REPRODUCTION
REVIEW

Healthy ageing and spermatogenesis

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Abstract

Delayed family planning and increased parental age increase the risk for infertility and impaired offspring health. While the impact of ageing on oogenesis is well studied, this is less understood on spermatogenesis. Assessing ageing effects on the male germline presents a challenge in differentiating between the effects of ageing-associated morbidities, infertility and 'pure' ageing. However, understanding the impact of ageing on male germ cells requires the separation of age from other factors. In this review, we therefore discuss the current knowledge on healthy ageing and spermatogenesis. Male ageing has been previously associated with declining sperm parameters, disrupted hormone secretion and increased time-to-pregnancy, among others. However, recent data show that healthy ageing does not deteriorate testicular function in terms of hormone production and spermatogenic output. In addition, intrinsic, age-dependent, highly specific processes occur in ageing germ cells that are clearly distinct from somatic ageing. Changes in spermatogonial stem cell populations indicate compensation for stem cell exhaustion. Alterations in the stem cell niche and molecular ageing signatures in sperm can be observed in ageing fertile men. DNA fragmentation rates as well as changes in DNA methylation patterns and increased telomere length are hallmarks of ageing sperm. Taken together, we propose a putative link between the re-activation of quiescent A_{dark} spermatogonia and molecular changes in aged sperm descending from these activated spermatogonia. We suggest a baseline of 'pure' age effects in male germ cells which can be used for subsequent studies in which the impact of infertility or co-morbidities will be studied.

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Introduction

Delaying parenthood until later ages has become increasingly common in industrialised countries, mainly due to socio-economic reasons (Mills *et al.* 2011). This delay is also reflected by the age of patients undergoing artificial reproductive techniques (ART). For example, the mean age for women and men undergoing *in vitro* fertilisation (IVF) treatments in Germany increased by more than 3 years from 1997 to 2019 (Blumenauer *et al.* 2020). In the United States of America, mean paternal age has increased from 27.4 to 30.9 years since the 1970s (Khandwala *et al.* 2017). The negative impact of increased parental age was mostly attributed to female age, and comparatively fewer studies evaluated the consequences of advanced paternal age. However, evidence is growing that increased paternal age also negatively affects fertility, pregnancy and children's health (Almeida *et al.* 2017, Khandwala *et al.* 2018).

Ageing is a multifactorial process characterised by various intrinsic and extrinsic factors. Besides age itself, disease and lifestyle can influence reproductive function and germ cell production. Although it is difficult to assess these factors separately in humans, this review tries to address 'pure' ageing effects on spermatogenesis in healthy men, which we define as ageing-associated effects caused only by age itself and not by age-related morbidities.

The production of male gametes, spermatogenesis, is a complex process regulated through endocrine and paracrine signals. Spermatogenesis occurs uninterrupted throughout adult life because of spermatogonial stem cells (SSCs), which either self-renew, thus maintaining the stem cell population, or give rise to daughter cells that enter spermatogenesis. Primates, including the human, present two types of undifferentiated type A spermatogonia that can be distinguished based on their morphology: A_{dark} and A_{pale}. Under physiological conditions, A_{dark} spermatogonia show very low proliferative activity and are seemingly only activated to divide after germ cell depletion due to testicular damage (van Alphen *et al.* 1988). It has been suggested that SSCs may be an A_{dark} subpopulation (Sharma *et al.* 2019, Caldeira-Brant *et al.* 2020). Spermatogonia entering spermatogenesis undergo a process that includes meiosis and spermiogenesis, which results in spermatozoa that are released into the tubular lumen.

Inititiation and maintenance of spermatogenesis essentially depends on a functional SSC population, which in turn requires a working stem cell niche and

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a microenvironment balancing and supporting self-renewal and germ cell differentiation. The niche is mainly composed of peritubular myoid cells (PMCs), at the base of the seminiferous tubule, and Sertoli cells, which support the different germ cell types. The latter are responsible for guaranteeing an immunologically privileged site, building the blood–testis barrier by tight and gap junctions. In this microenvironment, germ cell maintenance and maturation are regulated through various types of chemokines and cytokines, hormones and metabolites released by Sertoli cells. Because the human spermatogenic cycle constantly takes 72 days, ageing presumably does not affect the process of differentiating germ cells, but instead the long-living Sertoli cells and the SSC population are expected to be sensitive to ageing (Weinbauer *et al.* 2010).

So far, reproductive ageing has been studied mainly in patient cohorts, often lacking normalisation for confounding factors like infertility or ageing-related morbidities, both of which impact spermatogenesis. Here, we intend to predominantly focus on data from healthy men or men with normal spermatogenesis to review how ageing affects male reproductive function and gamete production. In brief, we will discuss the following topics:

- Reproductive ageing in healthy men/men with normal spermatogenesis
- Ageing-associated spermatogonial stem cell exhaustion
- Male germline genomic instability
- Telomeres in the male germline
- Age-associated sperm DNA methylation changes in sperm

Reproductive ageing in healthy men/men with normal spermatogenesis

The reproductive capacity of men declines with age, increasing the risk for infertility. Previous studies reported a decline in semen parameters and changes along the hypothalamic–pituitary–gonadal axis leading to reduced sexual and overall wellbeing (Sartorius & Nieschlag 2010, Almeida *et al.* 2017). Some studies reported adverse alterations in seminal fluid volume, sperm motility and morphology with increasing male age (Eskenazi *et al.* 2003, Beguería *et al.* 2014, Paoli *et al.* 2019). Regarding sperm concentration, reports range from an age-dependent decline (Auger *et al.* 1995, Luna *et al.* 2009), to no association with age (Irvine *et al.* 1996, Whitcomb *et al.* 2011) or even increased sperm concentration with age (Andolz *et al.* 1999, Beguería *et al.* 2014). This inconsistency might result from the absence of participant selection criteria and lack of control for confounding factors, such as abstinence time (Fisch *et al.* 1996). When semen parameters were studied in healthy men or proven fathers, no drastic effects with age were found (Nieschlag *et al.* 1982, Laurentino *et al.* 2020).

At the testicular level, studies described a general decrease in the number of male germ cells with increasing age in unselected cohorts (Holstein *et al.* 1988, Paniagua *et al.* 1991). Specifically, a reduction of A_{pale} spermatogonia was reported during the 6th decade of life and decrease of A_{dark} spermatogonia in the 8th decade (Nistal *et al.* 1987). Furthermore, the number of round spermatids in older men was found to be decreased in comparison to young men (Jiang *et al.* 2014). In contrast, in a study from our group selecting for qualitatively normal spermatogenesis, we could not confirm these findings and found spermatogenic output to remain constant over all age groups (Pohl *et al.* 2019).

Several studies have associated advanced paternal age with higher miscarriage rates, increased time to pregnancy and adverse clinical pregnancy outcome (in assisted reproduction treatments) (Rochebrochard & Thonneau 2002, Mutsaerts *et al.* 2012, Koh *et al.* 2013). In addition, increased parental age was associated with low birth weight, elevated rates of gestational diabetes and a low Apgar score – a scoring system to evaluate the state of health in newborn children. Increased male age alone (when adjusted for maternal age), however, is associated with an increased risk of premature birth and offspring suffering from seizures (Khandwala *et al.* 2018).

The aforementioned adverse consequences of increased paternal age can predominantly be associated with *de novo* mutations in germ cells arising from multiple mitotic divisions. As men age, the number of stem cell divisions increases, resulting in progressively accumulating DNA replication errors. For example, the production of sperm in a 20-year-old man is estimated to be preceded by ~190 cell divisions. This number assumingly rises to ~650 divisions by the age of 40 years (Goriely 2016).

In 1955, Penrose was the first to report a link between advanced paternal age and impaired health conditions in the offspring by describing a significantly increased mean age of fathers from children suffering from achondroplasia (Penrose 1955). This disease is part of the so-called paternal age effect (PAE)-disorders, a group of severe developmental disorders that also includes Apert syndrome, thanatotropic dysplasia and Costello syndrome (Goriely & Wilkie 2012). Besides that, PAE-disorders also include psychiatric conditions such as certain types of schizophrenia and autism (Kovac *et al.* 2013, Gromoll *et al.* 2016).

Developmental PAE-disorders are typically associated with *de novo* mutations in male germ cells, which show an increased risk for DNA-copy errors due to repeated cycles of spermatogenesis during the course of male life (Gao *et al.* 2016, Wu *et al.* 2020). A study on child–parent trios estimated an increase in the *de novo* mutation rate of about two mutations per year and a doubling of paternal mutations every 16.5 years (Kong *et al.* 2012). Conceivably, the increased *de novo* mutation rate leads to an enhanced risk for paternally

inherited congenital disorders (Crow 2000, Kong *et al.* 2012, Jónsson *et al.* 2017). Reduced accuracy of DNA replication and inefficient repair mechanisms in the germline have also been hypothesised to contribute to the accelerated mutation rate with increasing age (Chianese *et al.* 2014). However, recent genetic studies proposed the existence of a more complex mechanism behind PAE (Goldmann *et al.* 2016, Gratten *et al.* 2016, Rahbari *et al.* 2016), as the increase in *de novo* mutation rate is not sufficient to explain the paternal age effect of polygenic diseases such as neuropsychiatric disorders. Changes in DNA methylation and DNA fragmentation have been suggested to be involved in the aetiology of these complex disorders; however, the precise mechanism is not completely understood (Yatsenko & Turek 2018).

One of the recent concepts about *de novo* mutations – different from the idea of accumulation of copy errors only – is the 'selfish spermatogonial selection' hypothesis. It suggests that *de novo* mutations in genes for tyrosine kinase receptors, such as the fibroblast growth factor receptor 2 (FGFR2; found in patients with Apert syndrome) or its paralog FGFR3 (found in cases of achondroplasia) offer a selective advantage to spermatogonia carrying this mutation, causing their clonal expansion (Goriely *et al.* 2009, Maher *et al.* 2016).

According to Kitadate *et al.* (2019), spermatogonia compete for FGF2, a factor that is important for SSC homing and homeostasis regulation. It is conceivable that *FGFR2* mutations result in an enhanced response to FGF2 and that spermatogonia carrying such mutations outgrow WT spermatogonia due to this improved response. A similar mechanism can be envisioned for FGFR3 and its ligand, although this has not yet been evaluated. This would explain why undifferentiated spermatogonial clones are superior in terms of outgrowth but not in differentiation capacity. Functional studies in mice, however, did not unequivocally support the selfish selection hypothesis. While Apert syndrome *FGFR2* mutation enhanced competitiveness and stem cell fitness of affected spermatogonia in mice (Martin *et al.* 2014), no advantage could be observed for the Costello-syndrome causing HRAS mutation (Yamada *et al.* 2019). Furthermore, the rate of mutated clones found in studies on selfish spermatogonial selection was far beyond the incidences seen in the offspring. As this clonal outgrowth was only analysed in a cohort of old men but not in younger controls, the actual phenomenon remains to be elucidated in future studies. Functional studies in human testicular tissues are not available and descriptive studies are scarce due to technical limitations (Pohl *et al.* 2019).

Ageing-associated spermatogonial stem cell exhaustion

Adult stem cells mediate tissue homeostasis and regeneration during their lifetime. Thus, declining stem cell proliferation capacity and decreasing stem cell numbers could negatively influence homeostasis and regeneration in older ages. This is summarised under the term 'stem cell exhaustion', representing one of the hallmarks of ageing. Molecular impairment of the stem cell compartment, such as DNA damage, epigenetic changes and telomere attrition, also contributes to stem cell exhaustion (López-Otín *et al.* 2013, Krauss & de Haan 2016). In stem cell systems of high-turnover, for example, the seminiferous epithelium and the hematopoietic stem cell system, coexistence of reserve (quiescent) and active (proliferating) stem cell pools has been observed (Sharma *et al.* 2019).

Germ cell markers have been identified for different spermatogonial subpopulations (Kossack *et al.* 2013, Di Persio *et al.* 2017). A recent study provides evidence that the morphological criteria of human A_{dark} spermatogonia can define a population with stem cell characteristics, a finding that recalls observations from the 1960s (Clermont 1966, Caldeira-Brant *et al.* 2020). Although recent studies suggest new concepts on spermatogonial subpopulations based on single-cell RNA expression profiles, for example, the existence of transcriptional/developmental spermatogonial states characterised by bi-directional dynamics (Guo *et al.* 2018), no definite marker for SSCs has yet been identified. Therefore, studies on SSC exhaustion in the human testis are scarce.

Functional insights on SSCs were mainly obtained from studies on primate testes irradiation: van Alphen *et al.* (1988) reported an increased repopulation of the testis with A_{dark} spermatogonia following the irradiation-induced depletion of A_{pale} spermatogonia. This is similar to the activation of reserve stem cells occurring after somatic tissue damage, resulting in the replenishment of active stem cells lost by the lesions (Wilson *et al.* 2008, Li & Clevers 2010).

We detected increased rates of proliferating spermatogonia and re-activation of previously quiescent A_{dark} spermatogonia with increasing age in testicular tissues with full spermatogenesis (Pohl *et al.* 2019). This is in accordance with a study showing increased proliferative activity in spermatogonia with increased age (Codesal *et al.* 1989). This appears to be a common and conserved concept amongst taxa and tissues: a study in *D. melanogaster* showed hyperproliferation of intestinal stem cells in aged animals, proposed to be associated with high stress levels and deregulation of stress responses (Biteau *et al.* 2010). In addition, increased proliferation and loss of quiescence were shown for muscle stem cells in old mice (Chakkalakal *et al.* 2012). More recently, a study showed that murine SSCs cultured for a longer time (60 vs 5 months) proliferated more actively and that SSC self-renewal factors were enhanced (Kanatsu-Shinohara *et al.* 2019).

Whether the previously reported age-related germ cell loss in the human testis (Holstein *et al.* 1988, Kimura *et al.* 2003, Jiang *et al.* 2014) results from stem cell depletion due to increased proliferation and loss of

quiescent spermatogonia remains to be demonstrated (Li & Clevers 2010). Two possible conditions might trigger ageing-associated hyperproliferation: (i) increased demand for active and/or differentiating cells evoking increased proliferation or (ii) a loss of quiescence resulting in overall higher spermatogonia proliferative rates (Fig. 1). The first hypothesis would fit with the 'DNA damage theory of ageing' (see below), where increased proliferation rates can be seen as a compensatory mechanism in response to impaired stem cell function associated with age-dependent accumulation of DNA damage (Freitas & de Magalhães 2011). This concept concurs with the decreased spermatogenic efficiency detected in aged testis (Johnson *et al.* 1990, Pohl *et al.* 2019) and evidence showing that older transplanted SSCs maintain their stem cell activity but do not produce sperm (Kanatsu-Shinohara *et al.* 2003). In addition, increased proliferation might also be due to clonal dominance of stem cells with mutations conferring increased proliferation, that is 'selfish spermatogonial selection' (Goriely & Wilkie 2012). The second hypothesis, that is the loss of quiescence, was proposed as the main driving force of ageing in other stem cell systems, for example, muscle stem cells. Malfunction of the stem cell niche due to impaired FGF2 signalling disrupts the integrity of the stem cell niche and results in increased proliferation of stem cells (Chakkalakal *et al.* 2012). Similarly, it is conceivable that in the testis loss of quiescence in A_{dark} spermatogonia might be triggered by an altered germ cell niche (Pohl *et al.* 2019, Schmid *et al.* 2019). It was demonstrated that FGFs play a crucial role in SSC homing and homeostasis and that SSCs localise preferentially to areas with higher concentration of FGFs (Kitadate *et al.* 2019). In the aforementioned long-term mouse SSC culture approach, older SSCs showed altered responsiveness to SSC self-renewal factors FGF2 and GDNF (Kanatsu-Shinohara *et al.* 2003). Against this background, we might speculate that disturbances in SSC regulation, through FGFs or other mitogens, lead to loss of quiescence in SSCs with increasing age.

In fact, ageing-associated impairments were previously described in cells comprising the testicular stem cell niche, including Sertoli cells, Leydig cells and peritubular cells. Different studies from the 1980s demonstrated decreased Sertoli cell numbers (Johnson *et al.* 1984, Paniagua *et al.* 1987*a*) and morphological alterations in Sertoli cells, including secondary lysosomes, accumulation of lipid droplets and vacuoles in testes from men 65 years of age and older. As the authors also observed decreased germ cell numbers, they assumed that remnants of degenerated germ cells were being absorbed by Sertoli cells via phagocytosis (Paniagua *et al.* 1987*b*, 1991). A reduction in Sertoli cell numbers with ageing was also recently confirmed in men with normal spermatogenesis (Mularoni *et al.* 2020). Interestingly, in our study on men with normal spermatogenesis, we found increased Sertoli

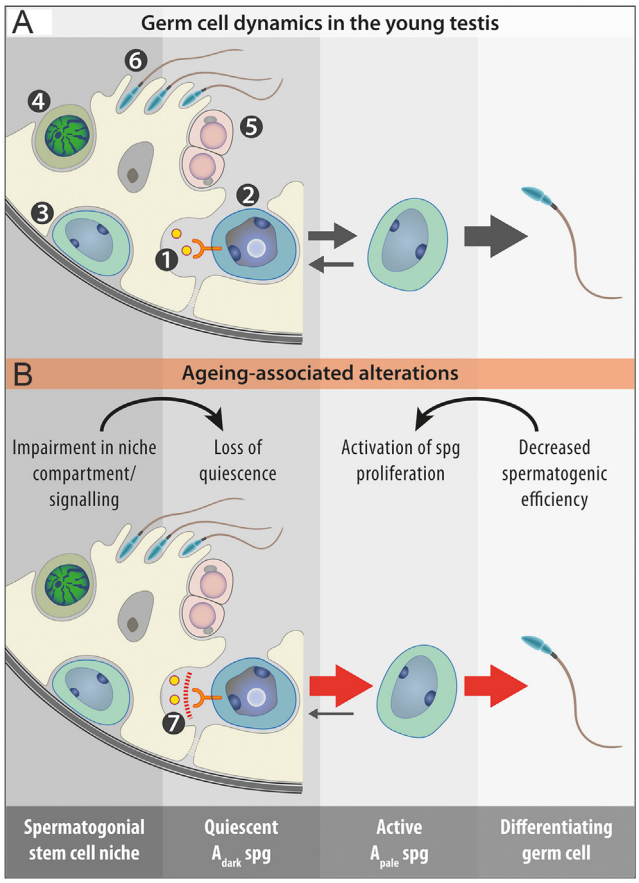

Figure 1 Concept of ageing-associated spermatogonial dynamics. (A) In young testis, the spermatogonial stem cell niche and its signalling (1) regulate the maintenance of an appropriate number of quiescent A_{dark} spermatogonia (spg). (2) There is a well-balanced situation of quiescent A_{dark} (reserve) and active A_{pale} spg (3) (self-renewing) contributing to the differentiation process into primary spermatocytes (4), secondary spermatocytes (5), and finally into sperm (6). Bold arrows indicate that there is increased cellular turnover towards the indicated direction, for example, differentiating germ cells. (B) Upon ageing, impairments in the stem cell niche and its signalling towards spg (7) results in loss of quiescence of A_{dark} spg. This and/or increased demand for differentiating germ cells due to ageing-dependent decreased functional efficiency causes increased proliferation of active A_{pale} spg, which in turn also leads to recruitment of reserve A_{dark} spg. Red arrows indicate a shifted balance towards the differentiating germ cells.

cell nuclei and nucleoli size already in men from 45 years of age onwards (Pohl *et al.* 2019). In a study on human fibroblasts, nucleoli size reflected ribosome biogenesis, and it was reported that ageing is associated with increased protein turnover indicative of impaired proteostasis (Buchwalter & Hetzer 2017).

Moreover, a recent study demonstrated ageing effects on Leydig cells and Sertoli cells in men with normal spermatogenesis (including disease-free human testicular tissue from organ donors). Mularoni *et al.* (2020) found reduced numbers of Leydig cells as well as Sertoli cells. Previously it was suggested that altered ultrastructural

morphology, that is multinucleation, vacuolisation and dedifferentiation, in Leydig cells was associated with reduced capacity of androgen production and/or endocrine signalling in the aged testis (Paniagua *et al.* 1991, Wang *et al.* 2017). However, in testis with normal spermatogenesis steroidogenic capacity of Leydig cells *in vitro* was not different between young and old men. Interestingly, the constant correlation between Leydig cell and Sertoli cell numbers at any age may hint towards a functional link between the two cell types (Mularoni *et al.* 2020).

Testicular peritubular cells contribute to the spermatogonial stem cell niche via secreted factors, including those of immunoregulatory functions (Mayer *et al.* 2016). In a primary cell culture approach, Schmid *et al.* (2019) reported a hampered mitochondrial network and an increased lysosome abundance in senescent human peritubular cells from men with normal spermatogenesis. Moreover, their proteomic analysis provided evidence for impaired proteostasis (Schmid *et al.* 2019). Furthermore, signs of altered inflammatory response have also been described in the testicular environment, for example, when increased numbers of macrophages and macrophages of irregular ultrastructure were observed in mice (Giannessi *et al.* 2005). In addition, elevated levels of the circulating proinflammatory cytokines IL-1β, IL-6 and TNFα have been reported in ageing men (Maggio *et al.* 2005). Under physiological conditions, interstitial macrophages are interacting with Leydig cells via direct cell–cell interactions whereas in aged testes, macrophages and Leydig cells lose their cytoplasmic connections (Giannessi *et al.* 2005).

Taken together, these observations underline the concept that testicular ageing is a result of the germ cell/spermatogonial stem cell niche impairments.

The question remains whether the ageing-associated stem cell exhaustion results from an increased demand for active/differentiating germ cells or from the assumed loss of quiescence, causative of age-related changes in proliferation and quiescence. Most likely the cause is a combination of both.

Genomic instability of the male germline

One of the ageing hallmarks in somatic cells derives from the 'DNA damage theory'. It states that ageing occurs due to an accumulation of DNA damage originating from external sources (e.g. radiation) or endogenous factors (e.g. reactive oxygen species; ROS). Accumulated DNA damage causes cellular functional decline and leads to cell loss through dysregulation of gene expression, impaired transcription, cell cycle arrest and apoptosis. Finally, the depletion of stem cells results in impaired tissue homeostasis and loss of regenerative capacity (Freitas & de Magalhães 2011).

Rodent testicular germ cells show increased levels of ROS and elevated DNA damage in spermatocytes (Selvaratnam *et al.* 2015), and increased levels of apoptotic spermatogonia with increasing age (Wang *et al.* 1999). In human testis, however, findings about DNA damage and apoptosis are inconclusive as both decreased apoptosis in spermatogonia (Kimura *et al.* 2003) and increased germ cell apoptosis (Jiang *et al.* 2014) have been reported in older men. These conflicting results might be attributed to differences in the methodology or confounding factors such as infertility.

DNA damage is closely related to genomic instability, one of the hallmarks of cellular ageing. With time, mechanisms that keep genome integrity become faulty, leading to an increase in genomic abnormalities and DNA breaks. In sperm, genomic instability has been the object of much debate, as sperm DNA damage, or fragmentation, is a common parameter used to evaluate the quality of a semen sample (Agarwal *et al.* 2020). An age-associated increase in DNA damage has been reported in sperm of ageing men and might originate in the SSCs. In haematopoietic stem cells, DNA damage accumulates during quiescence (Beerman *et al.* 2014). The transition from quiescence into active cell cycle, on the other hand, results in increased DNA damage (Walter *et al.* 2015). It can be hypothesised that ageing in human testis, which is associated with re-activation of quiescent reserve A_{dark} SSCs (Pohl *et al.* 2019) and with a decrease in the efficiency of DNA repair mechanisms (Yatsenko & Turek 2018), also leads to increased DNA damage in the germline and, therefore, in sperm.

Sperm DNA fragmentation has consequences to fertility, including lower pregnancy rates and higher miscarriage rates after IVF (Zhao *et al.* 2014). A recent study showed that radiation-induced sperm DNA fragmentation leads to genomic instability in the early embryo, chromosomal rearrangements and unequal cleavages resulting in chaotic mosaicism (Middelkamp *et al.* 2020). High sperm DNA fragmentation is more prevalent in infertile men and is commonly used to evaluate male infertility (Santi *et al.* 2018). Sperm DNA fragmentation is thought to arise in four ways:

1. Defects in sperm DNA compaction resulting from an abnormal protamination of sperm during spermiogenesis as histone-to-protamine transition involves the formation of double-strand breaks (DSBs; Laberge & Boissonneault 2005). If the subsequent repair is incomplete, the breaks may become permanent.
2. Abortive apoptosis – sperm exhibiting apoptotic markers such as externalised phosphatidylserine (PS) and the Fas/FasL system can be removed by Sertoli cells during spermatogenesis (Sakkas *et al.* 1999, Hai *et al.* 2014). When damaged germ cells escape the elimination and complete spermatogenesis, they can be released as mature sperm with DNA damage (Sakkas *et al.* 2003).
3. Insufficient anti-oxidants capacity to counteract ROS production during ageing (Gibb *et al.* 2020).

4. External factors, such as cancer treatment (Delessard *et al.* 2020), may also result in damage to the germline DNA, often through oxidative stress.

The three most widely used methods for evaluating sperm DNA fragmentation are SCSA (Sperm Chromatin Structure Assay), TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-nick end labelling) and the Comet assay. These methods do not distinguish between single (SSBs) and double-strand breaks (DSBs), with the exception of the Comet assay which, when performed under neutral conditions, is able to specifically detect DSBs. Additionally, immune detection of histone variant γ-H2AX has been used to quantify DSBs in sperm; however, since sperm is lacking in histones this quantification is very limited. It is therefore important to consider that each method quantifies different types of sperm DNA fragmentation. The distinction between SSBs and DSBs while evaluating sperm DNA fragmentation is important, as DSBs are thought to have a greater impact on fertilisation because they are more difficult to repair by the oocyte than SSBs (Sakkas & Alvarez 2010). The limited studies evaluating the two types of DNA damage nevertheless point to DSBs having a higher impact on reproductive outcome than SSBs (for a review, read Agarwal *et al.* 2020), indicating that measuring DSBs is more informative of the fertilisation capacity of sperm. However, until a method is developed that measures DSBs throughout the paternal genome it will be very difficult to evaluate the impact of each type of DNA break on reproductive outcomes.

Studies evaluating changes in DNA fragmentation with age have reached conflicting results. While most studies have found an increase in sperm DNA fragmentation with age (Wyrobek *et al.* 2006, Belloc *et al.* 2014, Rosiak-Gill *et al.* 2019, Evenson *et al.* 2020), some could not find the same association (Winkle *et al.* 2009, Brahem *et al.* 2011). This might be due to the different techniques used to evaluate sperm DNA fragmentation but may also originate from differences in study population, enrolment criteria for volunteers and sample processing. For example, most studies were performed on unselected or infertile men. Considering that men with abnormal sperm have higher risk for high sperm DNA fragmentation, the study outcome might have been confounded. In order to understand whether age by itself influences sperm DNA fragmentation, one must focus on studies including only fertile, normozoospermic or healthy men (Table 1 shows a summary of the study results). These few studies all show an increase in sperm DNA fragmentation with age. A recent study compared the patterns of DNA fragmentation increase in two cohorts, one of infertile and the other of healthy normozoospermic men (Evenson *et al.* 2020). Interestingly, the patterns observed were the same regardless of the fertility status, indicating that age is the main factor influencing the increase in DFI reported even in infertile cohorts.

The increase in sperm DNA fragmentation does not seem to be linear over the lifetime but shows an acceleration in the prevalence of abnormally fragmented sperm DNA with older age. In healthy men, we found an acceleration after the age of 56 years (Laurentino *et al.* 2020), while Evenson *et al.* (2020) identified a turning point around the age of 41.6 years. Interestingly, in both cohorts, the proportion of men presenting abnormally high DNA fragmentation increased with age. All men above 70 years in Wyrobek *et al.* (2006) and almost 80% of men above 66 years in our study presented

Table 1 Summary of studies evaluating the relationship between age and sperm DNA fragmentation in fertile, normozoospermic (NZ) or healthy (H) men.

Cohort				
n	Age, years	**Method**	**Association**	**Reference**
97 NZ	22–80	SCSA	Increase in %DFI with age, increase in the proportion of men with abnormal %DFI with age, five-fold increase in %DFI between 20 and 80 years.	Wyrobek *et al.* (2006)
*70 H, N-S	22–80	Comet	Increase in sperm DNA damage with age under alkaline conditions, but no change under neutral conditions	Schmid *et al.* (2007)
NZ, H	37 (6)¶	TUNEL	Weak positive correlation between per cent of sperm DNA fragmentation and age	Belloc *et al.* (2014)
198 H	18–84	SCSA	Increase in %DFI with age, increase in proportion of men with high %DFI with age	Laurentino *et al.* (2020)
80 H		TUNEL‡	Older men presented higher levels of sperm DNA fragmentation	Paoli *et al.* (2019)
40	20–40			
40	50–81			
119 NZ†		TUNEL	Older men had an increase in sperm showing abnormal levels of DNA fragmentation	Rosiak-Gill *et al.* (2019)
*87 NZ, H; 25.445 infertile men		SCSA	The patterns of %DFI increase with age were similar between healthy normozoospermic and infertile men	Evenson *et al.* (2020)
675 H‡		Halosperm	Men above 40 years of age present higher risk for abnormally high sperm DNA fragmentation	Gill *et al.* (2020)

*Subcohort of Wyrobek *et al.* (2006); †Part of a larger cohort; ¶Mean (S.D.); ‡Undergoing sperm assessment; ‡Under microscopic evaluation. N-S, non-smokers; y, years.

pathological levels using a similar methodology (Laurentino *et al*. 2020). However, even young men with normal sperm parameters can present abnormally high DNA fragmentation in their gametes; therefore, youth and normal sperm are not a guarantee of the genomic stability of the paternal genome.

One question that remains largely unanswered regards the regions in the paternal genome mostly affected by these breaks. Future studies will have to focus on determining not only genomic regions susceptible to oxidative damage but also DNA breakage in general, both in infertile and in aged men. This is of pivotal importance to understand the possible effects of sperm genomic instability on reproductive outcomes and on the offspring of men presenting high sperm DNA fragmentation.

Telomeres in sperm and the male germline

Telomeres are DNA hexameric tandem repeats (TTAGGG) that cap the chromosome extremities and help maintain genome integrity during each DNA replication cycle (Blasco 2007). Determination of telomere length (TL) is widely used to measure the biological age of somatic cells (López-Otín *et al*. 2013).

In contrast to somatic cells, sperm telomeres do not suffer age-associated attrition but increase telomere length with age in humans. Furthermore, telomerase activity was shown in human adult testis (Kim *et al*. 1994, Hiyama *et al*. 1995). Studies in mice indicate that telomerase activity is mostly restricted to spermatogonia (Prowse & Greider 1995) and expressed at higher levels in SSCs (Pech *et al*. 2015). This might also hold true for humans, as testis tissues with Sertoli cell-only syndrome show no telomerase activity, but tissues with full spermatogenesis present similar telomerase activity as those with an arrest at the spermatocyte stage (Fujisawa *et al*. 1998).

TL in somatic tissues is notoriously heterogeneous between different individuals (Okuda *et al*. 2002), and there is evidence that male germline TL can also be highly heterogeneous (Baird *et al*. 2006, Antunes *et al*. 2015). Kimura *et al*. (2008) have shown that the on-average longer telomeres in older men are due to subpopulations of sperm with longer telomeres and a possible reduction in those with shorter telomeres, further highlighting the heterogeneity in sperm TL. It is unknown whether the emerging populations of sperm carrying longer telomeres are due to the heterogeneous activity of telomerase in SSCs, a selective advantage of SSC lineages with longer telomeres (which become more prevalent with age), or to the re-activation of quiescent SSCs in the testis of older men (Pohl *et al*. 2019) with a higher TL baseline). While the concordance in blood TL between monozygotic twins remains as expected high regardless of age, it intriguingly increases between dizygotic twins with increasing paternal age (Hjelmborg *et al*. 2015). This would indicate the chances that two independent sperm present telomeres of similar length would increase with age, which gives credence to the second hypothesis, that quiescent SSCs with longer telomeres are activated at older ages. However, further studies are needed to decipher the mechanism by which sperm telomeres are tendentiously longer in older men and whether the heritability of telomere length from older fathers confers an evolutionary adaptive advantage (reviewed in Eisenberg 2011).

Because most studies evaluating telomere length in sperm were conducted in semen donors and some reports suggested a decrease in sperm production with age, sperm parameters were indicated as possible confounders. Indeed, some studies have reported that infertile men present shorter sperm telomeres than controls (Thilagavathi *et al*. 2013, Cariati *et al*. 2016) and that sperm telomere length is correlated with sperm counts (Ferlin *et al*. 2013). However, the correlation between sperm telomere length and age is also maintained in infertile men (Thilagavathi *et al*. 2013). Interestingly, one study found that samples with low sperm DNA fragmentation had longer telomeres, raising the question of whether genomic and telomeric stability are associated in sperm (Moskovtsev *et al*. 2010), although other studies failed to confirm this observation (Thilagavathi *et al*. 2013). Nevertheless, gene variants in two telomerase components (TERT and TEP1) have been associated with an increased risk of male infertility and, in the case of TEP1, with increased sperm DNA fragmentation (Yan *et al*. 2014). A study in men with normozoospermia also found a negative association of sperm telomere length with DNA fragmentation (Rocca *et al*. 2016). As age is associated with both an increase in sperm DNA fragmentation and mean sperm telomere length, this observation seems paradoxical. Re-evaluation of data on both these parameters obtained from a cohort of healthy ageing men (Laurentino *et al*. 2020) did not reveal an association between short sperm telomeres and high sperm DNA fragmentation (Fig. 2). On the contrary, there was a weak but significant positive correlation ($\rho = 0.21$; $P = 0.006$) between relative telomere length in sperm and %DFI, which was lost after correcting for age. Therefore, it appears that any influence of spermatogenic status on sperm telomere length is likely independent of sperm DNA fragmentation and age-related sperm telomere length changes. In any case, any study evaluating associations between sperm telomere length and sperm parameters should control for age as a possible confounder.

Age-associated epigenetic alterations in human sperm

Epigenetics can be defined as the changes to gene function that do not interfere directly with the DNA sequence. The most commonly studied epigenetic marks

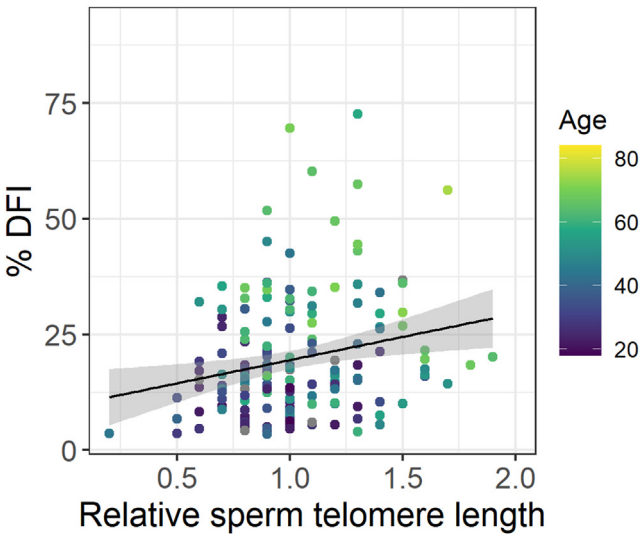

Figure 2 Scatter plot showing the relationship between the relative sperm telomere length and the per cent DNA fragmentation index in a cohort of healthy men (Laurentino *et al.* 2020). A positive association was found between the two variables, which was not significant after correction for age (shown as a colour gradient). A linear regression is shown in black with the 95% CI in grey shading.

are DNA methylation, post-translational modification of histones (e.g. methylation and phosphorylation) and non-coding RNAs (e.g. involvement of *XIST* in X chromosome inactivation). Epigenetic marks, alone or through the interaction of different marks, are involved in the regulation of gene expression.

Age-associated epigenetic alterations have been previously described in somatic tissues and cells, and epigenetic drift is in fact one of the main features of ageing in somatic tissues (López-Otín *et al.* 2013). Most studies in humans have focused on DNA methylation changes in diverse tissues and cell types, several of which have resulted in the development of epigenetic clocks allowing for calculation of biological or epigenetic age (Horvath & Raj 2018).

In comparison, until recently, age-related epigenetic alterations in sperm have been relatively neglected. Due to the scarcity of histones in human sperm (which are mostly substituted by protamines), only DNA methylation changes have been studied in ageing sperm. For this reason, we will focus exclusively on sperm DNA methylation changes with age. Jenkins *et al.* (2013) identified an increase in global 5-methylcytosine levels in sperm from the same men obtained 9–21 years apart and a correlation between age and 5-methylcytosine content in sperm. The same group evaluated the location of DNA methylation changes in sperm of fertile donors obtained 9–17 years apart and found that changes in sperm DNA methylation involve both increase and decrease in DNA methylation (Jenkins *et al.* 2014). They detected changes in 117 genes, significantly associated with schizophrenia and bipolar disorder. These findings seem to be corroborated by other studies in humans. Recently, a study also identified age-related changes in sperm DNA methylation in genes associated with neurodevelopmental disorders (Denomme *et al.* 2020) and in histone-rich regions of the paternal genome thought to be important for development (Hammoud *et al.* 2009). In our study on healthy ageing men, we found 254 differentially methylated regions (DMRs) in sperm between the two extreme age groups (Laurentino *et al.* 2020). Gene ontology analysis indicated an enrichment close to homeobox genes and genes involved in nervous system development. Taken together, these studies indicate a possible role of sperm epigenetic drift in the pathophysiology of neurodevelopmental disorders in the offspring of older men; however, a causal connection remains to be demonstrated in humans.

In animal models, hints were found associating sperm DNA methylation changes in older males and offspring phenotypes. Milekic *et al.* (2015) observed a loss of methylation in sperm and behavioural changes in the offspring of older mice and similar changes in DNA methylation in the brain of the offspring, indicating a possible inheritance of the sperm DNA methylation changes. The DMRs were enriched in genes associated with autism and schizophrenia. It is unknown how close the epigenetic changes in the sperm of animal models resemble those in humans, as differences in SSC systems and longevity might make the transfer of findings from rodents to humans difficult beyond a proof of principle level.

Attempts at applying epigenetic age predictors based on DNA methylation changes developed in somatic cells and tissues to sperm were unsuccessful, highlighting the differences in epigenetic drift occurring in the germline. Therefore, methylation-based age predictors have been developed specifically for human sperm. They are based on different techniques – methylation arrays (Jenkins *et al.* 2018), deep bisulphite sequencing (Laurentino *et al.* 2020), and pyrosequencing (Potabattula *et al.* 2020) – but demonstrate the reproducibility of DNA methylation changes with age and can potentially be used to track the influence of external factors on sperm age.

In order to be transmitted to the offspring, changes in sperm DNA methylation need to bypass the wave of genome-wide demethylation occurring after fertilisation. This reprogramming period results in a 'clean slate' by deleting prior epigenetic programming and setting new epigenetic marks necessary for development. Very few regions escape this genome-wide demethylation, notably imprinted genes. However, recent studies have shown that other regions in the genome are able to bypass this reprogramming and maintain the DNA methylation patterns inherited from the parents via the gametes' epigenomes (Tang *et al.* 2015). Jenkins *et al.* (2019) found no alterations in sperm DNA methylation regions between men with old or young grandparents, suggesting that age-associated DNA methylation changes

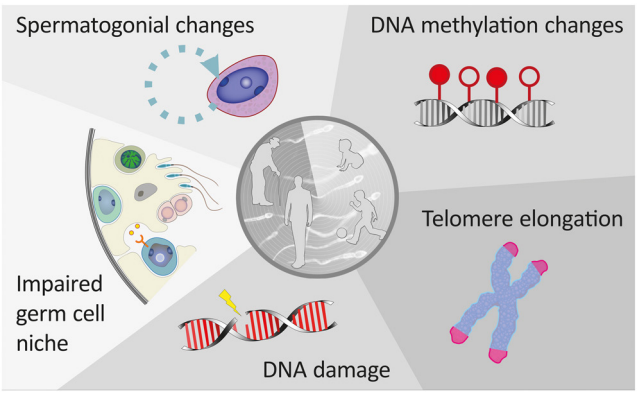

Figure 3 The five hallmarks of male germ cell ageing identified in healthy ageing men: DNA damage in male germ cells, increased telomere length in sperm, altered sperm DNA methylation, impaired germ cell/spermatogonial stem cell niche with hampered cellular signalling and altered spermatogonial dynamics accompanied by activation of (reserve) A_{dark} spermatogonia (represented by dashed arrow).

do not have transgenerational potential. As a proof-of-principle, we compared our healthy ageing sperm DMRs with available embryo methylome data and identified ten regions that might potentially escape the first wave of demethylation (Laurentino *et al.* 2020). Denomme *et al.* (2020) identified over 200 genes showing differential methylation with paternal age in both sperm and blastocysts, which were enriched in pathways involved in neurodevelopmental disorders. Despite the indications from several studies pointing in the direction of an association between age-associated DNA methylation changes in sperm with neurodevelopment, a functional proof of the influence of an aged-sperm epigenome on offspring health remains to be presented.

Concluding remarks

Considering the current knowledge, ageing itself has a major impact on spermatogenesis, gamete quality and presumably progeny health. It is conceivable that maintaining general health over lifetime ensures a nearly normal reproductive status in men, however, despite age-associated molecular changes occurring in sperm, for example, DNA fragmentation increase and DNA methylation pattern change. Moreover, to maintain a constant spermatogenic output, increased proliferation of spermatogonia can be observed in parallel with decreased spermatogenic efficiency. This increased proliferation of A_{dark} spermatogonia suggests an imbalanced SSC regulation in aged men, leading towards loss of quiescence. To which extent alterations in cells comprising the SSC niche might reflect ageing associated impairments in the stem cell niche has to be evaluated (Fig. 3).

The well-described increase in the mutation rate of male germ cells together with the observed changes in the testicular stem cells in telomere length, genomic stability and DNA methylation changes clearly indicate that the age of prospective fathers has to be considered seriously when considering progeny health. To date, only a few studies excluded confounders and investigated healthy aged men, and more information on healthy ageing of male germ cell is urgently required. These data sets are invaluable as they provide baseline values of pure age effects. Only based on such data sets can clinical studies be broadened into topics such as infertility and defined co-morbidities, as data obtained can be normalised against these 'reference' values.

Declaration of interest

Joachim Wistuba is an Associate Editor of Reproduction. Joachim Wistuba was not involved in the review or editorial process for this paper, on which he is listed as an author.

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Author contribution statement

E P, J G, J W, and S L surveyed and discussed the literature, wrote the manuscript, and conceptualised the images.

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