone binding protein and IGF-1 levels, which play a role in follicle growth and granulosa cell proliferation, appear to be lower in FF of women of advanced reproductive age [102, 151, 154]. Testosterone and androstenedione levels show a trend towards higher levels in younger women [102, 151, 153]. In summary, the studies above show that there are no major hormonal differences in FF of older women in spontaneous cycles, but hyperstimulation during IVF may unmask the reduced function of follicle somatic cells in this age group which may point to the underlying dysfunction of these cells in women of advanced reproductive age.

Reproductive aging is associated with redox imbalance and increased apoptosis in the local oocyte microenvironment

Reactive oxygen species (ROS) are byproducts of oxygen metabolism. The three major types are: superoxide (O_2^-) hydrogen peroxide (H_2O_2), and hydroxyl (OH^-). They affect many aspects of physiological reproductive processes from follicle growth to embryo implantation as key signaling molecules [155–158]. There is a delicate balance between ROS and a cell's scavenging ability to neutralize these molecules via enzymatic and non-enzymatic antioxidants [158]. The disruption of this homeostasis can lead to oxidative stress, where the excess levels of these substances damage nucleic acids, lipids, proteins, and carbohydrates and can result in cell death [158, 159]. Age-related increase in oocyte and ovary ROS levels combined with decreased antioxidant capacity was proposed as one of the major mechanisms of female reproductive aging [24, 160–162]. Recent studies demonstrate that this age associated redox imbalance is not restricted to the oocytes. There is an increase in ROS levels and a reduction in the expression of antioxidant genes or antioxidant levels in follicular fluid and/or cumulus and granulosa cells in mice, non-human primates, and humans [146, 163]. Single-cell RNA-Seq analysis of non-human primate granulosa cells from the whole ovary demonstrated that genes down-regulated with aging are enriched in oxidoreductase activity [163]. The same study also showed that ROS levels from FF of women undergoing IVF correlate with chronological age, and this is associated with the down-regulated expression of antioxidant genes (*Idh1*, *Prdx4*, and *Ndufb10*) in granulosa cells.

Several other studies also evaluated the age-related changes in the redox status of the cumulus cells and follicular fluid. The comparison of enzymatic antioxidant levels in FF demonstrates that older women (39–45 yo vs. 27–32 yo) show increased superoxide dismutase (SOD) and decreased glutathione transferase and catalase activities [164]. In contrast, two other studies demonstrated reduced SOD activity in cumulus cells [165] and in FF [166] with advanced reproductive age. However, catalase activity in FF was lower with age consistent with previous findings [166]. Lipid peroxidation, glutathione levels, and glutathione reductase activity are significantly higher in FF of women >37 yo compared to the younger group [166]. Another study shows that although the levels of non-enzymatic antioxidants are not different in FF between younger and older women, FF proteins of older women demonstrate increased levels of ROS related damage [167]. Glutathione S-transferase theta 1 is upregulated in granulosa cells with aging [168].

ROS generation can be promoted by advanced glycation end products (AGEs) [169, 170]. AGEs are formed as the result of non-enzymatic glycation of nucleic acids, lipids, or proteins [170]. AGEs damage tissues either directly through protein cross-linking or indirectly by binding to cell surface receptors for advanced glycation end products (RAGEs) [171]. AGEs interaction with RAGEs has been implicated in the pathogenesis of various age-related diseases [172, 173]. AGEs may also drive reproductive aging by reducing ovarian vascularization and promoting oxidative stress [174]. Significantly higher concentrations of AGEs are found in FF of aged cows [175]. Furthermore, exposure of oocytes from reproductively young and old cows to aged bovine FF or the addition of AGEs to the oocyte maturation medium leads to increased ROS levels, accelerated nuclear maturation, and significantly compromised oocyte developmental competence [175]. In humans, AGEs and RAGEs are detected on granulosa cells [176, 177] and RAGE expression on these cells appears to increase with advanced age [176]. Direct measurement of two AGEs (pentosidine and carboxymethyl lysin) in FF demonstrates that the concentrations of these substances are not significantly different in women ≥35 yo compared to younger women [104]. Soluble isoforms of RAGE (sRAGE) can bind to AGEs and may negate their effects by preventing AGE interaction with the surface receptors [178]. One study that measured sRAGE levels in FF did not observe a difference between women ≥35 yo and <35 yo [104]. However, a different study showed reduced sRAGE levels in older women (≥37 yo vs. <37 yo) [179]. Moreover, sRAGE levels were predictive of fertility treatment success in women of advanced reproductive age in this study [179]. The differences in the findings of these studies may be explained by the various age cut-offs (35 yo vs. 37 yo) used and different patient populations (Japan and China, respectively).

Table 1. Age associated changes in the local oocyte microenvironment. Arrows indicate changes with advanced maternal age compared to young.

Genetic & epigenetic	CC telomere length does not change with age and is longer than in WBCs
	CCs demonstrate "younger" methylation profile than the patient's chronological age or "epigenetic age" of WBCs
	↑ DNA double-strand breaks in CCs
	↓ mtDNA in CCs
	↑ mtDNA deletions in CCs
	↑ cf-mtDNA in FF
Transcriptome	↑ hypoxia stress response (NOS2, RORA, NR4A3), vasculature development (NR2F2, PTHLH), glycolysis (RALGAPA2, TBC1D4) and cAMP turnover genes (PDE4D) in CCs
	↑ creatine kinase B and peroxiredoxin 2 in CCs
	↑ angiogenic genes (ANGPTL4, LEPR, TGFBR3, and FGF2) in CCs
	↓ TGF-β signaling pathway genes (AMH, TGFB1, inhibin, activin receptor) in CCs
	↑ ECM genes (VCAN, TNFAIP6, PTX3, SDC4) in CCs
	Altered miRNA profile in CCs
	↓ lncRNAs with a possible role in CC-oocyte communication in CCs
	Altered miRNA profile in FF
Proteome	↑ fatty acid metabolism proteins (ACAT2, HSD17B4, ALDH9A1, MVK, CYP11A1, FDFT1) in CCs
	↓ oxidative phosphorylation (NDUFA1, UQCRC1, MT-ATP6, ATP5I, MT-ATP8), and post-transcriptional RNA processing proteins (KHSRP, SFPQ, DDX46, SNRPF, ADAR, NHPL1, U2AF2) in CCs
	↓ innate immunity (complement C3, C4), iron transport proteins (serotransferrin, hemopexin precursor), and kininogen in FF
Cytokines	↑ VEGF levels in FF
	↑ IL-3, IL-7, IL-15, TGFβ1, TGFβ3 and MIP-1 in FF
	↓ IL-1Ra and IL-5, ↑ IP-10 in FF
	↓ G-CSF, IL-8, ↑ IL-6, NGF in FF
Metabolome	↓ Glucose, ↑ lactate in FF
	↑ LDH in FF
	↓ D-aspartic acid in FF
	Altered levels of lipid metabolites and apolipoproteins in FF
	Altered expression of metabolic sensors (↓ SIRT3, SIRT5, ↑ SIRT1)
Hormones	↓ Inhibin, estradiol in FF during IVF, unaltered levels in spontaneous cycles
	↓ IGF-1, GHBP in FF
ROS	↑ ROS levels in FF
	↓ Expression of antioxidant genes
	↑ AGE, ↓ sRAGE in FF which likely promote ROS generation
Apoptosis	↑ Apoptosis in granulosa and cumulus cells

ROS – Reactive oxygen species; CC – cumulus cell; GC – granulosa cell; WBC – white blood cell; mtDNA – mitochondrial DNA; FF – follicular fluid; cf-mtDNA – cell free mitochondrial DNA; ECM – extracellular matrix; miRNA – micro-RNA; VEGF - Vascular endothelial growth factor; IL – Interleukin; TGF - Transforming growth factor; MIP - Macrophage inflammatory protein; IP - interferon-inducible protein; G-CSF - Granulocyte colony-stimulating factor; NGF - Nerve growth factor; LDH - lactate dehydrogenase; IGF-1 - Insulin-like growth factor-1; GHBP - Growth hormone-binding protein; AGE - Advanced glycation end products; sRAGE – soluble AGE receptor

Altogether, these studies are all consistent with the impaired redox balance of the local gamete microenvironment with advancing age, as evidenced by increased ROS levels, lipid peroxidation, and structural protein alterations. However, there is limited data on AGEs as well as significant heterogeneity on how the levels of specific antioxidants change with age. Such differences may be due to interfollicular variability or variation in patient characteristics (age, race/ethnicity, geographic region differences) and ovarian stimulation protocols used across studies. Although there are some data that oral supplementation of antioxidants may improve fertility outcomes with aging [146, 180–183], this evidence is also limited, and the majority of the investigations are focused on patients with diminished ovarian reserve. Therefore, more studies are needed to understand the role of redox homeostasis in reproductive aging, to dissect cellular signaling pathways of their action, and to determine whether antioxidants can modulate the progression of reproductive aging.

Impaired redox balance can lead to apoptosis. Apoptosis is highly regulated, programmed cell death which is the underlying mechanism of follicular atresia and ovarian aging [184, 185]. The incidence of granulosa cell apoptosis is one of the indicators of follicle health and is linked to fertility treatment outcomes [186, 187]. Granulosa cells in antral follicles of aged non-human primates show increased DNA damage and apoptosis [163]. Similarly, in humans the incidence of apoptosis in granulosa cells retrieved during IVF is positively correlated with age [188]. Cumulus cells also show increased apoptosis with age, and in patients >40 yo this is also associated with decreased fertilization [189]. Consistent with this, two additional studies reported that CCs from older IVF patients (>37 yo vs. <29 yo and ≥38 yo vs. <38 yo) have

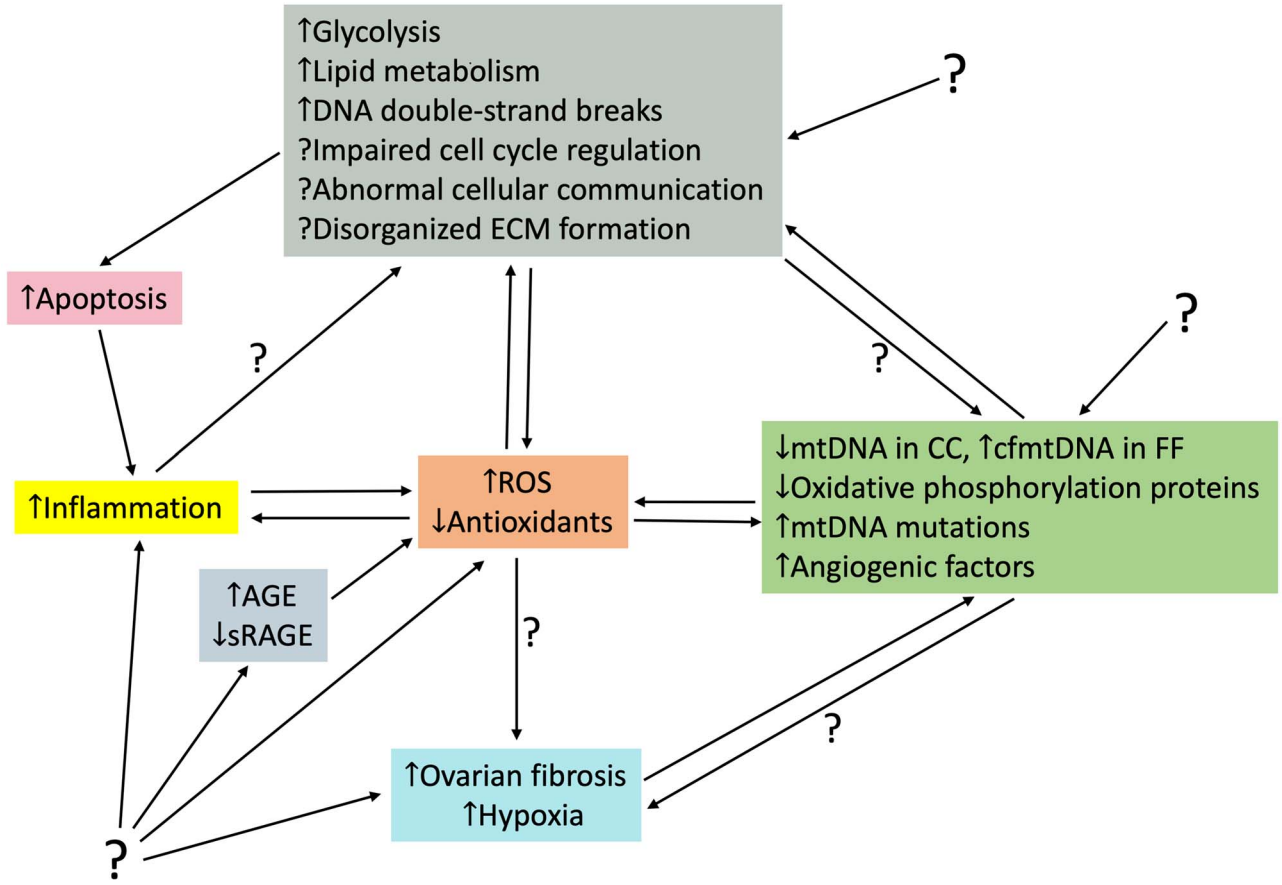

Figure 2. A schematic diagram of the age-related changes and their potential interactions in the local oocyte microenvironment. ROS, Reactive oxygen species; mtDNA, mitochondrial DNA; cf-mtDNA, cell-free mitochondrial DNA; ECM, extracellular matrix; AGE, Advanced glycation end products; sRAGE, soluble AGE receptor.

increased apoptosis rates [48, 190]. These data underscore the viability status of cumulus cells as a determinant of oocyte quality. It is not clear if the increased apoptosis in the local oocyte microenvironment is the cause or consequence of decreased gamete quality with advanced reproductive age, and this needs to be addressed in future studies.

Conclusions

Our systematic review of the literature on the age-related changes in the local oocyte microenvironment of antral follicles revealed that there are unique genetic, epigenetic, transcriptomic, proteomic, and metabolomic alterations in both the cumulus cells and follicular fluid (Table 1). A strength of this review is that the majority of the data are based in human and thus translationally relevant for the understanding of human reproductive aging and the discovery of new non-invasive markers of oocyte and embryo quality. However, there are several caveats, including that the findings are from the investigation of cumulus, mural granulosa cells, and follicular fluid obtained from ovarian stimulation cycles during IVF. Therefore, care must be taken in extrapolating the conclusions of these studies to spontaneous cycles. In addition, multiple variables (i.e., patient lifestyle, co-existent medical conditions, dietary intake, medications etc.) may affect the local oocyte microenvironment and these may not have been adequately controlled for in some studies. Furthermore, different age cut-offs were used when defining younger and older populations and this, combined with the heterogeneity in patient populations, affects generalizability of the findings. In the future, investigation of the local oocyte microenvironment in unstimulated cycles in well-defined reproductive aging animal models may eliminate some of these pitfalls.

The study of ovarian extracellular matrix during reproductive aging is an emerging frontier, and the available evidence demonstrates significant stromal alterations with advancing age [30, 31]. The ovarian ECM becomes stiffer with age, and based on the data reviewed herein, this is occurring concurrently with increased expression of hypoxia related genes. Therefore, follicular hypoxia due to ovarian stromal changes (decreased vascularization, increased fibrosis) and the associated increase in angiogenic factors may be an underlying mechanism of ovarian aging. However, it remains to be investigated whether fibrosis causes hypovascularization or vice versa. Despite the advances in understanding stromal changes in the whole ovary with age, the changes in the ECM of the local oocyte microenvironment have not been well defined and represent another important frontier. In response to ovulatory cues, including LH in natural cycles and hCG in IVF cycles, cumulus cells undergo expansion, synthesize large amounts of HA and disperse in this HA rich ECM. Cumulus expansion is required for ovulation and *in vivo* fertilization [43–45]. The various components and the spatial organization of the cumulus cell ECM, which includes pentraxin 3 and heavy chains of inter-α-trypsin inhibitor are relatively well described [191]. However, we only identified one study that addressed age-associated ECM changes in the local oocyte environment. FF levels of the ECM glycosaminoglycan, HA,

showed a trend of decreasing levels with advancing age (mean of 255.9 ng/ml for <30 yo, 197.9 ng/ml for 31–35 yo, and 142.1 ng/ml for 36–40 yo) but these were not significantly different [192]. However, higher HA levels were observed in FF of women with successful embryo implantation compared to the no pregnancy group, but the average age in this study population was overall young (31–33 yo). Therefore, age-related changes in molecular and biophysical properties of the oocyte microenvironment ECM and their effects on gamete quality and developmental competence remains to be investigated.

Several themes have emerged during our systematic review of the literature. In cumulus and mural granulosa cells, the aging clock moves slower for the telomeres and DNA methylation profile compared to leukocytes. The molecular mechanisms and physiological importance of this differential regulation is an interesting area of future investigation. Transcriptomic and proteomic data demonstrate the alterations in cytokine profiles, and hypoxia, angiogenesis, ECM, cellular communication, and cell regulation pathways. These disturbances are associated with metabolomic alterations and increased ROS generation, redox status imbalance, and increased apoptosis. It is not clear whether these changes are the cause or the consequence of reproductive aging and this question remains to be answered. It is possible that cellular dysfunction associated with aging (e.g., abnormalities in cellular communication, cell–ECM interaction, cell cycle regulation, mitochondrial dysfunction) leads to increased metabolism (carbohydrate and lipid), ROS generation, redox imbalance, change in inflammatory milieu, apoptosis, which in turn exacerbates age-related abnormalities, and a vicious cycle ensues, where the impact of these alterations gets progressively worse with time (Figure 2). This may explain the rapid decline in gamete quality in women after their mid-30s. The mechanisms of how these molecular changes in the local oocyte environment affect gamete quality, directly or indirectly (e.g., secondary to alterations in follicle vascularization, ovarian stromal changes), also need to be elucidated in the future, and these investigations may lead to the discovery of new therapeutic targets for patients with infertility, premature ovarian failure, and menopause. Another important question that remains unanswered is how these changes in the local oocyte environment affect the ovary in general, whether the effects of these alterations extend beyond the follicle and if they could play a role in the accelerated decline of follicle numbers with reproductive aging. Future studies also should take into account individual differences (e.g., genetic factors, obesity, nutrition, medical history, the impact of the environment) while investigating the alterations in the oocyte microenvironment with advancing chronological age.

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Conflict of interest

The authors have nothing to declare.

References

1. Ahmed TA, Ahmed SM, El-Gammal Z, Shouman S, Ahmed A, Mansour R, El-Badri N. Oocyte aging: the role of cellular and environmental factors and impact on female fertility. *Adv Exp Med Biol* 2020; **1247**:109–123.
2. Cloutier CT, Coxworth JE, Hawkes K. Age-related decline in ovarian follicle stocks differ between chimpanzees (pan troglodytes) and humans. *Age (Dordr)* 2015; **37**:9746.
3. Duncan FE, Confino R, Pavone ME. Female reproductive aging: from consequences to mechanisms, markers, and treatments. In: Ram JL, Conn PM (eds.), *Conn's Handbook of Models for Human Aging*. Amsterdam, Netherlands: Elsevier, 2018: 109–130.
4. Mara JN, Zhou LT, Larmore M, Johnson B, Ayiku R, Amargant F, Pritchard MT, Duncan FE. Ovulation and ovarian wound healing are impaired with advanced reproductive age. *Aging (Albany NY)* 2020; **12**:9686–9713.
5. Igarashi H, Takahashi T, Nagase S. Oocyte aging underlies female reproductive aging: biological mechanisms and therapeutic strategies. *Reprod Med Biol* 2015; **14**:159–169.
6. Esencan E, Simsek B, Seli E. Analysis of female demographics in the United States: life expectancy, education, employment, family building decisions, and fertility service utilization. *Curr Opin Obstet Gynecol* 2021; **33**:170–177.
7. Sauer MV. Reproduction at an advanced maternal age and maternal health. *Fertil Steril* 2015; **103**:1136–1143.
8. Hoekstra C, Zhao ZZ, Lambalk CB, Willemsen G, Martin NG, Boomsma DI, Montgomery GW. Dizygotic twinning. *Hum Reprod Update* 2008; **14**:37–47.
9. Flenady V, Koopmans L, Middleton P, Froen JF, Smith GC, Gibbons K, Coory M, Gordon A, Ellwood D, McIntyre HD, Fretts R, Ezzati M. Major risk factors for stillbirth in high-income countries: a systematic review and meta-analysis. *Lancet* 2011; **377**:1331–1340.
10. Aoyama K, Pinto R, Ray JG, Hill AD, Scales DC, Lapinsky SE, Hladunewich MA, Seaward GR, Fowler RA. Association of maternal age with severe maternal morbidity and mortality in Canada. *JAMA Netw Open* 2019; **2**:e199875.
11. Sheen JJ, Wright JD, Goffman D, Kern-Goldberger AR, Booker W, Siddiq Z, D'Alton ME, Friedman AM. Maternal age and risk for adverse outcomes. *Am J Obstet Gynecol* 2018; **219**: 390 e391–390 e315.
12. Hook EB. Rates of chromosome abnormalities at different maternal ages. *Obstet Gynecol* 1981; **58**:282–285.
13. Hook EB, Cross PK, Schreinemachers DM. Chromosomal abnormality rates at amniocentesis and in live-born infants. *JAMA* 1983; **249**:2034–2038.
14. Hollier LM, Leveno KJ, Kelly MA, DD M. CIntire, Cunningham F. G. Maternal age and malformations in singleton births. *Obstet Gynecol* 2000; **96**:701–706.
15. Reefhuis J, Honein MA. Maternal age and non-chromosomal birth defects, Atlanta–1968-2000: teenager or thirty-something, who is at risk. *Birth Defects Res A Clin Mol Teratol* 2004; **70**: 572–579.
16. Cleary-Goldman J, Malone FD, Vidaver J, Ball RH, Nyberg DA, Comstock CH, Saade GR, Eddleman KA, Klugman S, Dugoff L, Timor-Tritsch IE, Craigo SD *et al.* Impact of maternal age on obstetric outcome. *Obstet Gynecol* 2005; **105**:983–990.
17. Cooke A, Mills TA, Lavender T. Advanced maternal age: delayed childbearing is rarely a conscious choice a qualitative study of women's views and experiences. *Int J Nurs Stud* 2012; **49**:30–39.
18. McLernon DJ, Raja EA, Toner JP, Baker VL, Doody KJ, Seifer DB, Sparks AE, Wantman E, Lin PC, Bhattacharya S, Van Voorhis BJ. Predicting personalized cumulative live birth following in vitro fertilization. *Fertil Steril* 2021. Online ahead of print. PMID: 34674824.
19. Luke B, Brown MB, Wantman E, Lederman A, Gibbons W, Schattman GL, Lobo RA, Leach RE, Stern JE. Cumulative birth

rates with linked assisted reproductive technology cycles. *N Engl J Med* 2012; **366**:2483–2491.
20. Duncan FE, Hornick JE, Lampson MA, Schultz RM, Shea LD, Woodruff TK. Chromosome cohesion decreases in human eggs with advanced maternal age. *Aging Cell* 2012; **11**:1121–1124.
21. Beverley R, Snook ML, Brieno-Enriquez MA. Meiotic cohesin and variants associated with human reproductive aging and disease. *Front Cell Dev Biol* 2021; **9**:710033.
22. Yuan L, Yin P, Yan H, Zhong X, Ren C, Li K, Heng BC, Zhang W, Tong G. Single-cell transcriptome analysis of human oocyte ageing. *J Cell Mol Med* 2021; **25**:6289–6303.
23. Babayev E, Seli E. Oocyte mitochondrial function and reproduction. *Curr Opin Obstet Gynecol* 2015; **27**:175–181.
24. Wang L, Tang J, Wang L, Tan F, Song H, Zhou J, Li F. Oxidative stress in oocyte aging and female reproduction. *J Cell Physiol* 2021; **236**:7966–7983.
25. Kinnear HM, Tomaszewski CE, Chang FL, Moravek MB, Xu M, Padmanabhan V, Shikanov A. The ovarian stroma as a new frontier. *Reproduction* 2020; **160**:R25–R39.
26. Foley KG, Pritchard MT, Duncan FE. Macrophage-derived multinucleated giant cells: hallmarks of the aging ovary. *Reproduction* 2021; **161**:V5–V9.
27. Huang Y, Hu C, Ye H, Luo R, Fu X, Li X, Huang J, Chen W, Zheng Y. Inflamm-aging: a new mechanism affecting premature ovarian insufficiency. *J Immunol Res* 2019; **2019**:8069898.
28. Lliberos C, Liew SH, Mansell A, Hutt KJ. The inflammasome contributes to depletion of the ovarian reserve during aging in mice. *Front Cell Dev Biol* 2020; **8**:628473.
29. Briley SM, Jasti S, McCracken JM, Hornick JE, Fegley B, Pritchard MT, Duncan FE. Reproductive age-associated fibrosis in the stroma of the mammalian ovary. *Reproduction* 2016; **152**: 245–260.
30. Amargant F, Manuel SL, Tu Q, Parkes WS, Rivas F, Zhou LT, Rowley JE, Villanueva CE, Hornick JE, Shekhawat GS, Wei JJ, Pavone ME *et al.* Ovarian stiffness increases with age in the mammalian ovary and depends on collagen and hyaluronan matrices. *Aging Cell* 2020; **19**:e13259.
31. Ouni E, Peaucelle A, Haas KT, Van Kerk O, Dolmans MM, Tuuri T, Otala M, Amorim CA. A blueprint of the topology and mechanics of the human ovary for next-generation bioengineering and diagnosis. *Nat Commun* 2021; **12**:5603.
32. Landry DA, Vaishnav HT, Vanderhyden BC. The significance of ovarian fibrosis. *Oncotarget* 2020; **11**:4366–4370.
33. Umehara T, Richards JS, Shimada M. The stromal fibrosis in aging ovary. *Aging (Albany NY)* 2018; **10**:9–10.
34. Wartosch L, Schindler K, Schuh M, Gruhn JR, Hoffmann ER, McCoy RC, Xing J. Origins and mechanisms leading to aneuploidy in human eggs. *Prenat Diagn* 2021; **41**:620–630.
35. Hsueh AJ, Kawamura K, Cheng Y, Fauser BC. Intraovarian control of early folliculogenesis. *Endocr Rev* 2015; **36**:1–24.
36. McGee EA, Hsueh AJ. Initial and cyclic recruitment of ovarian follicles. *Endocr Rev* 2000; **21**:200–214.
37. Edwards RG. Follicular fluid. *J Reprod Fertil* 1974; **37**:189–219.
38. Rodgers RJ, Irving-Rodgers HF. Formation of the ovarian follicular antrum and follicular fluid. *Biol Reprod* 2010; **82**:1021–1029.
39. Baena V, Terasaki M. Three-dimensional organization of transzonal projections and other cytoplasmic extensions in the mouse ovarian follicle. *Sci Rep* 2019; **9**:1262.
40. Diaz FJ, Wigglesworth K, Eppig JJ. Oocytes determine cumulus cell lineage in mouse ovarian follicles. *J Cell Sci* 2007; **120**: 1330–1340.
41. Pedersen T, Peters H. Proposal for a classification of oocytes and follicles in the mouse ovary. *J Reprod Fertil* 1968; **17**:555–557.
42. Dumesic DA, Meldrum DR, Katz-Jaffe MG, Krisher RL, Schoolcraft WB. Oocyte environment: follicular fluid and cumulus cells are critical for oocyte health. *Fertil Steril* 2015; **103**:303–316.
43. Wigglesworth K, Lee KB, O'Brien MJ, Peng J, Matzuk MM, Eppig JJ. Bidirectional communication between oocytes and ovarian follicular somatic cells is required for meiotic arrest of mammalian oocytes. *Proc Natl Acad Sci U S A* 2013; **110**:E3723–E3729.
44. Chen L, Russell PT, Larsen WJ. Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. *Mol Reprod Dev* 1993; **34**:87–93.
45. Varani S, Elvin JA, Yan C, DeMayo J, DeMayo FJ, Horton HF, Byrne MC, Matzuk MM. Knockout of pentraxin 3, a downstream target of growth differentiation factor-9, causes female subfertility. *Mol Endocrinol* 2002; **16**:1154–1167.
46. Fulop C, Szanto S, Mukhopadhyay D, Bardos T, Kamath RV, Rugg MS, Day AJ, Salustri A, Hascall VC, Glant TT, Mikecz K. Impaired cumulus mucification and female sterility in tumor necrosis factor-induced protein-6 deficient mice. *Development* 2003; **130**:2253–2261.
47. Ouzzani M, Hammady H, Fedorowicz Z, Elmagarmid A. Rayyan-a web and mobile app for systematic reviews. *Syst Rev* 2016; **5**:210.
48. Sun XL, Jiang H, Han DX, Fu Y, Liu JB, Gao Y, Hu SM, Yuan B, Zhang JB. The activated DNA double-strand break repair pathway in cumulus cells from aging patients may be used as a convincing predictor of poor outcomes after in vitro fertilization-embryo transfer treatment. *PLoS One* 2018; **13**:e0204524.
49. Varela E, Blasco MA. 2009 nobel prize in physiology or medicine: telomeres and telomerase. *Oncogene* 2010; **29**:1561–1565.
50. Wellinger RJ. In the end, what's the problem? *Mol Cell* 2014; **53**: 855–856.
51. Keefe DL, Liu L. Telomeres and reproductive aging. *Reprod Fertil Dev* 2009; **21**:10–14.
52. Rocca MS, Foresta C, Ferlin A. Telomere length: lights and shadows on their role in human reproduction. *Biol Reprod* 2019; **100**:305–317.
53. Kalmbach KH, Antunes DMF, Dracxler RC, Knier TW, Seth-Smith ML, Wang F, Liu L, Keefe DL. Telomeres and human reproduction. *Fertil Steril* 2013; **99**:23–29.
54. Vasilopoulos E, Fragkiadaki P, Kalliora C, Fragou D, Docea AO, Vakonaki E, Tsoukalas D, Calina D, Buga AM, Georgiadis G, Mamoulakis C, Makrigiannakis A *et al.* The association of female and male infertility with telomere length (review). *Int J Mol Med* 2019; **44**:375–389.
55. Liu L, Franco S, Spyropoulos B, Moens PB, Blasco MA, Keefe DL. Irregular telomeres impair meiotic synapsis and recombination in mice. *Proc Natl Acad Sci U S A* 2004; **101**:6496–6501.
56. Hanson BM, Tao X, Zhan Y, Kim JG, Klimczak AM, Herlihy NS Jr, Scott RT, Seli E. Shorter telomere length of white blood cells is associated with higher rates of aneuploidy among infertile women undergoing in vitro fertilization. *Fertil Steril* 2021; **115**: 957–965.
57. Lara-Molina EE, Franasiak JM, Marin D, Tao X, Díaz-Gimeno P, Florensa M, Martin M, Seli E, Pellicer A. Cumulus cells have longer telomeres than leukocytes in reproductive-age women. *Fertil Steril* 2020; **113**:217–223.
58. Morin SJ, Tao X, Marin D, Zhan Y, Landis J, Bedard J, Scott RT, Seli E. DNA methylation-based age prediction and telomere length in white blood cells and cumulus cells of infertile women with normal or poor response to ovarian stimulation. *Aging* 2018; **10**:3761–3773.
59. Pasquariello R, Ermisch AF, Silva E, McCormick S, Logsdon D, Barfield JP, Schoolcraft WB, Krisher RL. Alterations in oocyte mitochondrial number and function are related to spindle defects and occur with maternal aging in mice and humans. *Biol Reprod* 2019; **100**:971–981.
60. Bentov Y, Casper RF. The aging oocyte–can mitochondrial function be improved. *Fertil Steril* 2013; **99**:18–22.
61. Rebolledo-Jaramillo B, Su MS, Stoler N, McElhoe JA, Dickins B, Blankenberg D, Korneliussen TS, Chiaromonte F, Nielsen R, Holland MM, Paul IM, Nekrutenko A *et al.* Maternal age effect

and severe germ-line bottleneck in the inheritance of human mitochondrial DNA. *Proc Natl Acad Sci U S A* 2014; **111**: 15474–15479.
62. Yang SC, Yu EJ, Park JK, Kim TH, Eum JH, Paek SK, Hwang JY, Lyu SW, Kim JY, Lee WS, Yoon TK, Song H *et al.* The ratio of mitochondrial DNA to genomic DNA copy number in cumulus cell May serve as a biomarker of embryo quality in IVF cycles. *Reprod Sci* 2021; **28**:2495–2502.
63. Tsai HD, Hsieh YY, Hsieh JN, Chang CC, Yang CY, Yang JG, Cheng WL, Tsai FJ, Liu CS. Mitochondria DNA deletion and copy numbers of cumulus cells associated with in vitro fertilization outcomes. *J Reprod Med* 2010; **55**:491–497.
64. Liu Y, Shen Q, Zhao X, Zou M, Shao S, Li J, Ren X, Zhang L. Cell-free mitochondrial DNA in human follicular fluid: a promising bio-marker of blastocyst developmental potential in women undergoing assisted reproductive technology. *Reprod Biol Endocrinol* 2019; **17**:54.
65. Bernstein BE, Meissner A, Lander ES. The mammalian epigenome. *Cell* 2007; **128**:669–681.
66. Horvath S. DNA methylation age of human tissues and cell types. *Genome Biol* 2013; **14**:R115.
67. Field AE, Robertson NA, Wang T, Havas A, Ideker T, Adams PD. DNA methylation clocks in aging: categories, causes, and consequences. *Mol Cell* 2018; **71**:882–895.
68. Christiansen L, Lenart A, Tan Q, Vaupel JW, Aviv A, McGue M, Christensen K. DNA methylation age is associated with mortality in a longitudinal Danish twin study. *Aging Cell* 2016; **15**: 149–154.
69. Perna L, Zhang Y, Mons U, Holleczek B, Saum KU, Brenner H. Epigenetic age acceleration predicts cancer, cardiovascular, and all-cause mortality in a German case cohort. *Clin Epigenetics* 2016; **8**:64.
70. Hanson BM, Tao X, Zhan Y, Jenkins TG, Morin SJ, Scott RT, Seli EU. Young women with poor ovarian response exhibit epigenetic age acceleration based on evaluation of white blood cells using a DNA methylation-derived age prediction model. *Hum Reprod* 2020; **35**:2579–2588.
71. Olsen KW, Castillo-Fernandez J, Zedeler A, Freiesleben NC, Bungum M, Chan AC, Cardona A, Perry JRB, Skouby SO, Borup R, Hoffmann ER, Kelsey G *et al.* A distinctive epigenetic ageing profile in human granulosa cells. *Hum Reprod* 2020; **35**: 1332–1345.
72. Uyar A, Torrealday S, Seli E. Cumulus and granulosa cell markers of oocyte and embryo quality. *Fertil Steril* 2013; **99**:979–997.
73. Mishina T, Tabata N, Hayashi T, Yoshimura M, Umeda M, Mori M, Ikawa Y, Hamada H, Nikaido I, Kitajima TS. Single-oocyte transcriptome analysis reveals aging-associated effects influenced by life stage and calorie restriction. *Aging Cell* 2021; **20**: e13428.
74. Lee MS, Liu CH, Lee TH, Wu HM, Huang CC, Huang LS, Chen CM, Cheng EH. Association of creatin kinase B and peroxiredoxin 2 expression with age and embryo quality in cumulus cells. *J Assist Reprod Genet* 2010; **27**:629–639.
75. Adriaenssens T, Wathlet S, Segers I, Verheyen G, De Vos A, Van der Elst J, Coucke W, Devroey P, Smitz J. Cumulus cell gene expression is associated with oocyte developmental quality and influenced by patient and treatment characteristics. *Hum Reprod* 2010; **25**:1259–1270.
76. Al-Edani T, Assou S, Ferrières A, Bringer Deutsch S, Gala A, Lecellier CH, Aït-Ahmed O, Hamamah S. Female aging alters expression of human cumulus cells genes that are essential for oocyte quality. *Biomed Res Int* 2014; **2014**:964614.
77. Li Y, Li RQ, Ou SB, Zhang NF, Ren L, Wei LN, Zhang QX, Yang DZ. Increased GDF9 and BMP15 mRNA levels in cumulus granulosa cells correlate with oocyte maturation, fertilization, and embryo quality in humans. *Reprod Biol Endocrinol* 2014; **12**:81.
78. Merhi Z, Thornton K, Bonney E, Cipolla MJ, Charron MJ, Buyuk E. Ovarian kisspeptin expression is related to age and to monocyte chemoattractant protein-1. *J Assist Reprod Genet* 2016; **33**:535–543.
79. Ribeiro A, Freitas C, Matos L, Gouveia A, Gomes F, Silva Carvalho JL, Almeida H. Age-related expression of TGF beta family receptors in human cumulus oophorus cells. *J Assist Reprod Genet* 2017; **34**:1121–1129.
80. Shen Q, Chen M, Zhao X, Liu Y, Ren X, Zhang L. Versican expression level in cumulus cells is associated with human oocyte developmental competence. *Syst Biol Reprod Med* 2020; **66**: 176–184.
81. Blasco V, Pinto FM, Fernández-Atucha A, González-Ravina C, Fernández-Sánchez M, Candenas L. Female infertility is associated with an altered expression of the neurokinin B/neurokinin B receptor and kisspeptin/kisspeptin receptor systems in ovarian granulosa and cumulus cells. *Fertil Steril* 2020; **114**:869–878.
82. Molinari E, Bar H, Pyle AM, Patrizio P. Transcriptome analysis of human cumulus cells reveals hypoxia as the main determinant of follicular senescence. *Mol Hum Reprod* 2016; **22**:866–876.
83. Dell'Aversana C, Cuomo F, Longobardi S, D'Hooghe T, Caprio F, Franci G, Santonastaso M, Colacurci N, Barone S, Pisaturo V, Valerio D, Altucci L. Age-related miRNome landscape of cumulus oophorus cells during controlled ovarian stimulation protocols in IVF cycles. *Hum Reprod* 2021; **36**:1310–1325.
84. Bouckenheimer J, Fauque P, Lecellier CH, Bruno C, Commes T, Lemaître JM, De Vos J, Assou S. Differential long non-coding RNA expression profiles in human oocytes and cumulus cells. *Sci Rep* 2018; **8**:2202.
85. Chen D, Zhang Z, Chen B, Ji D, Hao Y, Zhou P, Wei Z, Cao Y. Altered microRNA and Piwi-interacting RNA profiles in cumulus cells from patients with diminished ovarian reserve. *Biol Reprod* 2017; **97**:91–103.
86. Liu T, Zhang L, Joo D, Sun SC. NF-kappaB signaling in inflammation. *Signal Transduct Target Ther* 2017; **2**:e17023.
87. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007; **9**:654–659.
88. Santonocito M, Vento M, Guglielmino MR, Battaglia R, Wahlgren J, Ragusa M, Barbagallo D, Borzi P, Rizzari S, Maugeri M, Scollo P, Tatone C *et al.* Molecular characterization of exosomes and their microRNA cargo in human follicular fluid: bioinformatic analysis reveals that exosomal microRNAs control pathways involved in follicular maturation. *Fertil Steril* 2014; **102**:1751, e1751–1761.
89. Di Pietro C. Exosome-mediated communication in the ovarian follicle. *J Assist Reprod Genet* 2016; **33**:303–311.
90. Diez-Fraile A, Lammens T, Tilleman K, Witkowski W, Verhasselt B, De Sutter P, Benoit Y, Espeel M, D'Herde K. Age-associated differential microRNA levels in human follicular fluid reveal pathways potentially determining fertility and success of in vitro fertilization. *Hum Fertil (Camb)* 2014; **17**:90–98.
91. Moreno JM, Núñez MJ, Quiñonero A, Martínez S, de la Orden M, Simón C, Pellicer A, Díaz-García C, Domínguez F. Follicular fluid and mural granulosa cells microRNA profiles vary in in vitro fertilization patients depending on their age and oocyte maturation stage. *Fertil Steril* 2015; **104**:1037–1046.e1031.
92. Battaglia R, Musumeci P, Ragusa M, Barbagallo D, Scalia M, Zimbone M, Lo Faro JM, Borzì P, Scollo P, Purrello M, Vento EM, Di Pietro C. Ovarian aging increases small extracellular vesicle CD81(+) release in human follicular fluid and influences miRNA profiles. *Aging (Albany NY)* 2020; **12**:12324–12341.
93. McReynolds S, Dzieciatkowska M, McCallie BR, Mitchell SD, Stevens J, Hansen K, Schoolcraft WB, Katz-Jaffe MG. Impact of maternal aging on the molecular signature of human cumulus cells. *Fertil Steril* 2012; **98**:1574–1580.e1575.
94. Hashemitabar M, Bahmanzadeh M, Mostafaie A, Orazizadeh M, Farimani M, Nikbakht R. A proteomic analysis of human follicular fluid: comparison between younger and older women with normal FSH levels. *Int J Mol Sci* 2014; **15**:17518–17540.

95. Jarkovska K, Martinkova J, Liskova L, Halada P, Moos J, Rezabek K, Gadher SJ, Kovarova H. Proteome mining of human follicular fluid reveals a crucial role of complement cascade and key biological pathways in women undergoing in vitro fertilization. *J Proteome Res* 2010; **9**:1289–1301.
96. Miller YI, Smith A, Morgan WT, Shaklai N. Role of hemopexin in protection of low-density lipoprotein against hemoglobin-induced oxidation. *Biochemistry* 1996; **35**:13112–13117.
97. Tilly JL. Molecular and genetic basis of normal and toxicant-induced apoptosis in female germ cells. *Toxicol Lett* 1998; **102-103**:497–501.
98. Guo YL, Colman RW. Two faces of high-molecular-weight kininogen (HK) in angiogenesis: bradykinin turns it on and cleaved HK (HKa) turns it off. *J Thromb Haemost* 2005; **3**: 670–676.
99. Lee A, Christenson LK, Stouffer RL, Burry KA, Patton PE. Vascular endothelial growth factor levels in serum and follicular fluid of patients undergoing in vitro fertilization. *Fertil Steril* 1997; **68**: 305–311.
100. Friedman CI, Danforth DR, Herbosa-Encarnacion C, Arbogast L, Alak BM, Seifer DB. Follicular fluid vascular endothelial growth factor concentrations are elevated in women of advanced reproductive age undergoing ovulation induction. *Fertil Steril* 1997; **68**: 607–612.
101. Manau D, Balasch J, Jiménez W, Fábregues F, Civico S, Casamitjana R, Creus M, Vanrell JA. Follicular fluid concentrations of adrenomedullin, vascular endothelial growth factor and nitric oxide in IVF cycles: relationship to ovarian response. *Hum Reprod* 2000; **15**:1295–1299.
102. Klein NA, Battaglia DE, Woodruff TK, Padmanabhan V, Giudice LC, Bremner WJ, Soules MR. Ovarian follicular concentrations of activin, follistatin, inhibin, insulin-like growth factor I (IGF-I), IGF-II, IGF-binding protein-2 (IGFBP-2), IGFBP-3, and vascular endothelial growth factor in spontaneous menstrual cycles of normal women of advanced reproductive age. *J Clin Endocrinol Metab* 2000; **85**:4520–5.
103. Kawano Y, Zeineh Hasan K, Fukuda J, Mine S, Miyakawa I. Production of vascular endothelial growth factor and angiogenic factor in human follicular fluid. *Mol Cell Endocrinol* 2003; **202**: 19–23.
104. Fujii EY, Nakayama M. The measurements of RAGE, VEGF, and AGEs in the plasma and follicular fluid of reproductive women: the influence of aging. *Fertil Steril* 2010; **94**:694–700.
105. Monteleone P, Giovanni Artini P, Simi G, Casarosa E, Cela V, Genazzani AR. Follicular fluid VEGF levels directly correlate with perifollicular blood flow in normoresponder patients undergoing IVF. *J Assist Reprod Genet* 2008; **25**:183–186.
106. Palmer BF, Clegg DJ. Oxygen sensing and metabolic homeostasis. *Mol Cell Endocrinol* 2014; **397**:51–58.
107. Moncayo HE, Penz-Koza A, Marth C, Gastl G, Herold M, Moncayo R. Vascular endothelial growth factor in serum and in the follicular fluid of patients undergoing hormonal stimulation for in-vitro fertilization. *Hum Reprod* 1998; **13**:3310–3314.
108. Lam PM, Haines C. Vascular endothelial growth factor plays more than an angiogenic role in the female reproductive system. *Fertil Steril* 2005; **84**:1775–1778.
109. Van Blerkom J, Antczak M, Schrader R. The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. *Hum Reprod* 1997; **12**:1047–1055.
110. Machlin JH, Barishansky SJ, Kelsh J, Larmore MJ, Johnson BW, Pritchard MT, Pavone ME, Duncan FE. Fibroinflammatory signatures increase with age in the human ovary and follicular fluid. *Int J Mol Sci* 2021; **22**:4902.
111. Piccinni MP, Vicenti R, Logiodice F, Fabbri R, Kullolli O, Pallecchi M, Paradisi R, Danza G, Macciocca M, Lombardelli L, Seracchioli R. Description of the follicular fluid cytokine and hormone profiles in human physiological natural cycles. *J Clin Endocrinol Metab* 2021; **106**:e721–e738.
112. Bouet PE, Boueilh T, de la Barca JMC, Boucret L, Blanchard S, Ferré-L'Hotellier V, Jeannin P, Descamps P, Procaccio V, Reynier P, May-Panloup P. The cytokine profile of follicular fluid changes during ovarian ageing. *J Gynecol Obstet Hum Reprod* 2020; **49**:101704.
113. Cakiroglu Y, Saltik A, Yuceturk A, Karaosmanoglu O, Kopuk SY, Scott RT, Tiras B, Seli E. Effects of intraovarian injection of autologous platelet rich plasma on ovarian reserve and IVF outcome parameters in women with primary ovarian insufficiency. *Aging (Albany NY)* 2020; **12**:10211–10222.
114. Pellicer A, Garrido N, Albert C, Navarro J, Remohí J, Simón C. Cytokines in older patients undergoing in vitro fertilization: the relationship to the response to controlled ovarian hyperstimulation. *J Assist Reprod Genet* 1999; **16**:247–252.
115. Lédée N, Lombroso R, Lombardelli L, Selva J, Dubanchet S, Chaouat G, Frankenne F, Foidart JM, Maggi E, Romagnani S, Ville Y, Piccinni MP. Cytokines and chemokines in follicular fluids and potential of the corresponding embryo: the role of granulocyte colony-stimulating factor. *Hum Reprod* 2008; **23**: 2001–2009.
116. Malizia BA, Wook YS, Penzias AS, Usheva A. The human ovarian follicular fluid level of interleukin-8 is associated with follicular size and patient age. *Fertil Steril* 2010; **93**:537–543.
117. Yang H, Pang H, Miao C. Ovarian IL-1α and IL-1β levels are associated with primary ovarian insufficiency. *Int J Clin Exp Pathol* 2018; **11**:4711–4717.
118. Palumbo MA, Giuffrida E, Gulino FA, Leonardi E, Cantarella G, Bernardini R. Nerve growth factor (NGF) levels in follicular fluid of infertile patients undergoing to in vitro fertilization (IVF) cycle. *Gynecol Endocrinol* 2013; **29**:1002–1004.
119. Han M, Park SB, Park BJ. Lower growth factor expression in follicular fluid undergone in-vitro fertilization. *Clin Exp Reprod Med* 2011; **38**:210–215.
120. Merhi Z, Buyuk E, Berger DS, Zapantis A, Israel DD, Chua JS, Jindal S. Leptin suppresses anti-Mullerian hormone gene expression through the JAK2/STAT3 pathway in luteinized granulosa cells of women undergoing IVF. *Hum Reprod* 2013; **28**:1661–1669.
121. Llaneza-Suarez D, Llaneza P, Gonzalez C, De-La-Fuente P, Garcia-Ochoa C, Garrido P, Castanon V, Perez-Lopez FR. Assessment of follicular fluid leptin levels and insulin resistance as outcome predictors in women undergoing in vitro fertilization-intracytoplasmic sperm injection. *Fertil Steril* 2014; **102**:1619–1625.
122. Asimakopoulos B, Nikolettos N, Papachristou DN, Simopoulou M, Al-Hasani S, Diedrich K. Follicular fluid levels of vascular endothelial growth factor and leptin are associated with pregnancy outcome of normal women participating in intracytoplasmic sperm injection cycles. *Physiol Res* 2005; **54**:263–270.
123. Russell DL, Gilchrist RB, Brown HM, Thompson JG. Bidirectional communication between cumulus cells and the oocyte: old hands and new players? *Theriogenology* 2016; **86**:62–68.
124. Su YQ, Sugiura K, Eppig JJ. Mouse oocyte control of granulosa cell development and function: paracrine regulation of cumulus cell metabolism. *Semin Reprod Med* 2009; **27**:32–42.
125. Sugiura K, Su YQ, Diaz FJ, Pangas SA, Sharma S, Wigglesworth K, O'Brien MJ, Matzuk MM, Shimasaki S, Eppig JJ. Oocyte-derived BMP15 and FGFs cooperate to promote glycolysis in cumulus cells. *Development* 2007; **134**:2593–2603.
126. Gilchrist RB, Lane M, Thompson JG. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum Reprod Update* 2008; **14**:159–177.
127. Buccione R, Schroeder AC, Eppig JJ. Interactions between somatic cells and germ cells throughout mammalian oogenesis. *Biol Reprod* 1990; **43**:543–547.
128. Leese HJ, Barton AM. Production of pyruvate by isolated mouse cumulus cells. *J Exp Zool* 1985; **234**:231–236.

129. Donahue RP, Stern S. Follicular cell support of oocyte maturation: production of pyruvate in vitro. *J Reprod Fertil* 1968; **17**: 395–398.
130. Pacella L, Zander-Fox DL, Armstrong DT, Lane M. Women with reduced ovarian reserve or advanced maternal age have an altered follicular environment. *Fertil Steril* 2012; **98**:986–994:e981–e982.
131. Adiga SK, Kumar P, Bijoor SN. Lactate dehydrogenase estimation in follicular fluid: correlation with patient age, follicle size and super ovulation in ART cycles. *Eur J Obstet Gynecol Reprod Biol* 2002; **105**:150–154.
132. Dogan B, Karaer A, Tuncay G, Tecellioglu N, Mumcu A. High-resolution (1)H-NMR spectroscopy indicates variations in metabolomics profile of follicular fluid from women with advanced maternal age. *J Assist Reprod Genet* 2020; **37**:321–330.
133. Eppig JJ, Pendola FL, Wigglesworth K, Pendola JK. Mouse oocytes regulate metabolic cooperativity between granulosa cells and oocytes: amino acid transport. *Biol Reprod* 2005; **73**: 351–357.
134. D'Aniello A, Di Cosmo A, Di Cristo C, Annunziato L, Petrucelli L, Fisher G. Involvement of D-aspartic acid in the synthesis of testosterone in rat testes. *Life Sci* 1996; **59**:97–104.
135. D'Aniello A, Di Fiore MM, Fisher GH, Milone A, Seleni A, D'Aniello S, Perna AF, Ingrosso D. Occurrence of D-aspartic acid and N-methyl-D-aspartic acid in rat neuroendocrine tissues and their role in the modulation of luteinizing hormone and growth hormone release. *FASEB J* 2000; **14**:699–714.
136. D'Aniello G, Tolino A, D'Aniello A, Errico F, Fisher GH, Di Fiore MM. The role of D-aspartic acid and N-methyl-D-aspartic acid in the regulation of prolactin release. *Endocrinology* 2000; **141**: 3862–3870.
137. D'Aniello G, Grieco N, Di Filippo MA, Cappiello F, Topo E, D'Aniello E, Ronsini S. Reproductive implication of D-aspartic acid in human pre-ovulatory follicular fluid. *Hum Reprod* 2007; **22**:3178–3183.
138. Valsangkar D, Downs SM. A requirement for fatty acid oxidation in the hormone-induced meiotic maturation of mouse oocytes. *Biol Reprod* 2013; **89**:43.
139. Zhang X, Wang T, Song J, Deng J, Sun Z. Study on follicular fluid metabolomics components at different ages based on lipid metabolism. *Reprod Biol Endocrinol* 2020; **18**:42.
140. Cordeiro FB, Montani DA, Pilau EJ, Gozzo FC, Fraietta R, Turco EGL. Ovarian environment aging: follicular fluid lipidomic and related metabolic pathways. *J Assist Reprod Genet* 2018; **35**: 1385–1393.
141. Von Wald T, Monisova Y, Hacker MR, Yoo SW, Penzias AS, Reindollar RR, Usheva A. Age-related variations in follicular apolipoproteins may influence human oocyte maturation and fertility potential. *Fertil Steril* 2010; **93**:2354–2361.
142. Peng L, Yuan Z, Ling H, Fukasawa K, Robertson K, Olashaw N, Koomen J, Chen J, Lane WS, Seto E. SIRT1 deacetylates the DNA methyltransferase 1 (DNMT1) protein and alters its activities. *Mol Cell Biol* 2011; **31**:4720–4734.
143. Mao Z, Hine C, Tian X, Van Meter M, Au M, Vaidya A, Seluanov A, Gorbunova V. SIRT6 promotes DNA repair under stress by activating PARP1. *Science* 2011; **332**:1443–1446.
144. Pacella-Ince L, Zander-Fox DL, Lan M. Mitochondrial SIRT3 and its target glutamate dehydrogenase are altered in follicular cells of women with reduced ovarian reserve or advanced maternal age. *Hum Reprod* 2014; **29**:1490–1499.
145. Pacella-Ince L, Zander-Fox DL, Lane M. Mitochondrial SIRT5 is present in follicular cells and is altered by reduced ovarian reserve and advanced maternal age. *Reprod Fertil Dev* 2014; **26**: 1072–1083.
146. Ben-Meir A, Kim K, McQuaid R, Esfandiari N, Bentov Y, Casper RF, Jurisicova A. Co-enzyme Q10 supplementation rescues cumulus cells dysfunction in a maternal aging model. *Antioxidants (Basel, Switzerland)* 2019; **8**:58.
147. Valerio D, Luddi A, De Leo V, Labella D, Longobardi S, Piomboni P. SA1/SA2 cohesion proteins and SIRT1-NAD+ deacetylase modulate telomere homeostasis in cumulus cells and are eligible biomarkers of ovarian aging. *Hum Reprod* 2018; **33**:887–894.
148. Dionyssiou-Asteriou A, Drakakis P, Loutradis D. Follicular fluid inhibin levels in relation to age in patients in an in vitro fertilization and embryo transfer programme. *Eur J Obstet Gynecol Reprod Biol* 1993; **51**:55–61.
149. Pellicer A, Marí M, de los Santos MJ, Simón C, Remohí J, Tarín JJ. Effects of aging on the human ovary: the secretion of immunoreactive alpha-inhibin and progesterone. *Fertil Steril* 1994; **61**:663–668.
150. Chang CL, Wang TH, Horng SG, Wu HM, Wang HS, Soong YK. The concentration of inhibin B in follicular fluid: relation to oocyte maturation and embryo development. *Hum Reprod* 2002; **17**:1724–1728.
151. Klein NA, Battaglia DE, Miller PB, Branigan EF, Giudice LC, Soules MR. Ovarian follicular development and the follicular fluid hormones and growth factors in normal women of advanced reproductive age. *J Clin Endocrinol Metab* 1996; **81**:1946–1951.
152. Klein NA, Illingworth PJ, Groome NP, McNeilly AS, Battaglia DE, Soules MR. Decreased inhibin B secretion is associated with the monotropic FSH rise in older, ovulatory women: a study of serum and follicular fluid levels of dimeric inhibin a and B in spontaneous menstrual cycles. *J Clin Endocrinol Metab* 1996; **81**: 2742–2745.
153. Yokota Y, Yokota M, Yokota H, Ishikawa Y, Sato S, Araki Y. Relationship between follicular fluid hormone levels, embryo quality, and maternal age during in vitro fertilization after the short or long protocol with a gonadotropin releasing hormone agonist. *Reprod Med Biol* 2003; **2**:165–169.
154. Amit T, Dirnfeld M, Barkey RJ, Peleg I, Hacham H, Abramovici H, Blumenfeld Z. Growth hormone-binding protein (GH-BP) levels in follicular fluid from human preovulatory follicles: correlation with serum GH-BP levels. *J Clin Endocrinol Metab* 1993; **77**:33–39.
155. Kala M, Shaikh MV, Nivsarkar M. Equilibrium between antioxidants and reactive oxygen species: a requisite for oocyte development and maturation. *Reprod Med Biol* 2017; **16**: 28–35.
156. Suzuki T, Sugino N, Fukaya T, Sugiyama S, Uda T, Takaya R, Yajima A, Sasano H. Superoxide dismutase in normal cycling human ovaries: immunohistochemical localization and characterization. *Fertil Steril* 1999; **72**:720–726.
157. Jozwik M, Wolczynski S, Jozwik M, Szamatowicz M. Oxidative stress markers in preovulatory follicular fluid in humans. *Mol Hum Reprod* 1999; **5**:409–413.
158. Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. *Reprod Biol Endocrinol* 2005; **3**:28.
159. Sies H. Oxidative stress: oxidants and antioxidants. *Exp Physiol* 1997; **82**:291–295.
160. Sasaki H, Hamatani T, Kamijo S, Iwai M, Kobanawa M, Ogawa S, Miyado K, Tanaka M. Impact of oxidative stress on age-associated decline in oocyte developmental competence. *Front Endocrinol (Lausanne)* 2019; **10**:811.
161. Tarín JJ, Pérez-Albalá S, Cano A. Oral antioxidants counteract the negative effects of female aging on oocyte quantity and quality in the mouse. *Mol Reprod Dev* 2002; **61**:385–397.
162. Devine PJ, Perreault SD, Luderer U. Roles of reactive oxygen species and antioxidants in ovarian toxicity. *Biol Reprod* 2012; **86**:27.
163. Wang S, Zheng Y, Li J, Yu Y, Zhang W, Song M, Liu Z, Min Z, Hu H, Jing Y, He X, Sun L *et al.* Single-cell transcriptomic atlas of primate ovarian aging. *Cell* 2020; **180**:585, e519–600.
164. Carbone MC, Tatone C, Delle Monache S, Marci R, Caserta D, Colonna R, Amicarelli F. Antioxidant enzymatic defences in human follicular fluid: characterization and age-dependent changes. *Mol Hum Reprod* 2003; **9**:639–643.

165. Matos L, Stevenson D, Gomes F, Silva-Carvalho JL, Almeida H. Superoxide dismutase expression in human cumulus oophorus cells. *Mol Hum Reprod* 2009; **15**:411–419.
166. Debbarh H, Louanjli N, Aboulmaouahib S, Jamil M, Ahbbas L, Kaarouch I, Sefrioui O, Cadi R. Antioxidant activities and lipid peroxidation status in human follicular fluid: age-dependent change. *Zygote* 2021; **29**:490–494.
167. Luddi A, Governini L, Capaldo A, Campanella G, De Leo V, Piomboni P, Morgante G. Characterization of the age-dependent changes in antioxidant defenses and protein's sulfhydryl/carbonyl stress in human follicular fluid. *Antioxidants (Basel, Switzerland)* 2020; **9**:927.
168. Ito M, Muraki M, Takahashi Y, Imai M, Tsukui T, Yamakawa N, Nakagawa K, Ohgi S, Horikawa T, Iwasaki W, Iida A, Nishi Y *et al.* Glutathione S-transferase theta 1 expressed in granulosa cells as a biomarker for oocyte quality in age-related infertility. *Fertil Steril* 2008; **90**:1026–1035.
169. Uribarri J, Cai W, Peppa M, Goodman S, Ferrucci L, Striker G, Vlassara H. Circulating glycotoxins and dietary advanced glycation endproducts: two links to inflammatory response, oxidative stress, and aging. *J Gerontol A Biol Sci Med Sci* 2007; **62**: 427–433.
170. Rungratanawanich W, Qu Y, Wang X, Essa MM, Song BJ. Advanced glycation end products (AGEs) and other adducts in aging-related diseases and alcohol-mediated tissue injury. *Exp Mol Med* 2021; **53**:168–188.
171. Schmidt AM, Yan SD, Yan SF, Stern DM. The biology of the receptor for advanced glycation end products and its ligands. *Biochim Biophys Acta* 2000; **1498**:99–111.
172. Byun K, Yoo Y, Son M, Lee J, Jeong GB, Park YM, Salekdeh GH, Lee B. Advanced glycation end-products produced systemically and by macrophages: a common contributor to inflammation and degenerative diseases. *Pharmacol Ther* 2017; **177**:44–55.
173. Abate G, Marziano M, Rungratanawanich W, Memo M, Uberti D. Nutrition and AGE-ing: focusing on Alzheimer's disease. *Oxidative Med Cell Longev* 2017; **2017**:7039816.
174. Tatone C, Amicarelli F, Carbone MC, Monteleone P, Caserta D, Marci R, Artini PG, Piomboni P, Focarelli R. Cellular and molecular aspects of ovarian follicle ageing. *Hum Reprod Update* 2008; **14**:131–142.
175. Takeo A, Kimura K, Shirasuna K, Kuwayama T, Iwata H. Age-associated deterioration in follicular fluid induces a decline in bovine oocyte quality. *Reprod Fertil Dev* 2017; **29**: 759–767.
176. Stensen MH, Tanbo T, Storeng R, Fedorcsak P. Advanced glycation end products and their receptor contribute to ovarian ageing. *Hum Reprod* 2014; **29**:125–134.
177. Diamanti-Kandarakis E, Piperi C, Patsouris E, Korkolopoulou P, Panidis D, Pawelczyk L, Papavassiliou AG, Duleba AJ. Immunohistochemical localization of advanced glycation end-products (AGEs) and their receptor (RAGE) in polycystic and normal ovaries. *Histochem Cell Biol* 2007; **127**:581–589.
178. Hudson BI, Carter AM, Harja E, Kalea AZ, Arriero M, Yang H, Grant PJ, Schmidt AM. Identification, classification, and expression of RAGE gene splice variants. *FASEB J* 2008; **22**: 1572–1580.
179. Li YJ, Chen JH, Sun P, Li JJ, Liang XY. Intrafollicular soluble RAGE benefits embryo development and predicts clinical pregnancy in infertile patients of advanced maternal age undergoing in vitro fertilization. Journal of Huazhong University of Science and Technology. Medical sciences = Hua zhong ke ji da xue xue bao. Yi xue Ying De wen ban = Huazhong keji daxue xuebao. *Yixue Yingdewen ban* 2017; **37**:243–247.
180. Tamura H, Jozaki M, Tanabe M, Shirafuta Y, Mihara Y, Shinagawa M, Tamura I, Maekawa R, Sato S, Taketani T, Takasaki A, Reiter RJ *et al.* Importance of melatonin in assisted reproductive technology and ovarian aging. *Int J Mol Sci* 2020; **21**:1135.
181. Zhang M, Lu Y, Chen Y, Zhang Y, Xiong B. Insufficiency of melatonin in follicular fluid is a reversible cause for advanced maternal age-related aneuploidy in oocytes. *Redox Biol* 2020; **28**:101327.
182. Song C, Peng W, Yin S, Zhao J, Fu B, Zhang J, Mao T, Wu H, Zhang Y. Melatonin improves age-induced fertility decline and attenuates ovarian mitochondrial oxidative stress in mice. *Sci Rep* 2016; **6**:35165.
183. Zhang Y, Zhang C, Shu J, Guo J, Chang HM, Leung PCK, Sheng JZ, Huang H. Adjuvant treatment strategies in ovarian stimulation for poor responders undergoing IVF: a systematic review and network meta-analysis. *Hum Reprod Update* 2020; **26**:247–263.
184. Regan SLP, Knight PG, Yovich JL, Leung Y, Arfuso F, Dharmarajan A. Granulosa cell apoptosis in the ovarian follicle-a changing view. *Front Endocrinol (Lausanne)* 2018; **9**:61.
185. Matsuda F, Inoue N, Manabe N, Ohkura S. Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells. *J Reprod Dev* 2012; **58**:44–50.
186. Oosterhuis GJ, Michgelsen HW, Lambalk CB, Schoemaker J, Vermes I. Apoptotic cell death in human granulosa-lutein cells: a possible indicator of in vitro fertilization outcome. *Fertil Steril* 1998; **70**:747–749.
187. Nakahara K, Saito H, Saito T, Ito M, Ohta N, Sakai N, Tezuka N, Hiroi M, Watanabe H. Incidence of apoptotic bodies in membrana granulosa of the patients participating in an in vitro fertilization program. *Fertil Steril* 1997; **67**:302–308.
188. Sadraie SH, Saito H, Kaneko T, Saito T, Hiroi M. Effects of aging on ovarian fecundity in terms of the incidence of apoptotic granulosa cells. *J Assist Reprod Genet* 2000; **17**:168–173.
189. Lee KS, Joo BS, Na YJ, Yoon MS, Choi OH, Kim WW. Cumulus cells apoptosis as an indicator to predict the quality of oocytes and the outcome of IVF-ET. *J Assist Reprod Genet* 2001; **18**:490–498.
190. Moffatt O, Drury S, Tomlinson M, Afnan M, Sakkas D. The apoptotic profile of human cumulus cells changes with patient age and after exposure to sperm but not in relation to oocyte maturity. *Fertil Steril* 2002; **77**:1006–1011.
191. Salustri A, Campagnolo L, Klinger FG, Camaioni A. Molecular organization and mechanical properties of the hyaluronan matrix surrounding the mammalian oocyte. *Matrix Biol* 2019; **78-79**: 11–23.
192. Babayan A, Neuer A, Dieterle S, Bongiovanni AM, Witkin SS. Hyaluronan in follicular fluid and embryo implantation following in vitro fertilization and embryo transfer. *J Assist Reprod Genet* 2008; **25**:473–476.