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ORIGINAL ARTICLE *Andrology*

# Age-related changes in human Leydig cell status

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**STUDY QUESTION:** What are the consequences of ageing on human Leydig cell number and hormonal function?

**SUMMARY ANSWER:** Leydig cell number significantly decreases in parallel with *INSL3* expression and Sertoli cell number in aged men, yet the *in vitro* Leydig cell androgenic potential does not appear to be compromised by advancing age.

**WHAT IS KNOWN ALREADY:** There is extensive evidence that ageing is accompanied by decline in serum testosterone levels, a general involution of testis morphology and reduced spermatogenic function. A few studies have previously addressed single features of the human aged testis phenotype one at a time, but mostly in tissue from patients with prostate cancer.

**STUDY DESIGN, SIZE, DURATION:** This comprehensive study examined testis morphology, Leydig cell and Sertoli cell number, steroidogenic enzyme expression, *INSL3* expression and androgen secretion by testicular fragments *in vitro*. The majority of these endpoints were concomitantly evaluated in the same individuals that all displayed complete spermatogenesis.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** Testis biopsies were obtained from 15 heart beating organ donors (age range: 19–85 years) and 24 patients (age range: 19–45 years) with complete spermatogenesis. Leydig cells and Sertoli cells were counted following identification by immunohistochemical staining of specific cell markers. Gene expression analysis of *INSL3* and steroidogenic enzymes was carried out by qRT-PCR. Secretion of 17-OH-progesterone, dehydroepiandrosterone, androstenedione and testosterone by *in vitro* cultured testis fragments was measured by LC-MS/MS. All endpoints were analysed in relation to age.

**MAIN RESULTS AND THE ROLE OF CHANCE:** Increasing age was negatively associated with Leydig cell number ($R = -0.49$; $P < 0.01$) and concomitantly with the Sertoli cell population size ($R = -0.55$; $P < 0.001$). A positive correlation ($R = 0.57$; $P < 0.001$) between Sertoli cell and Leydig cell numbers was detected at all ages, indicating that somatic cell attrition is a relevant cellular manifestation of human testis status during ageing. *INSL3* mRNA expression ($R = -0.52$; $P < 0.05$) changed in parallel with Leydig cell number and age. Importantly, steroidogenic capacity of Leydig cells in cultured testis tissue fragments from young and old donors did not differ. Consistently, age did not influence the mRNA expression of steroidogenic enzymes. The described changes in Leydig cell phenotype with ageing are strengthened by the fact that the different age-related effects were mostly evaluated in tissue from the same men.

**LIMITATIONS, REASONS FOR CAUTION:** *In vitro* androgen production analysis could not be correlated with *in vivo* hormone values of the organ donors. In addition, the number of samples was relatively small and there was scarce information about the concomitant presence of potential confounding variables.

**WIDER IMPLICATIONS OF THE FINDINGS:** This study provides a novel insight into the effects of ageing on human Leydig cell status. The correlation between Leydig cell number and Sertoli cell number at any age implies a connection between these two cell types, which may be of particular relevance in understanding male reproductive disorders in the elderly. However aged Leydig cells do not lose

their *in vitro* ability to produce androgens. Our data have implications in the understanding of the physiological role and regulation of intratesticular sex steroid levels during the complex process of ageing in humans.

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**Key words:** Leydig cells / ageing / human / testis / steroidogenesis

# Introduction

Ageing has a significant negative impact on testis function in humans, with both spermatogenesis and steroidogenesis being progressively impaired, yet without a definite age at which the testicular involution begins. Importantly, age-related changes in testicular function display a pronounced interindividual variation reflecting the overall health status of aged men which is influenced by environmental, lifestyle and medical factors (Sartorius *et al.*, 2012; Kelsey *et al.*, 2014). There is general consensus on the gradual slow decline of serum testosterone concentrations in ageing men with apparent good health, after the age of 40 years, despite unchanged or slightly increased luteinising hormone (LH) levels (Kaufman and Vermeulen, 2005; Perheentupa and Huhtaniemi, 2009; Camacho *et al.*, 2013). These phenomena highlight an alteration of the hypothalamic–pituitary–testicular axis function in elderly men (Kaufman and Vermeulen, 2005; Wu *et al.*, 2008; Veldhuis *et al.*, 2009). Testicular endocrine dysfunction during ageing is also evidenced by a significant lower secretion of the non-steroidogenic hormone, INSL3, by Leydig cells of older men compared to young ones (Anand-Ivell *et al.*, 2006). Reduced production of both testosterone and INSL3 with advancing age points to a direct impairment of Leydig cell function and/or quantity. In contrast to the large body of *in vivo* evidence consistently showing age-related androgen failure, contradictory findings have been reported in the small number of studies examining Leydig cell population change with increasing age. Some studies have described no change (Paniagua *et al.*, 1987a; Petersen *et al.*, 2015), while others have reported a reduction in both Leydig and interstitial cell number (Kaler and Neaves, 1978; Neaves *et al.*, 1984). Moreover, research focusing on the effect of age *per se* upon Leydig cell function and the underlying regulatory mechanisms is lacking, likely related to the inadequate access to fresh, disease-free human testicular tissue.

This issue has intense clinical interest since the declining androgen status in ageing men is one of the symptoms of the so-called late onset hypogonadism (LOH). LOH is associated with increased adiposity, decreased libido, erectile dysfunction, decreased muscle mass, depressed mood, osteopenia and loss of vitality, resulting in significant detriment to the quality of life of older men (Surampudi *et al.*, 2012; Huhtaniemi, 2014). To solve or reduce symptoms of testosterone deficiency, replacement therapy is used in hypogonadal men, which provides several benefits, but also has potential risks to various organs including prostate and testis as well as the blood and cardiovascular and respiratory systems (Gagliano-Juca and Basaria, 2019; Santella *et al.*, 2019). A better understanding of the factors causing reduced testicular function with age could provide important input to the development of new and alternative approaches for treatment of Leydig cell insufficiency.

In addition to the impairment of interstitial cells in the testis, a possible involution of the spermatogenic compartment in elderly men has been reported, including a reduction in both Sertoli cell and sperm number and quality with advancing age (Johnson *et al.*, 1984, 2015; Neaves *et al.*, 1984; Jiang *et al.*, 2014; Petersen *et al.*, 2015). Moreover, morphological characteristics of seminiferous tubules, including volume, length and thickness of boundary tissue and seminiferous epithelium, exhibit significant alterations in aged individuals (Johnson *et al.*, 1986; Paniagua *et al.*, 1987b; Santiago *et al.*, 2019).

While age-related cellular and molecular changes in the human testis have not been extensively studied, rodent models have proven to be highly informative. The long-lived Brown Norway rats, which do not develop tumours of reproductive tract and do not become obese with age, have been used to study male reproductive ageing (Beattie *et al.*, 2015; Wang *et al.*, 2017). In these rats, serum testosterone levels decrease with age, as in men, but without loss of Leydig cells or change in serum LH levels (Wang *et al.*, 1993; Chen *et al.*, 1994). However, by using both the whole testis and isolated cells, functional deficits of aged Leydig cells were identified, including reductions in LH-stimulated cAMP production (Chen *et al.*, 2002), reduced expression and activity of steroidogenic enzymes (Luo *et al.*, 1996), as well as reduced expression of steroidogenic acute regulatory protein (STAR) and the outer mitochondrial membrane cholesterol-binding translocator protein (TSPO) (Chung *et al.*, 2013). Increased oxidative stress appeared to be responsible for at least some of the changes in the steroidogenic pathway in ageing rats (Wang *et al.*, 2017). An interesting model of premature ageing is the Cisd2-deficient mouse, whose testicular phenotype has been recently characterized (Curley *et al.*, 2019). At 6 months of age, these animals showed decreased circulating testosterone, reduced Leydig and Sertoli cell number and decreased testicular steroidogenic gene expression. However, the restrictive ablation of Cisd2 in either Leydig cells or Sertoli cells did not affect Leydig cell function, suggesting that age-related testicular dysfunction is not entirely driven by intrinsic ageing of Leydig cells or Sertoli cells *per se*.

In this study, we investigated the testicular ageing phenotype in a significant number of men, using non-pathological testicular biopsies from heart beating organ donors without major health problems and from patients referred to the andrology clinic due to infertility with normal spermatogenesis and hormone levels. We have analysed multiple features of testicular function in the same individuals, including quantitative histology of the parenchyma, Leydig and Sertoli cell numbers, steroidogenic and *INSL3* gene expression and *in vitro* androgen production in response to gonadotropins, providing the first comprehensive picture of age-associated changes of human Leydig cell physiology.

# Materials and methods

## Tissue collection

Testicular biopsies were obtained from 15 heart beating organ donors (age range: 19–85 years, median 52 years) at the Hospital Policlinico

Umberto I at the Sapienza University of Rome. The cause of death of the donors was mainly hemorrhage, stroke or accidents. Tissue samples were collected February 2011 to November 2017 during the multiorgan harvesting surgical procedures, that include perfusion through the aorta of the abdominal organs as well as simultaneous drainage of the perfusion fluid (Celsior solution) (Muciaccia *et al.*, 2013). After collection, biopsies were transported to the laboratory in ice-cold phosphate buffered saline (PBS) and processed within one hour. One part of each biopsy was fixed for light microscopy, another was kept fresh to be used in *in vitro* short-term organ culture, and some of the remaining sample was snap-frozen and stored at −80°C for further gene expression analysis, while some was placed in cryovials with a cryoprotective solution containing sterile Minimum Essential Medium (MEM), DMSO (1.1 M) fetal bovine serum (20%) and sucrose (0.15 M). Cryovials were frozen in dry ice for 15 min, then transferred to −80°C for 24–48 h and finally into liquid nitrogen.

Additional testicular material from 24 patients (age range: 19–45 years, median 32 years) was included in the study. The material had been obtained from patients referred to the andrology clinic at Department of Growth and Reproduction at Rigshospitalet, Copenhagen, mainly due to fertility problems. All biopsies were taken for diagnostic purposes only. The material had been fixed, paraffin-embedded and stored after evaluation. Suitable biopsy samples were identified in the department's patient database with the inclusion criteria that assessment of the biopsy showed tubules with complete spermatogenesis and serum hormone levels (testosterone, SHBG, inhibin B, oestradiol, LH and FSH) within the normal range. Of the 24 patients, 15 had been referred due to couple infertility (11 had obstructive azoospermia, 3 had normal sperm counts (total counts 44–139 mill) and one had reduced semen quality and biopsies performed to rule out presence of germ cell neoplasia *in situ*). The remaining nine patients had been unilaterally orchiectomized due to testis cancer: seven had total sperm counts at follow-up (total counts 140–475 mill) and two had reduced semen quality.

## Ethical approval

The use of organ donor material was approved by the ethics committee of the Hospital Policlinico Umberto I, according to national guidelines for organ donation as issued by the Italian Ministry of Public Health. For each sample, the free and informed consent of the family concerned was obtained.

Patient tissue was used in accordance with the Helsinki Declaration following approval from the local medical research ethics committee (permit no. H-3-2013-175). Patients visiting the Department of Growth and Reproduction at Copenhagen University Hospital (Rigshospitalet), gave their written and oral consent prior to any left-over biopsy tissue being used for research studies.

## Histological analysis

Testicular biopsy fragments from organ donors were fixed in Bouin's fixative or 10% buffered formalin solution, dehydrated and then paraffin embedded. Sections of 5 µm thickness were stained with Carmalum and examined under a light microscope. Images were acquired with a digital camera (Axiocam 503 colour, Zeiss) using Zen Lite 2 software.

For the purpose of quantifying morphological changes, four separate histological sections taken from different regions of each testicular biopsy were entirely scanned with NanoZoomer 2.0 HT (Hamamatsu Photonics, Germany). Using the NDP view software (version1.2.36) (Hamamatsu Photonics, Germany), each histological section occupied by seminiferous tubules was quantified and the area occupied by interstitium was obtained by subtracting the area occupied by seminiferous tubules from the total section area.

Leydig cell micronodules were identified as previously described (Holm *et al.*, 2003). For micronodule distribution quantification, micronodule area was measured in at least five separated histological sections for each donor and expressed as percentage of total section area.

Testis biopsies from the Danish patients were obtained by an open surgical procedure in the process of diagnostic work-up of infertility. The tissue samples were fixed immediately in modified Stieve fixative (GR fixative, composition: 200 ml 37% Formaldehyde, 40 ml acetic acid added to 1 L of 0.05 M phosphate buffer, pH 7.4), dehydrated and embedded in paraffin. The biopsies were then cut into multiple serial sections divided among at least 10 glass slides and stained with haematoxylin–eosin or periodic-acid Schiff. In addition, several slides (at the beginning and end of the series) were stained by immunohistochemistry (according to the procedure described below) for MAGE-A4 antigen to visualise spermatogonia and early primary spermatocytes, and for placental-like alkaline phosphatase (PLAP) or D2-40 antigen (Podoplanin) to exclude the presence of germ cell neoplasia *in situ* (Rajpert-De Meyts *et al.*, 2015). The sections were scanned with NanoZoomer (Hamamatsu Photonics, Germany) and examined by two experienced evaluators using the NDPview software. After the histopathological evaluation, the microscopic slides and paraffin blocks with the residual tissue were stored in the department's tissue archive.

## Immunohistochemical staining for Leydig and Sertoli cell number assessment

Immunohistochemistry was performed using two different protocols depending on the source of biopsies.

### *Donor tissue*

Paraffin sections of 5 µm thickness from samples fixed in 10% buffered formalin solution were deparaffinized, rehydrated and processed for antigen retrieval in citrate buffer (pH 7.8; UCS Diagnostic, Italy) in a microwave oven at 700 W, three times, 3 min each. Endogenous peroxidase activity was quenched with $H_2O_2$ followed by blocking in Super block (UltraTek HRP Anti-polyvalent kit, ScyTek Laboratories, UT, USA). Slides were incubated for 2 h at RT with primary antibodies at the following dilutions: goat polyclonal anti-3β-HSD (1:150, Santa Cruz sc-30820, lot no. H2009), rabbit monoclonal anti-SOX9 (1:300, Cell Signaling, 82630, lot no. 0001). After washing, sections were processed using UltraTek HRP Anti-polyvalent kit (ScyTek laboratories, UT, USA) and peroxidase activity was revealed using diaminobenzidine (Roche, Italy).

### *Biopsies from andrological patients*

Paraffin sections of 5 µm thickness from samples fixed in modified Stieve fixative were deparaffinized, rehydrated and incubated in TEG buffer (10 mM Tris, 0.5 mM EGTA, pH 9.0) for antigen retrieval in a

pressure cooker at 110°C for 30 min. After quenching with methanol and blocking with a solution containing 20% horse serum ImmPRESS kit MP-7405 (Vector Laboratories, CA, USA) and 5% BSA, sections were incubated overnight with primary antibodies at the following dilutions: rabbit polyclonal anti-SOX9 (dilution 1:100 000, Millipore AB5535, lot no. 2757163), rabbit monoclonal anti-CYP17A1 (1:300, Abcam ab134910, lot no. GR95872-10). After washing, sections were incubated with secondary antibody (ImmPRESS HRP reagent: Anti-Rabbit MP7401, Vector Laboratories, CA, USA). Peroxidase activity was revealed using ImmPact DAB (Vector Laboratories, UT, USA).

In both protocols, negative controls were performed by omitting primary antibodies and replacing primary antibodies with isotype IgG. Nuclei were counterstained with Mayer Haematoxylin (Sigma-Aldrich, Italy).

### *Cell counting*

3β-HSD/CYP17A1 positive Leydig cells and SOX9-positive nuclei of Sertoli cells were counted in 20 optical fields of 0.28 $mm^2$ area randomly chosen on testicular sections from different regions of the biopsies, using both light microscopy (40× objective) and scans captured by NanoZoomer 2.0 HT and analysed using the software NDP view version 1.2.36 (Hamamatsu Photonics, Germany). Immunopositive Leydig cells were counted when the nucleus was clearly visible. A small percentage (∼1.4%) of Sertoli cells, identified on the basis of nuclear morphology, were SOX9 negative, and remained constant during ageing. Cell number counting was repeated twice, after two independent immunohistochemistry experiments.

## *In vitro* culture of testis fragments

Cryopreserved testicular samples were thawed at 37°C in a water bath for 2 min, washed twice in alpha MEM (Life Technologies, Italy) and then incubated for 2 h at 34°C to dilute the cryoprotectant and equilibrate in the culture medium. Both frozen/thawed and fresh (immediately after collection) tissues were cut into ∼2 mm fragments and immersed into the culture medium containing alfa MEM supplemented with glutamine, non-essential amino-acids, penicillin-streptomycin and gentamicin. Recombinant hLH (Luveris 75 UI, Merck Serono, Italy) and rhCG (Ovitrelle 250 micrograms, Merck Serono, Italy) were added to the culture medium as indicated in the figure text. Testicular fragments in triplicate were cultured for 3 h at 34°C in humidified atmosphere of 5% $CO_2$ in air. At the end of incubation, media were collected, centrifuged at 960*g* for 10 min at 4°C and stored at −20°C for hormone analysis. Testis fragments were homogenized in RIPA buffer (150 mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 50 mM Tris pH 8 and the following protease inhibitors: 10 mg/ml aprotinin, 10 mg/ml leupeptin, 4 mg/ml pepstatin, 100 mM PMSF), sonicated and centrifuged at 18 000*g* for 5 min. Protein in the supernatants were quantified by the bicinchoninic method (Pierce, IL, USA).

## Hormone analysis

17-OH-progesterone (17-OH-P), dehydroepiandrosterone (DHEA) androstenedione (A) and Testosterone (T) secreted into the culture media were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the Centre for Applied Biomedical Research of the Alma Mater Studiorum, University of Bologna—S. Orsola-Polyclinic, Bologna, Italy. The assay was adapted from Mezzullo *et al.* (2017). Volumes of 500 µl were processed and quantified by isotopic dilution. The quantitation range was between 0.01 and 20, 0.02 and 20, 0.39 and 50 and 0.08 and 20 ng/ml for T, A, DHEA and 17-OH-P, respectively. Intra- and inter-assay imprecision were assessed at 5.7 and 4.7%, 5.8 and 10.4%, 7.7 and 12.1%, 7.9 and 14.5% for T, A, DHEA and 17-OH-P, respectively.

In some experiments, T and 17-OH-P were also quantified by ELISA according to the manufacturer's instruction for T: ADI-900-065, ENZO (Life Sciences, NY, USA) (intra- and inter-assay coefficient of variation <10% and <12%, respectively) and for 17-OH-P: KA4015, Abnova (Germany) (both intra- and inter-assay coefficients of variation within 8%). Steroid production was normalized to the protein level in the testicular fragments.

## RNA extraction and qRT-PCR

Total RNA from frozen samples was extracted using Trizol reagent and quantified by Nano-drop spectrometry. RNA quality was checked running an aliquot (500 ng) of each RNA sample on a denaturing 1% agarose gel stained with Nancy-520 (Sigma-Aldrich, Italy). cDNA was synthesized from 2 µg RNA using High Capacity RNA-to-cDNA kit (Applied Biosystems, Life Technologies, Italy), following manufacturer's instructions. qRT-PCR assays were run on 7500 Real Time PCR System (Applied Biosystems, Life Technologies, Italy) using SYBR Green (Euroclone, Italy) with a reaction volume of 20 µl. Each reaction consisted of 10 µl 2X FluoCycle II SYBR Master Mix, 20 ng of cDNA, final concentration of 300 nM each forward and reverse primers and $ddH_2O$. Transcript levels were measured with two-step thermal cycling conditions: initiation at 95°C for 5′, then 45 cycles of 95°C for 15 s and 60°C for 60 s (fluorescence measurement). Primers used are listed in Supplementary Table SI. Data were analysed with $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using RPS29 to normalise gene expression levels, as previously validated (Svingen *et al.*, 2014). Data are presented as the fold change in gene expression relative to donor no. 20, a 50 years old, chosen as the calibrator because the median age of the group was 52 years. Each sample was run in duplicate and experiments were repeated twice following two separate retrotranscriptions.

## Statistical analysis

Initial correlations between data sets were analysed using the Pearson correlation coefficient. When data were assessed to differ markedly from a bivariate normal distribution by Sigma Plot software, Spearman rank correlation was used with *P*-value calculated by a randomization test. For testing differences between groups Student's *t*-test and one-way ANOVA test were used. When data were assessed to differ markedly from a bivariate normal distribution by Sigma Plot software, Mann–Whitney test was used.

All statistical analyses were carried out using Sigma Plot 11 software (Systat Software, Inc, CA, USA). *P*-value <0.05 was considered significant.

# Results

## Morphological changes in testicular parenchyma during human ageing

We first evaluated the morphology of testicular parenchyma in young (age range: 19–49 years) and elderly (age range: 50–85 years) organ donors. Carmalum stained sections were evaluated by light microscopy. All samples appeared normal with both tubular and interstitial compartments represented. Testes of the young men displayed compact seminiferous tubules with germ cells at all stages of differentiation and a rather dense interstitium (Fig. 1A and B). In contrast, testicular parenchyma of elderly men showed fewer tubules with complete spermatogenesis, the presence of empty and sclerotic tubules and fibrotic tissue in a loosen interstitium (Fig. 1C and D). A thickening of the peritubular layer was also typically found in older men (Supplementary Fig. S1). The histological changes were quantified by determining the proportion of testicular parenchyma occupied by both the tubular and interstitial compartments, respectively. When comparing these in testes from young versus elderly (Fig. 1E) it was evident that the area occupied by seminiferous tubules significantly decreased in aged men, whereas the area of the space between seminiferous tubules (interstitium) significantly increased in older donors (Fig. 1F). These data are in agreement with previous observations (Johnson *et al.*, 1986; Paniagua *et al.*, 1987a, b; Santiago *et al.*, 2019), and confirmed that this cohort of healthy organ donors could be used to examine age-related effects of testicular function.

## Leydig cell number declines with increasing age in men

Given that fibrotic tissue was consistently observed in interstitial compartment in older men, we investigated whether Leydig cell number changed with age. We performed immunohistochemical analysis of testicular sections using specific Leydig cell markers for the steroidogenic pathway enzymes 3βHSD or P450C17/CYP17A1 (Supplementary Fig. S2). A similar number of cells were counted when Leydig cells were immunostained with either 3βHSD or P450C17/CYP17A1 antibodies in the same sample (data not shown). Leydig cell number per given microscopic field was evaluated in testis biopsies from both organ donors (n = 13) and patients with complete spermatogenesis and normal testicular function (n = 24). Interestingly, the correlation analysis showed a significant decrease in Leydig cell number with advancing age of men (Fig. 2A). In particular, Leydig cell number did not change in the age range from 19 to 40–45 years and thereafter significantly declined (Table I). These results indicate that ageing has a negative impact on the size of the human Leydig cell population, which is the major producer of testosterone.

## Leydig cell micronodules are similarly distributed in young and elderly men

Clusters of 15 or more Leydig cells in the testicular parenchyma, termed 'micronodules', are regarded as histological sign of testicular failure. Micronodule frequency has been correlated with various spermatogenic disorders, including testicular cancer and infertility (Holm *et al.*, 2003; Lottrup *et al.*, 2017). Following our observation of substantial histological alterations in testicular biopsies of aged individuals, we reasoned that Leydig cell micronodule number would be affected by age. We therefore assessed the area occupied by Leydig cell micronodules/section and expressed as percentage of total testis section area in 16 biopsies from organ donors of different ages. The distribution of Leydig cell micronodules did not correlate with age (Fig. 2B). In addition, when three classes of micronodules with different size (Leydig cell number/micronodule: 15–19; 20–30 and >30) were identified, counted in each section and compared between young and elderly groups, the frequency of the three classes was similar (Fig. 2C), although nodules with 15–19 Leydig cells appeared to decrease, while nodules with >30 Leydig cells tended to increase in testicular samples from the elderly compared to the young.

## Sertoli cell number significantly declines in ageing men and positively correlates with Leydig cell number at all ages

Previous studies in mice have demonstrated that Leydig cell number strongly correlates with Sertoli cell number at all ages (Rebourcet *et al.*, 2014a,b, 2017). However, this relationship has not previously been analysed in human samples. To examine this in detail, we first assessed Sertoli cell number/field in the same histological sections from 37 testicular biopsies initially used to evaluate the number of Leydig cells. Sertoli cells were identified by immunohistochemical detection of SOX9, a specific marker of this cell type (Supplementary Fig. S2). We detected a significantly negative correlation between Sertoli cell number and age (Fig. 3A). The pattern of decline was very similar to that of Leydig cells. No change in Sertoli cell number from 19 to 40–45 years and a significant decrease thereafter (Table I). Therefore, we next analysed the direct relationship between the two cell populations in each donor or subject testis biopsy, by examining the correlation coefficient. There was a highly significant positive correlation (rho = 0.57; $P < 0.001$) between the two cell population sizes at all ages (Fig. 3B), indicating that in humans there is an interdependence between Leydig cells and Sertoli cells in the entire investigated age-interval.

## Analysis of Leydig cell function during ageing

### *INSL3, but not LHCGR, gene expression declines in aged men*

The observation of an age-dependent loss of Leydig cells (Fig. 2A) prompted us to analyse the expression of a non-steroidogenic specific product of Leydig cells, INSL3, which mainly reflects the number of Leydig cells and their differentiation status (Ivell and Anand-Ivell, 2009; Steggink *et al.*, 2019). Interestingly, the results from qRT-PCR expression analysis in testis biopsies from organ donors of different ages showed that *INSL3* transcript levels were negatively correlated with age (rho= −0.52, $P < 0.05$) (Fig. 4A). In contrast, the expression of *LHCGR* mRNA in the same testicular biopsies was not correlated with age ($P = 0.59$, rho =−0.15) (Fig. 4B).

### *Steroidogenic gene expression is not altered in ageing*

Next, we focused on Leydig cell steroidogenic function during ageing by analysing the expression of the following genes of the steroidogenic machinery: *StAR*, *CYP11A1*, *CYP17A1*, *3β-HSD2*, *17β-HSD3*, in testis biopsies from the organ donors of different ages, using qRT-PCR. When

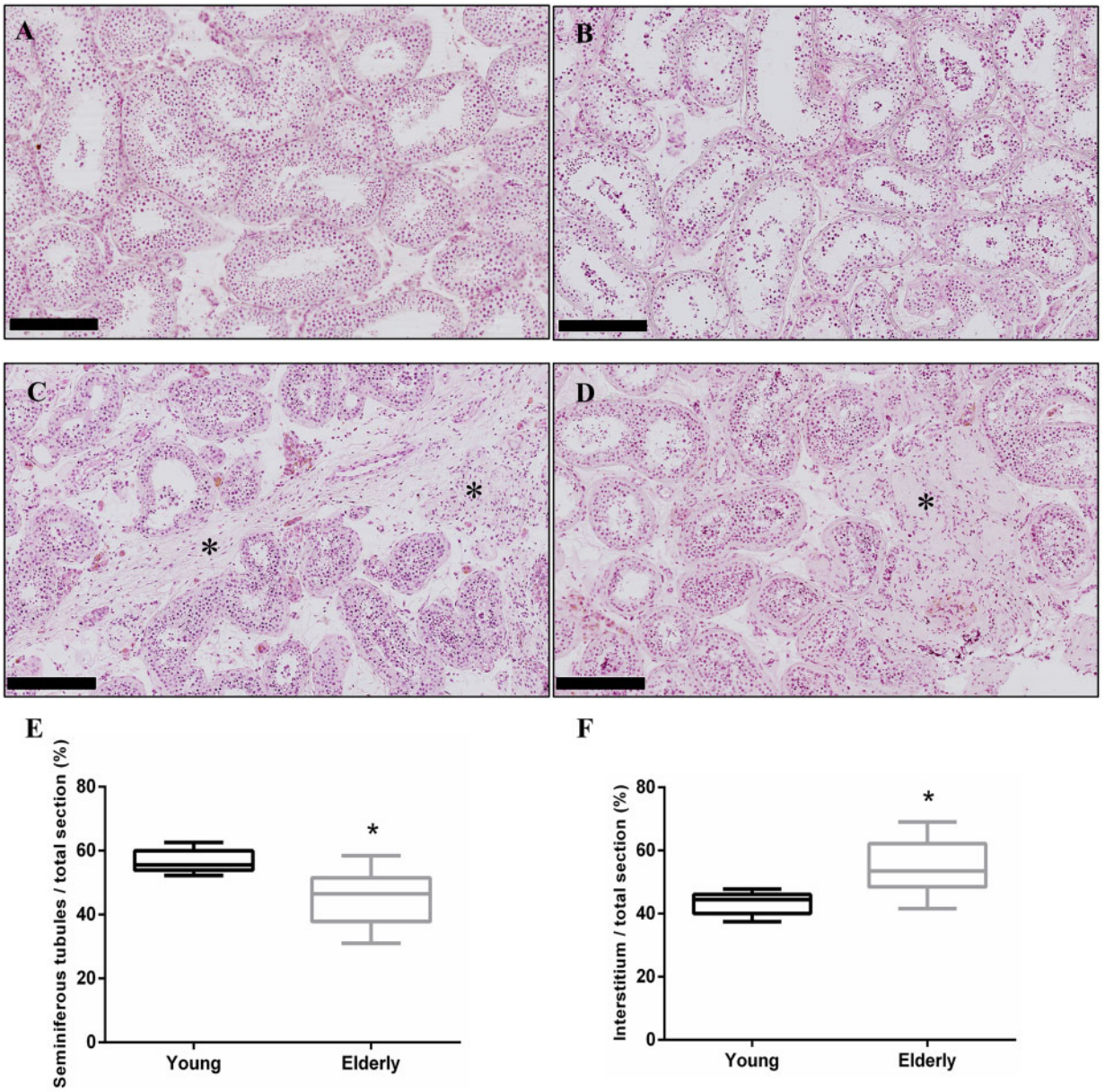


**Figure 1. Histological analysis of testicular biopsies from organ donors of different ages.** (**A**) 19 years, (**B**) 35 years, (**C**) 75 years and (**D**) 85 years. Sections representative of age-associated morphological features were stained with Carmalum. Asterisks indicate fibrotic tissue. Bars indicate 250 µm. (**E**, **F**) Percentages of seminiferous tubule area/section and interstitium area/section were determined and compared between young (n = 5, age range: 19–41 years) and elderly donors (n = 9, age range: 53–85 years) *$P < 0.05$.

association between the expression of each gene and donor age was analysed, no significant change in the expression of any of the examined genes was observed with ageing (Table II). These results are indicative of a steady expression of the essential steroidogenic steps occurring in Leydig cells despite the increasing age.

### *Steroid production by* in vitro *cultured human testis fragments in basal and stimulated conditions*

In light of the constancy of steroidogenic enzyme expression with age, we addressed the question of whether Leydig cell androgen production was reduced during ageing. To this end, testicular fragments of cryopreserved biopsies from organ donors were cultured *in vitro* for 3 h in basal conditions. Initially, the histological morphology of the tissue was examined both after cryopreservation and at the beginning and end of the culture period. No alterations were seen in tubular or interstitial compartments, validating the use of the organ culture method to maintain the structure of the testicular tissue *in vitro* for short-term cultures (data not shown). To assess the ability of Leydig cells to produce androgens, four steroids secreted into the media were simultaneously measured by mass spectrometry and

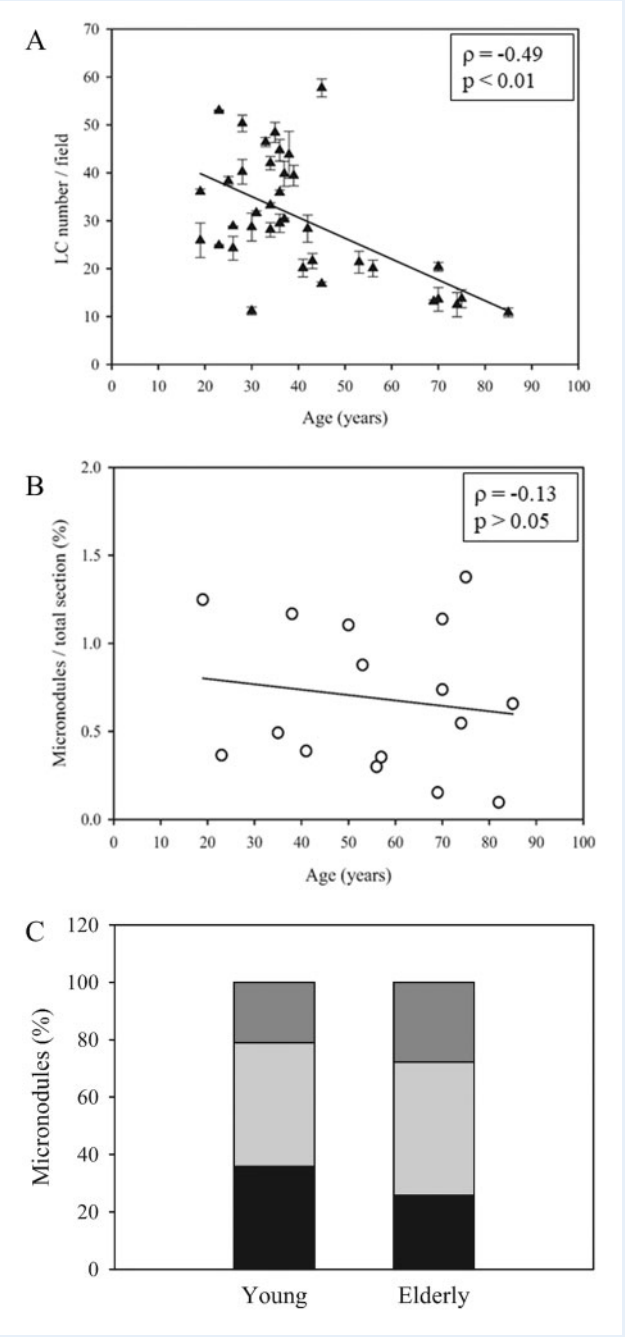


**Figure 2. Leydig cells.** (**A**) Correlation between age and Leydig cell number/field. Leydig cells were identified by immunohistochemical staining (see Supplementary Fig. S2) and counted on testis biopsies from organ donors (n = 13) and patients with complete spermatogenesis and reproductive hormone levels within the normal range (n = 24) of different ages. Spearman correlation test revealed a significant decline of Leydig cell number with advancing age. (**B**) Leydig cell micronodule distribution in testis biopsies from organ donors of different ages. Correlation between area occupied by micronodules/total section area and age. The presence of Leydig cell micronodules was similar in young and old men. (**C**) Frequency of the three classes of Leydig cell micronodules in testis sections from young and elderly organ donors. $P > 0.05$ as assessed using $\chi^2$ test; 15–19 cells/micronodule, black bars; 20–29 cells/micronodule, light grey bars; ≥30 cells/micronodule, dark grey bars.

normalized to the protein amount of the tissue fragment: 17-OH-progesterone (17-OH-P), dehydroepiandrosterone (DHEA), androstenedione (A) and testosterone (T) (Fig. 5). Interestingly, all four steroids were detectable in the collected culture media, but with concentrations of A were two times lower than those of the other three hormones. It is worth noting that there was considerable individual variation in steroid production regardless of age. The effect of age on the secretion of each measured steroid was examined by a correlation analysis, but no significant relationship was found, although 17-OH-P and T showed a weak negative trend (Fig. 5 A and D), whereas DHEA and A showed a positive trend (Fig.5 B and C). These findings indicate that ageing does not impact on the baseline steroidogenic capacity of Leydig cells *in vitro*.

Next, a possible age-related change in stimulated testosterone production by Leydig cells *in vitro* was investigated. Testicular fragments from cryopreserved biopsies were cultured in the absence or presence of rhLH or rhCG and secretion of T was determined. No significant response to gonadotropins was detected in eight organ donors of different ages examined, except in a 74-year-old donor responsive to LH and an 85-year-old donor responsive to hCG (Fig. 5E and F). The observed lack of gonadotropin-mediated stimulation of androgen production in cryopreserved testis fragments prompted us to investigate whether tissue cryopreservation negatively affected steroidogenic capability of Leydig cells and their hormone response. Similar experiments using fresh biopsies were therefore set-up immediately after organ collection. However, baseline steroid secretion was not correlated with age in these experiments (Fig. 6A–D), similar to the findings obtained with cryopreserved tissue. It is of note that A and DHEA levels measured in the culture media were ∼10 times lower than T and 17-OH-P, and all four steroids showed considerable interindividual variation. In the organ cultures with fresh testis tissue, rhLH did not stimulate T production in tissue from ten organ donors of different ages, except in a 57-year-old donor. In contrast, an hCG-dependent T increase was observed in four out of ten organ donors (Fig. 6E and F). Together these results demonstrated that: (i) ageing did not compromise baseline Leydig cell steroidogenesis; (ii) the response to gonadotropins was highly variable and independent of donor age; (iii) hCG was more potent than LH in eliciting an increase in T secretion *in vitro* and (iv) freezing testicular tissue negatively impacted the Leydig cell response to gonadotropins *in vitro*.

## Cryopreservation of testis biopsies changes the steroid profile compared with fresh tissue

Given that androstenedione and dehydroepiandrosterone were found in higher concentrations in the culture media from cryopreserved testicular fragments compared to those from fresh tissue, we investigated whether testis cryopreservation might alter the pattern of testicular steroid products. With this aim, tissue from nine donors of different ages was divided and either cultured directly (fresh tissue) or was cryopreserved and subsequently cultured. The basal levels of 17-OH-P, DHEA, A and T were determined and compared between fresh and cryopreserved tissue, irrespective of the donor age. T production was significantly higher in fresh tissue cultures, whereas A and DHEA levels were lower (Supplementary Fig. S3). The 17-OH-P production was

**Table I Leydig cell and Sertoli cell numbers during ageing.**

| Age (years) | 19–29 | 30–40 | 41–45 | 46–85 |
|---|---|---|---|---|
| n | 9 | 15 | 5 | 8 |
| Leydig cell number/field (mean + SEM) | $35.7 \pm 3.4^{a}$ | $35.5 \pm 2.4^{b}$ | $28.9 \pm 6.7^{c}$ | $15.7 \pm 1.4^{d}$ |
| Sertoli cell number/field (mean ± SEM) | $108.4 \pm 6.9^{e}$ | $110.0 \pm 5.8^{f}$ | $89.7 \pm 4.9^{g}$ | $72.9 \pm 4.1^{h}$ |

Statistical significance was calculated by ANOVA: a versus b, n.s.; a versus c, n.s.; a versus d, $P < 0.05$; b versus c, n.s.; b versus d, $P < 0.05$; c versus d, n.s. e versus f, n.s.; e versus g, n.s.; e versus h, $P < 0.05$; f versus g, n.s.; f versus h, $P < 0.05$; g versus h, n.s.

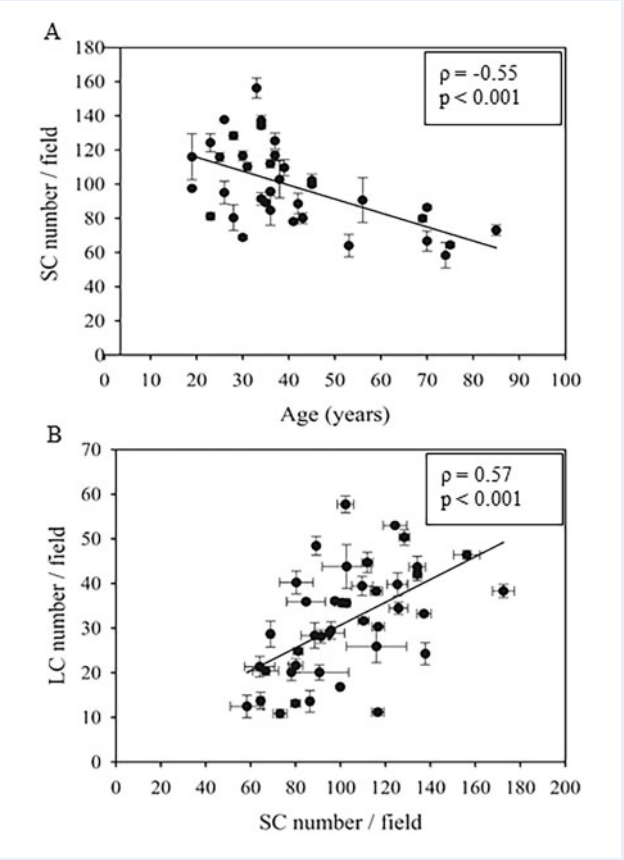


**Figure 3. Sertoli cells.** (**A**) Correlation between age and Sertoli cell number/field. Sertoli cells were identified by immunohistochemical staining (see Supplementary Fig. S2) and counted on testis biopsies from organ donors (n = 13) and patients with complete spermatogenesis and reproductive hormone levels within the normal range (n = 24) of different ages. Spearman correlation test revealed a significant decline of Sertoli cell number with advancing age. (**B**) Correlation between Sertoli cell number and Leydig cell number. Both cell types were counted in the same testis sections. A highly significant relationship between the two cell populations in each sample was observed.

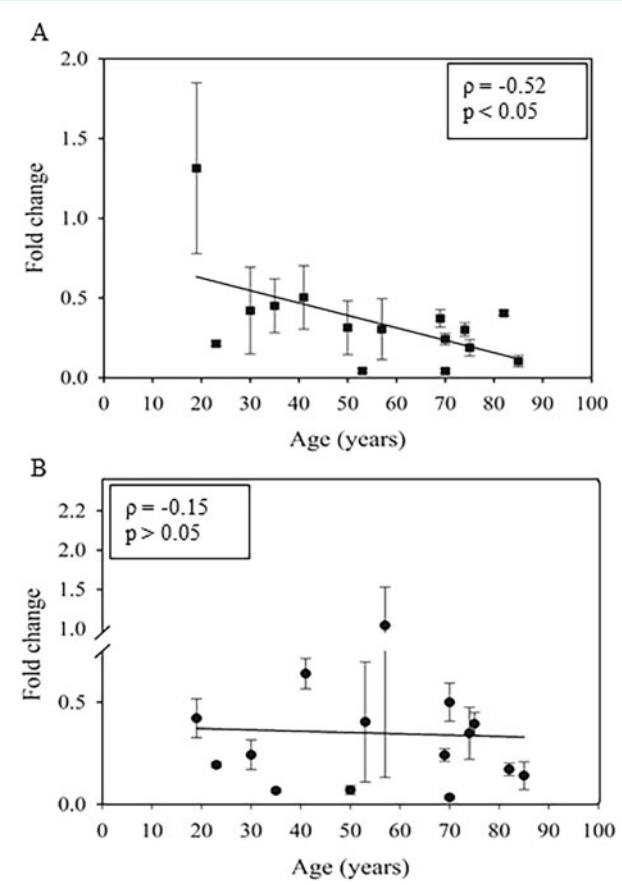


**Figure 4. Correlation between specific Leydig cell marker gene expression and age.** (**A**) *INSL3*; (**B**) *LHCGR*. RNA was extracted from testis biopsies of organ donors (n = 15) of different ages and analysed by qRT-PCR. *INSL3* mRNA expression negatively correlated with age, whereas no correlation was found between LHCGR mRNA expression and age.

not altered between fresh and cryopreserved tissue. These data revealed an alteration in steroidogenesis after cryopreservation of testicular biopsies, possibly related to a change in enzymatic activities of P450c17, 3β-HSD2 and 17β-HSD3.

## Discussion

In this first comprehensive study addressing the impact of age on human testis status, multiple aspects, including morphology, cell numbers and endocrine functions were concomitantly evaluated in the same individuals. Given that one of the male ageing hallmarks is the decline of serum T, we particularly explored the age-associated changes in

**Table II Correlation analysis between steroidogenic pathway genes and age.**

| Gene | Rho | *P*-value |
|---|---|---|
| *StAR* | −0.03 | 0.91 |
| *CYP11A1* | 0.02 | 0.92 |
| *CYP17A1* | 0.27 | 0.31 |
| *3β-HSD2* | 0.24 | 0.38 |
| *17β-HSD3* | −0.41 | 0.13 |

Rho coefficients and *P*-values are given.

Leydig cells. We found that Leydig cell number decreased during ageing in parallel with reduced *INSL3* expression, yet the testis steroidogenic capacity *in vitro* was not compromised with advancing age. Interestingly, we found also a decrease in Sertoli cell population size during ageing.

In the literature there are a few studies describing age-related changes, most examining only single features of human testis physiology, leading to fragmentary description of the ongoing process (Santiago *et al.*, 2019). Moreover, given the scarce availability of fresh, disease-free human adult testicular tissue, most studies have analysed testicular biopsies obtained from patients with prostate cancer, which may introduce bias in some of the previously reported results. In the present study, we mainly used testis biopsies, which all displayed normal spermatogenesis, from heart beating organ donors of different ages, with no history of overt clinically relevant pathologies. However, the tissue from this source is limited, resulting in a relatively small number of samples included in the study.

Testicular ageing in humans is characterized by changes in the morphology of the parenchyma, with gradual appearance of sclerosed tubules and tubules showing numerous morphological abnormalities in the germ cells (Paniagua *et al.*, 1987a, b), present findings which are associated with a decrease in sperm production (Neaves *et al.*, 1984). In contrast, age-related histological alterations in the interstitial compartment have been examined in less detail. We report here alterations in testicular architecture in elderly men compared to young ones, with the presence of fibrotic tissue in the interstitium and damaged tubules, as well as quantitative evidence of a significant interstitial area increase with age, accompanied by loss of area occupied by seminiferous tubules. These results reinforce the notion that ageing alters testicular morphological characteristics.

Previously, it has been suggested that the androgen deficit in ageing men might reflect a decline in the Leydig cell population (Kaler and Neaves, 1978). A few studies have addressed this issue, using only morphological criteria for Leydig cell identification and conflicting results have been reported, likely due to the relatively small number of subjects examined (Kaler and Neaves, 1978; Neaves *et al.*, 1984, 1985; Paniagua *et al.*, 1987a; Petersen *et al.*, 2015). In particular, only one of these studies demonstrated a decrease of Leydig cell number paralleled by plasma testosterone levels measured simultaneously in the same men (Paniagua *et al.*, 1987a). Here, we specifically identified Leydig cells by immunohistochemistry and determined their number in biopsies from individuals of different ages, which all had complete spermatogenesis (in the biopsy evaluation). Our data demonstrated that the size of the Leydig cell population significantly declined in advanced age. Importantly, this reduction positively correlated with a significant age-related change in Sertoli cell number in the same individuals, supporting the notion that human testis ageing is associated with a physiological senescence of somatic cells (Pohl *et al.*, 2019). Interestingly, our results reveal a direct close relationship between numbers of Leydig cells and Sertoli cells in each subject, at all ages. Consistent with our observations, studies in a mouse model following diphtheria toxin mediated cell ablation (Rebourcet *et al.*, 2014a, 2017) reported that reduced Sertoli cell number resulted in a parallel decline in Leydig cell population size, pointing to the concept that Sertoli cells are essential for the maintenance of Leydig cells in the adult. In contrast, in the aged Brown Norway rat, attrition of Sertoli cell number occurs. However, the age associated reduced serum testosterone secretion could not be explained by a reduction in Leydig cell number (Wang *et al.*, 1993; Midzak *et al.*, 2009), suggesting that other mechanisms controlling the size of Leydig cell population may be involved.

Consistent with the age-dependent loss of Leydig cells, we detected that the expression of *INSL3* mRNA in testicular tissue was significantly lower in elderly men. Indeed, it is well established that circulating INSL3 levels are only dependent on Leydig cell number and their differentiation status in humans as well in rats (Bay *et al.*, 2005; Anand-Ivell *et al.*, 2009; Ivell and Anand-Ivell, 2009). In agreement with our data, ageing men have significantly reduced peripheral INSL3 concentrations (Anand-Ivell *et al.*, 2006) and INSL3 expression, at both the mRNA and protein levels, is severely attenuated also in the older rats (Paust *et al.*, 2002).

The finding of a negative correlation between age and Leydig cell number in the present study would argue in favour of Leydig cell quantity contribution to the testosterone deficiency observed in many elderly subjects. Unexpectedly, when we analysed *in vitro* androgen secretion by cryopreserved testis fragments, we found that the intrinsic steroidogenic capacity of aged Leydig cells remained stable. Given that serum testosterone concentrations of the organ donors were unobtainable, the *in vitro* organ culture of testis fragments was used as an experimental model to study the age effect on Leydig cell steroidogenesis. This strategy has the advantages that a small amount of tissue is required and the *in vivo* situation is mimicked, as the cell to cell interactions and the structural integrity of both interstitial and tubular compartments are preserved (Boitani *et al.*, 1993, 1995). Importantly, this approach allowed us to assess the amounts of four steroids (17-OH-P, DHEA, A, T) secreted by testis fragments. In line with the evidence that *in vitro* Leydig cell androgen production did not decline with age, mRNA expression of genes required for Leydig cell androgen synthesis in testis biopsies from the same organ donors was also found to be stable regardless of age. Thus, our data suggest that the decrease in Leydig cell number with ageing may be compensated by the retained or possibly even increased intrinsic steroidogenic potential in the remaining Leydig cells. However, the significant impact of the changes in health and lifestyle factors on individual androgen status should be noted when considering the *in vivo* age-associated Leydig cell dysfunction.

In a recent study, Curley *et al.* (2019) generated a mouse model of premature ageing which has a characteristic testicular ageing phenotype with reduced Leydig and Sertoli cell number, decreased circulating testosterone and decreased transcriptional expression of steroidogenic

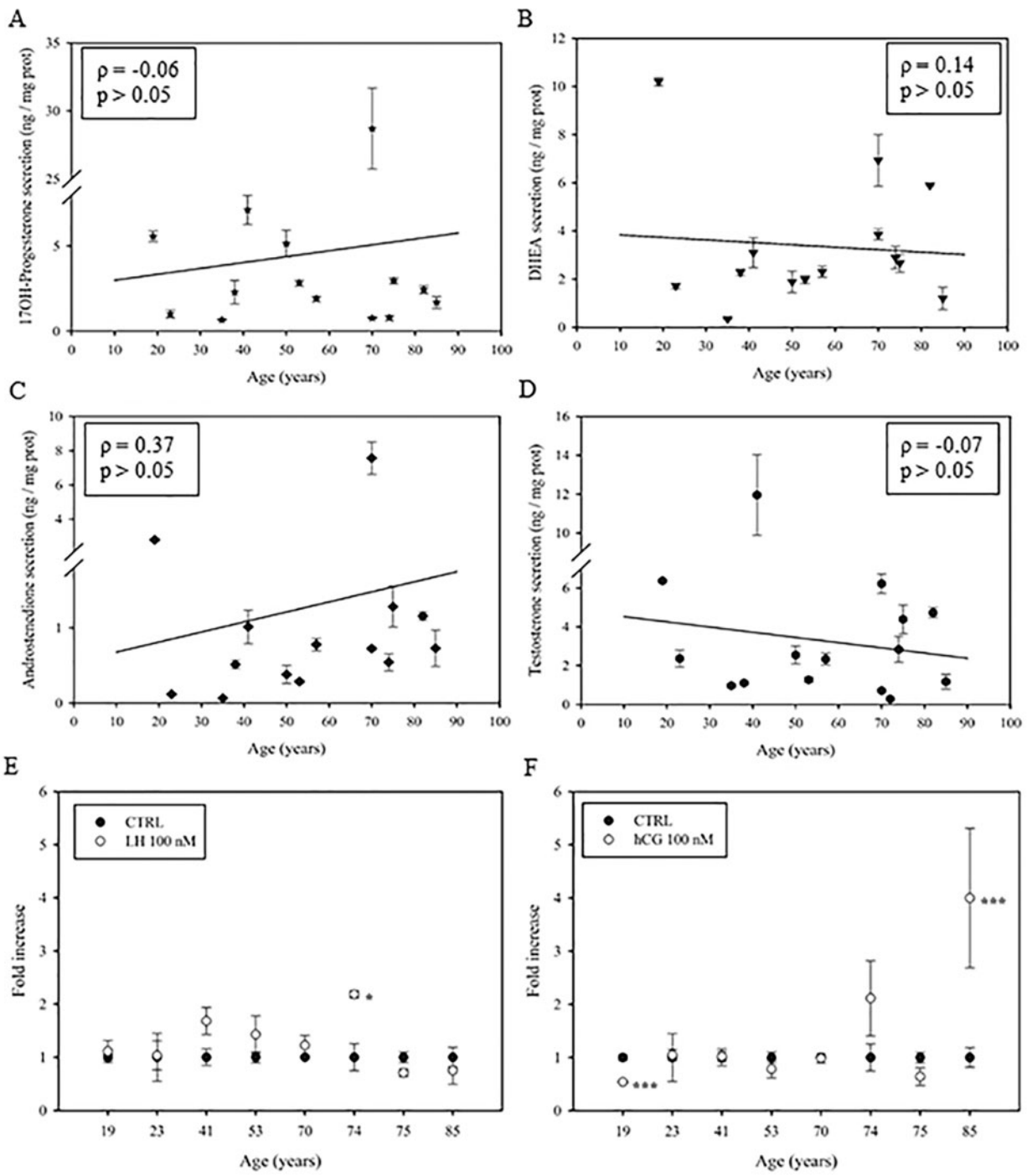


**Figure 5.** ***In vitro*** **androgen secretion by tissue fragments obtained from cryopreserved testis biopsies of organ donors of different ages.** Testicular fragments from organ donors (n = 14) were cultured for 3 h under basal and stimulated conditions and four steroids were simultaneously measured into the media by LC-MS/MS and normalized to the protein level in the testicular fragment. To investigate steroid levels and age, Spearman correlation test was performed. (**A**) 17-OH-progesterone; (**B**) DHEA; (**C**) androstenedione; (**D**) testosterone. No significant age-related changes in basal androgen production were observed. (**E**, **F**) Testis fragments from organ donors (n = 8) were cultured for 3 h in the presence or absence of rhLH or rhCG and testosterone secreted was measured. Overall, no significant response to stimulation was found. LC-MS/MS, liquid chromatography-tandem mass spectrometry. DHEA, dehydroepiandrosterone.

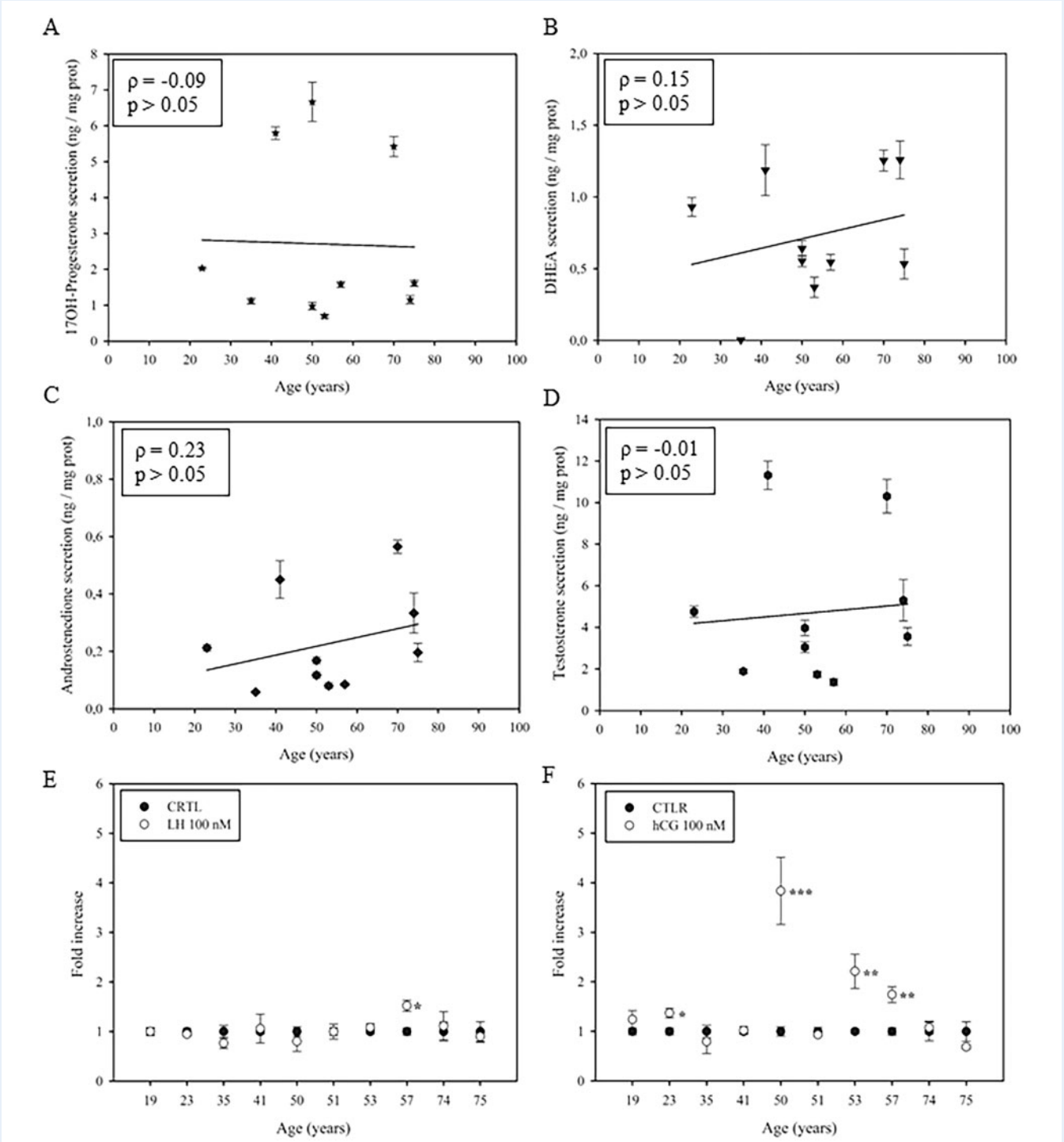


**Figure 6.** ***In vitro*** **androgen secretion by tissue fragments obtained from fresh testis biopsies of organ donors of different ages.** Immediately after collection, testicular fragments from organ donors (n = 10) were cultured for 3 h under basal and stimulated conditions and four steroids were simultaneously measured into the media by LC-MS/MS and normalized to the protein level in the testicular fragment. To investigate steroid levels and age, Spearman correlation test was performed. (**A**) 17-OH-progesterone; (**B**) DHEA; (**C**) androstenedione; (**D**) testosterone. No significant age-related changes in basal androgen production were observed. (**E**, **F**) Fresh testis fragments (n = 10) were cultured for 3 h in the presence or absence of rhLH or rhCG and testosterone secreted was measured. No significant response to rhLH was found, whereas hCG caused a significant increase in testosterone production in biopsies from four out of ten organ donors. DHEA, dehydroepiandrosterone.

enzymes. Importantly, when premature ageing was selectively induced in either Leydig cells or Sertoli cells, no testicular dysfunction was observed, indicating that extrinsic factors changing with age, rather than a cell intrinsic pathway, drive the functional impairment of Leydig cell with age. Indeed a variety of environmental factors, lifestyle habits and vascular dysfunction, often in combination, have the capability to interfere with human testis function at the individual level (Sartorius *et al.*, 2012). In line with this, an increased between-subject variation in total testosterone levels with advancing age has been observed after age of 40 years (Kelsey *et al.*, 2014). Consistently, our study highlights a pronounced variability among individuals at all ages as a major characteristic observed for all parameters tested. Individual differences may exist in the extent to which men, with frail general health and irrespective of age, are more susceptible to the risk of developing androgen-related disorders over the lifespan.

In this study, *in vitro* androgen production was examined using both fresh testicular biopsies and cryopreserved tissue. Lower testosterone levels and higher androstenedione and DHEA levels were observed in cryopreserved samples, suggesting alterations in 3βHSD2 and 17βHSD3 enzymatic activities. Importantly, also the response to hCG-mediated stimulation of T production *in vitro* differed between fresh and cryopreserved tissue, with a higher number of fresh tissue samples responding, showing significantly higher T levels after hCG treatment compared to cryopreserved tissue.

Another interesting finding in this work was that human Leydig cells responded differently to the two recombinant gonadotropins, LH and hCG in *in vitro* culture. Whether the two gonadotropins might differ also in eliciting intracellular signaling remains to be explored. The fact that hCG was more efficient than LH at the same concentration is in line with recent studies demonstrating a higher potency of rhCG compared to rLH in human granulosa cells, mouse primary Leydig cells and Leydig tumour cells (Casarini *et al.*, 2012; Riccetti *et al.*, 2017a,b).

Together our data deepen the understanding of human testis ageing, providing new evidence of a correlation between Leydig and Sertoli cell numbers at all ages, both declining with advancing age. Of particular importance is the original observation that *in vitro* Leydig cells do not lose their steroidogenic capacity during ageing, implying that the *in vivo* androgen profile in elderly men is influenced by the complex combination of both intrinsic and extrinsic factors.

## Supplementary data

Supplementary data are available at *Human Reproduction* on-line.

## Data availability

The data underlying this article are available in the article and in its Supplementary Material.

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## Authors' roles

C.B. planned and designed the study. V.M., V.E., S.D.P. and J.E.N. were responsible for study execution and data acquisition. G.S. and G.B. provided testis biopsies from organ donors. N.J., A.J. and E.R.-D.M. provided testis sections from patients referred to the andrological clinic. F.F., M.M., U.P. and C.P. were responsible for androgen assessment by LC-MS/MS. N.J., A.J., E.R.-D.M., V.M., E.V and C.B. participated in critical discussion. V.M. and C.B. analysed the data and wrote the manuscript.

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## Conflict of interest

The authors have no conflicts of interest.

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