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Mini-Review

Age-related Changes in the Adrenal Cortex: Insights and Implications

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Abstract

Aging is characterized by a gradual decline in physiological function. This process affects all organs including the adrenal cortex, which normally functions to produce essential steroid hormones including mineralocorticoids, glucocorticoids, and androgens. With increasing age, features such as reduced adrenal cortex size, altered zonation, and increased myeloid immune cell infiltration substantially alter the structure and function of the adrenal cortex. Many of these hallmark features of adrenal cortex aging occur both in males and females, yet are more enhanced in males. Hormonally, a substantial reduction in adrenal androgens is a key feature of aging, which is accompanied by modest changes in aldosterone and cortisol. These hormonal changes are associated with various pathological consequences including impaired immune responses, decreased bone health, and accelerated age-related diseases. One of the most notable changes with adrenal aging is the increased incidence of adrenal tumors, which is sex dimorphic with a higher prevalence in females. Increased adrenal tumorigenesis with age is likely driven by both an increase in genetic mutations as well as remodeling of the tissue microenvironment. Novel antiaging strategies offer a promising avenue to mitigate adrenal aging and alleviate age-associated pathologies, including adrenal tumors.

Key Words: adrenocortical, aging, cellular senescence, senescence-associated secretory phenotype (SASP), aging and cancer, adrenal tumor, steroidogenesis, sexual dimorphism, longevity, antiaging

Abbreviations: ACC, adrenocortical carcinoma; ACTH, adrenocorticotrophic hormone; AD, Alzheimer disease; APAs, aldosterone-producing adenomas; APMs, adrenal-producing micronodules; CDKi, cyclin-dependent kinase inhibitor; cKO, conditional knockout; DHEA-S, dehydroepiandrosterone/sulfate; DHT, dihydrotestosterone; ER, endoplasmic reticulum; HPA, hypothalamic-pituitary-adrenal; IL, interleukin; mo, month; SASP, senescence-associated secretory phenotype; wk, week; y, year; zF, zona fasciculata; zG, zona glomerulosa; zR, zona reticularis.

Aging is a complex biological process that refers to the progressive decline in physiological function [1]. Age-related alterations in tissue structure and integrity are a ubiquitous phenomenon that affect all organs, including the adrenal gland [2]. The adrenal is an essential endocrine organ that is situated above the kidneys and functions to produce many critical hormones [3]. The adrenal is composed of 2 compartments of distinct embryological origin: the cortex and the medulla, which are surrounded by an outer mesenchymal capsule layer. In this review, we will focus on age-related changes specifically within the adrenal cortex, which is subdivided into 3 functionally and histologically distinct zones that are replenished through a widely accepted centripetal migration model [4-7]. The outermost zone, the zona glomerulosa (zG), is responsible for the production of mineralocorticoids that regulate salt and water balance. The intermediate zone, the zona fasciculata (zF), produces glucocorticoids in response to adrenocorticotropin (ACTH) under the regulation of the hypothalamic-pituitary-adrenal (HPA) axis. Finally, the innermost zone, the zona reticularis (zR), produces adrenal androgens, including dehydroepiandrosterone (DHEA) and its sulfated form, DHEA-S [3]. As we age, the adrenal gland undergoes changes that disrupt its ability to maintain homeostatic hormone levels, which can significantly affect overall health and well-being. Thus, researching adrenal aging and interventions to delay the onset of age-associated adrenal pathologies has the potential to help increase endocrine function and improve health span.

Studying the effects of aging on the normal adrenal gland is a challenging task. First, information on "healthy" aged human adrenal glands in the literature is scarce. While rodents are the most commonly used model organism to investigate these phenomena, standard laboratory strains lack a functional zR due to silencing of *Cyp17a1* after birth [8, 9], which limits our use of these models to study adrenal androgens [10]. Moreover, the mouse adrenal cortex contains an additional X-zone [11], which is of fetal origin [12]. The functional significance of the X-zone is incompletely understood (see review [13]), but it regresses in males after puberty and in females after the first pregnancy [11, 12, 14]. Secondly, conducting in vivo aging studies is expensive and requires long study timelines, which highlights the current challenge in determining the exact onset of age-related changes. While this may be clearer in human studies, there is ongoing debate in the scientific community regarding the appropriate time point for aging in laboratory animals. A common approach is to use age equivalencies to humans. For example, a 3- to 6-month-old C57BL/6J mouse is roughly equivalent to a 20- to

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30-year-old human, while a 10- to 14-month-old mouse is approximately aged 38 to 47 years, and an 18- to 24-month-old mouse is approximately aged 56 to 69 in human [15]. Despite the difficulties associated with defining aging time points, much of the research presented in this review focuses on mice that are older than 1 year.

Overall, this review aims to consolidate current knowledge regarding adrenal aging studies conducted in animal models and present what is known in the context of clinical data (Fig. 1). We seek to highlight areas that require further investigation and discuss potential therapeutic interventions for antiaging strategies, including those highlighted by the recent scientific statement on Hormones and Aging from the Endocrine Society [16]. One crucial factor that we will consider throughout this review is that male and female adrenal glands exhibit an aging dimorphism. This sex difference is clinically relevant given that many age-associated adrenal pathologies exhibit a female sex bias [17]. Thus, understanding sex-dependent mechanisms of adrenal aging may provide novel insight into disease etiology.

Structural Changes in the Aged Adrenal

The fetal adrenal gland experiences rapid growth in utero due to expansion of the fetal zone. After birth, the fetal zone atrophies and the definitive zone remodels to form the adult cortex [18]. Ultimately the adrenal increases in size again during postnatal maturation (from week 2-3), and this is conserved across most species, including *Mus musculus*, *Rattus norvegicus*, and *Homo sapiens* [19, 20]. However, in studies as early as 1960, adrenal weight was found to significantly decrease during aging in a sex-dimorphic manner [21, 22]. In this study, raw adrenal weight peaked in male mice at 14 weeks, whereas in females, it peaked later at 26 weeks. After peak growth, adrenal weight dramatically declined in males, but regressed at a slower rate in females. This trend is also observed in various mammalian models and human clinical samples where adrenal weight decreases in an aged setting and is accelerated in males [23, 24]. Additionally, data from our own laboratory confirm that adult C57BL/6J female adrenals are larger when compared to that of males, and male adrenal weight significantly decreases with advanced aging up to 78 weeks [25]. Given that weight changes between male and female adrenals are first observed at puberty in mice, it is tempting to speculate on the role of sex hormones in age-related adrenal changes [19, 26]. Recent literature suggests a dichotomous role of male sex hormones in adrenal aging, whereby androgens have been shown to suppress proliferation *and* enhance immune-mediated degeneration. Specifically, Grabek et al [27] demonstrated that androgens repress recruitment and proliferation of capsular stem cells, leading to decreased proliferation and cortical cell renewal in males compared to females. In addition to the known sex differences in X-zone retention [28], this could provide an explanation as to why female adrenals are larger post puberty. However, the dynamics of capsular stem cell proliferation in adrenal aging has not been evaluated, which is of particular interest given the decline in androgen levels with age [29]. Recent studies have also uncovered a novel role for androgens in regulating the recruitment and activity of phagocytic immune cells to the adrenal cortex [25, 30]. These data demonstrate that androgen deprivation following castration decreases infiltration of CD68-positive myeloid cells that normally function to clear away damaged adrenal cortex cells. Coupled with enhanced proliferation, this blunted immune response in a low-androgen environment results in significant adrenal enlargement. Complementary data show treatment with the androgen receptor agonist dihydrotestosterone (DHT) promotes myeloid cell infiltration and subsequently accelerates adrenal cortex regression [30]. Furthermore, our study found an accumulation of CD68-positive myeloid cells in aging mice, particularly at the cortical-medullary boundary, where the longest-lived adrenocortical cells reside. This effect was significantly elevated in males compared with females [25], further supporting a key role for sex hormones in modulating the immune microenvironment during adrenal aging. Immune-mediated regression of the aged adrenal will be discussed in greater detail later in this review.

Given the known species-dependent differences in adrenal biology, it is important to evaluate the observed age-associated changes in the human adrenal. This has been investigated in healthy human adrenal tissue whereby cortical area was evaluated using zone-specific markers [31]. This study compared adrenals from male and female participants younger and older than 50 years. In older individuals, there were significant reductions in the zR as per CYB5A staining, and a concomitant increase in the zF as per VSNL1 and HSD3B2 calculated area. Additional changes were shown when evaluating males only, whereby there was also a decrease in zG according to VSNL1- and CYP11B2-positive staining. The findings in relation to zR reduction have previously been observed in an independent cohort of young vs old male human adrenal specimens [32], and age-related changes in HSD3B2 expression in the adrenal have been observed in a study of male and female human adrenals [33]. While changes in zR synthesized hormones with aging have been widely documented, the relative changes in zG and zF hormones are still under investigation. These initial insights into human adrenal aging highlight the complexity of both sex-specific differences during aging, and the importance of elucidating the relationship between structural and functional differences within the aged adrenal cortex.

Overall, there are many structural changes associated with advanced aging in the adrenal cortex (Table 1) [34-40]. While some of these changes have been associated with changes in functional hormone output, the physiological effect of many of these changes remain unexplored.

'Inflammaging' in the Adrenal

"*Inflammaging*" is a term used to describe the low-grade, chronic inflammatory response associated with age-related diseases [41]. This tissue-dependent and heterogeneous response is driven by proinflammatory cytokines such as tumor necrosis factor, interleukin-6 (IL-6), and interleukin-1 (IL-1) [42]. Immune cells and cytokines that contribute to aging in other major organs have been partially characterized, yet remain less well studied in the adrenal [43].

The adrenal cortex is often described as a highly immunosuppressed environment due to the presence of glucocorticoids [44]. However, initial findings by Rhodin noted the abundance of macrophages in rat adrenals, and this was subsequently confirmed by Hume et al in 1984, who found high levels of F4/80 positive macrophages in the mouse adrenal cortex [45, 46]. Subsequent studies, including those using advanced methodologies such as single-cell RNA sequencing and

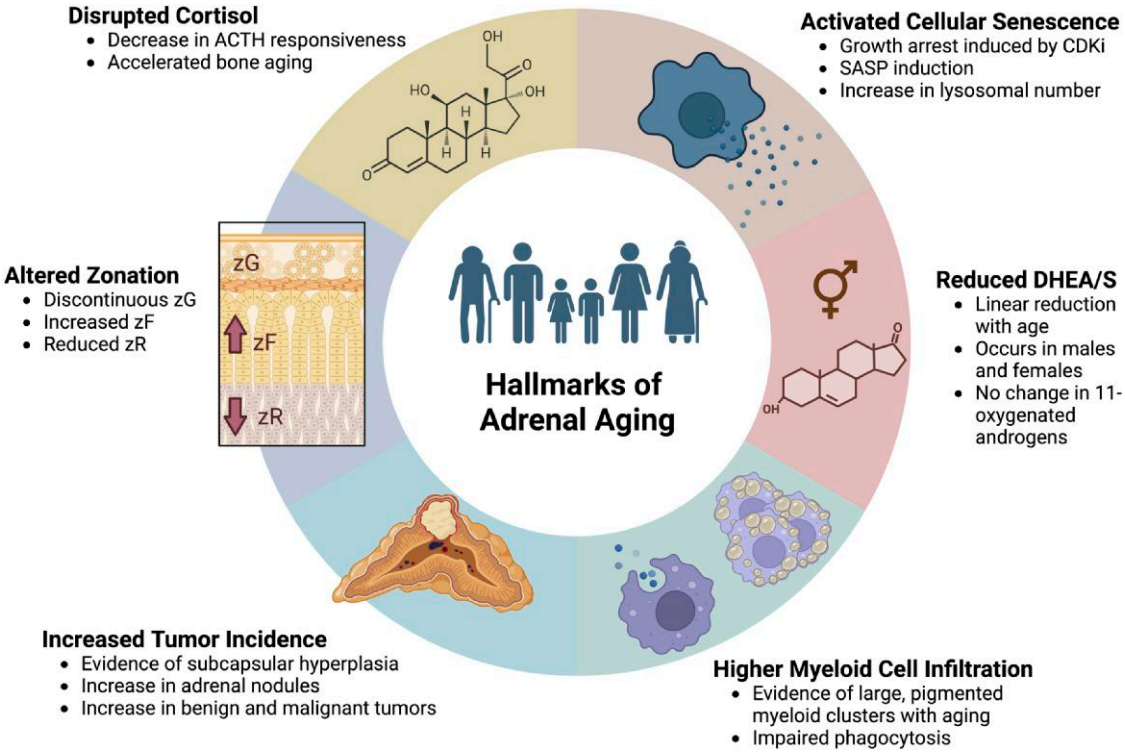

Figure 1. Hallmarks of adrenal aging. ACTH, adrenocorticotropic hormone; CDKi, cyclin-dependent kinase inhibitor; DHEA-S, dehydroepiandrosterone-sulfate; SASP, senescence-associated secretory phenotype; zF, zona fasciculata; zG: zona glomerulosa; zR, zona reticularis. Schematic created with BioRender.com.

lineage tracing, have identified multiple immune cell types in the adrenal cortex. While diverse immune cell types can be found, these new approaches have identified macrophages as the most abundant cell type, but also highlight other prominent myeloid cell types such as dendritic cells, neutrophils, and monocytes [25, 47, 48]. Myeloid cells have traditionally been found at the cortical-medullary boundary and are thought to be involved in phagocytosis-mediated clearance of apoptotic cortical cells [27, 30, 40, 48]. Phagocytosis is classically considered immunologically silent and is critical for maintaining homeostasis [49], which may be an important mechanism that contributes to normal homeostatic turnover of the adrenal cortex, as well as X-zone regression. However, in an aged context, phagocytosis often becomes inefficient and dysfunctional, leading to an accumulation of cellular debris that initiates a proinflammatory response [47, 50, 51]. This process is better understood in the setting of age-related macular degeneration, in which phagocytosis is reduced by 80% in aged mice, and defects in macrophage recruitment and function result in retinal pathologies [51-53]. In the adrenal, phagocytosis ligands and receptors such as Galectin-3, TREM2, and MERTK have been shown to be highly expressed and involved in the clearance of damaged cells [25, 30]. Expression of *Trem2* is significantly reduced in old (aged 27 months) compared to young (aged 6 months) mouse adrenals [54]; however, the dependency on this pathway for efficient phagocytosis in the adrenal has not been evaluated. Notably, treatment of inflamed adrenals with pexidartinib [55], an inhibitor of colony-stimulating factor 1 receptor (CSF1F), dramatically decreased infiltrating myeloid cells and increased adrenal weight [30]. Given that immune dysfunction is associated with age-related diseases, therapeutic interventions such as pexidartinib are attractive options. However, it is important to consider the balance between detrimental pathological inflammation and effective clearance of harmful toxins [43, 56].

Although there are few comprehensive studies evaluating "inflammaging" in the adrenal cortex, one consistent historical observation is the accumulation of large, pigmented multinucleated cell clusters in the inner adrenal cortex with increased age [34, 35, 57]. These cells were initially characterized by high lipid staining and brown degeneration, but have since been shown to be positive for myeloid cell markers such as CD68, CD11c, CD11b, and F4/80 [25, 40]. Recent studies have revealed that adrenocortical myeloid cell clusters, a significant feature of adrenal aging, are more frequent in males compared with females. These clusters begin to appear at age 4 months and progressively accumulate up to age 30 months, indicating a substantial age-dependent change in adrenal structure and function [35]. A study conducted in our laboratory demonstrated the presence of CD68-positive myeloid cell clusters in control mice at ages 44 and 52 weeks, an effect that was highly accelerated in males [25]. Using multiplex immunohistochemistry, we conducted further analysis on multinucleated cell clusters and identified diverse subpopulations including F4/80-positive resident macrophages, CD11c-positive dendritic cells, and CD11b-positive monocyte-derived macrophages. While not mutually exclusive, these markers can serve as general phenotypic indicators for each subpopulation [58]. While the functional effects of these giant myeloid clusters in the adrenal gland are unknown, a very similar phenotype has been observed in the aging ovary and is associated with increased IL-6 secretion [59]. As proinflammatory immune cell clusters accumulate in aging tissues,

Table 1. Structural changes in the aging mouse adrenal

Study description	Sample cohort	Findings	References
Comparison of cellular structure in old adrenals of various mouse strains	♀ Strains: cd, be, and C57BL/6J Old: aged 1 y Senile: average age 21 mo	• Evidence of adrenal degeneration (mostly zG and zF) found in 17% of old and 51% of senile mice • Brown degeneration and connective tissue replacement higher in senile mice • Accessory nodule incidence higher in females and senile mice	[34]
Histochemistry and ultrastructure of lipid pigment in adrenals of aging mice	♀C57BL/10 mice Time points: 4, 8, 20, and 30 mo	• Adrenal pigments visible at inner cortex after sexual maturity and increases with age • Adrenal pigments positive for many lipids and proteins commonly known as "age pigments" (including lipofuscin) • At 20 mo, observations of multinucleated giant cells at distal region noted with large lipid droplets and swollen mitochondria	[35]
Structure of the endoplasmic reticulum in adrenals of senile mice	♂ DKK mice Mature: aged 6 mo Senile: aged 23 mo	• Lamellar structure in linear, curved, horseshoe, or concentric shape observed in zR of senile mice • Lamellar structures suspected to be flattened smooth ER cisternae • Structures were found on occasion in young mice and similar structures were found in mouse adrenals treated with cholesterol synthesis inhibitors	[36]
Subcapsular hyperplasia in aged mouse adrenal glands	♂ ICR mice Time points: 2, 4, 13, 19, and 25 mo	• Subcapsular hyperplasia incidence increased from 0% at 2 mo to 59% in males and 91% in females at 19 mo • Hyperplasic lesions abundant in fusiform cells with some polygonal cells in the center • Lipid droplets confirmed by Oil red O and Sudan black were found in cytoplasm of both cell types	[37]
Investigation of subcellular hyperplasia and mast cell infiltration in aged mouse adrenal cortex	♂ A/J, BALB/c, C2H/He, C57BL/6, DBA/2J, IQI/Jic, WHT/Ht mice aged 13-15 mo	• In all mouse strains, females demonstrated higher levels of subcapsular hyperplasia at age 13-15 mo. This was more pronounced in C57BL/6, DBA/2J, and IQI/Jic mice • Using toluidine staining, all mouse strains assessed showed a positive correlation between mast cell infiltration and subcapsular hyperplasia with higher numbers detected in females compared to males	[38]
Degeneration and mitochondrial damage in adrenal aging	C57BL/6J mice Aged 5-110 wk at various intervals No sex indicated	• Mitochondrial dysfunction accumulates in aging mouse adrenals denoted by COX and SDH costaining from 5-, 50-, 75- and 110-week-old mice • An increase in adrenal picrosirius red staining indicates collagen accumulate over time in mice at age 37, 84, and 115 wk compared to 5-wk-old mice	[39]
Understanding endothelial dynamics in adrenal aging	♂ C57BL/6J mice Young: aged 2-8 wk Old: aged 56-70 wk Human adrenals Young: aged 18-20 y Old: aged 70-80 y	• No change was found in vessel density or number of arteries between young and old mouse adrenals • Significant increase in macrophages (F4/80) in old vs young adrenals in mice • Comparison of old vs young human adrenals shows no significant difference in vasculature and recapitulates increase in F4/80 staining demonstrated in mice	[40]

Abbreviations: ER, endoplasmic reticulum; zF, zona fasciculata; zG, zona glomerulosa; zR, zona reticularis; wk, week; mo, month; y, year.

their cytokines may further potentiate a pathological inflammatory immune response, leading to increased age-associated damage. Removal or resolution of these immune clusters in an aged setting may alleviate age-related degeneration of the adrenal cortex.

Given that the adrenal cortex functions to produce steroid hormones, there is a complex crosstalk with the immune system. In 1950, a study by Selye and Stone [60] demonstrated transformation of the adrenal cortex to lipid-rich, myeloid-like tissue through exogenous supplementation of testosterone, thyroxine, and lyophilized anterior pituitary. While this study used a crude cocktail of stimulants, it speaks to the ability of hormones to recruit and differentiate progenitor immune cells in the adrenal. Recent findings by our laboratory and Wilmouth et al, although more than 70 years later, complement this historic observation by showing androgen deprivation reduces myeloid cell infiltration, and conversely, DHT treatment promotes myeloid cell accumulation in the mouse adrenal cortex [25, 30]. Additional studies highlight the adrenal as a master regulator of immune recruitment under stressed conditions. For example, in obesity mouse models, males exhibit higher levels of proinflammatory monocytes and macrophages compared to females, an effect that is abrogated following surgical castration or genetic androgen receptor ablation [61]. Additionally, acute high-dose lipopolysaccharide is lethal in adrenalectomized mice due to the

lack of glucocorticoids that normally act as an essential immunoregulatory response [62]. This indicates that the modality of inflammatory stress and associated cytokines can induce variable outcomes, which must be considered in the context of chronic diseases associated with aging. Perturbations in the immune-hormone axis in an aged adrenal cortex context is not well understood; however, in patients with adrenal insufficiency, natural killer cell function is impaired and associated with increased mortality [63]. These data highlight the need for further investigation of immune dysregulation and hormonal action in the aging adrenal cortex.

Adrenal Hormones and Aging

In the aged adrenal, there are significant changes in the production of mineralocorticoids, glucocorticoids, and androgens, which affect normal physiology. First, the availability of circulating steroid precursor as cholesterol-rich lipoproteins increases with age [64]. Importantly, expression of P450scc (CYP11A1) in each adrenal cortex zone is maintained during aging, indicating that the potential conversion of cholesterol to steroid precursors is stable over time [65]. Measurements of steroid hormones and their respective metabolites are routinely completed in humans, and there have been many detailed reviews speculating the aging steroid metabolome (for a detailed clinical analysis, see [66-69]). These studies consistently observe a substantial reduction in adrenal androgens with increasing age, yet more modest changes in aldosterone and cortisol levels.

Many studies show a progressive decrease in aldosterone over time, which can be somewhat attributed to a decrease in zG area and CYP11B2 expression [31, 70]. In addition, there is an age-associated increase in the prevalence of adrenal-producing micronodules (APMs), previously known as aldosterone-producing cell clusters [71, 72]. Somatic mutations detected in postmortem, nonhypertensive APMs largely consist of *CACNA1D* mutations (34%) and are absent of *KCNJ5* mutations [71]. An additional study of APMs from a cohort with clinically diagnosed primary aldosteronism also had no *KCNJ5* mutations [73]. Further stratification of these APMs using mass spectrometry identified 2 distinct subgroups, one of which was predicted to be more similar to aldosterone-producing adenomas (APAs) and has been proposed as a potential precursor. Interestingly, this group of APMs was significantly older compared to the non–APA-associated cluster [71]. Further exploration of these subtypes and mutation status in the context of aging would be useful to evaluate whether APMs are precursors to APAs. In a cohort of human adrenals, CYP11B2 expression was shown to decrease with age. This study also noted a discontinuous and sporadic CYP11B2 expression pattern with advanced aging [31, 66, 70]. Given that this decrease in CYP11B2 is coincident with the prevalence of APMs, it would be useful to correlate CYP11B2 expression levels with aldosterone secretion to evaluate the functional significance of this histological finding in aging [70]. While some APMs have been detected through a pathological finding (eg, hyperaldosteronism), many are found only post mortem, which precludes the ability to further investigate their biochemical and clinical significance [73]. Accessing adrenal tissue and matched steroid data to better understand this relationship would be useful, but is challenging given that an adrenalectomy is rare in the absence of an overt clinical phenotype.

Age-related changes in cortisol levels have been extensively studied, with conflicting results reported in the literature. While some studies observe a gradual increase in cortisol with aging, others find no change, despite an age-associated increase in the glucocorticoid precursor, 11-deoxycortisol [31, 74, 75]. These contradictory findings have been somewhat attributed to methodological differences, such as sample collection time, which is a critical factor given the importance of circadian regulation of steroid hormone production. Additionally, some studies show that the secretion pattern and HPA axis can be impaired in aging, leading to decreased ACTH responsiveness [74, 76]. Animal models, particularly mice, have provided valuable insights into age-related effects of elevated cortisol. An overall decrease in the ratio of ACTH to corticosterone has been recapitulated in aged mice up to 100 weeks, with a reduction of approximately 40% shown from week 15 to week 100. This highlights the value of using mouse models to study high glucocorticoids and decreased responsiveness to ACTH in an aged setting [77, 78]. Additionally, high glucocorticoid levels alone have been shown to decrease bone mineral density and bone vasculature in aged male and female mice (up to 31 and 25 months, respectively) [79]. These features of bone aging are commonly found in patients with glucocorticoid excess resulting from either long-term supplementation or excess production [80, 81].

The relationship between elevated glucocorticoid levels and various age-associated diseases remains unclear, as it is challenging to distinguish whether the high levels of glucocorticoids are a contributing factor to the pathogenesis of these diseases, or a consequence of their progression. However, a recent longitudinal study showed that elevated urinary free cortisol levels predict the development of Alzheimer disease (AD) an average of 6 years before onset [82]. Whether glucocorticoids are causative or correlative in AD is not clear from this study; however, in vivo work indicates that blocking the conversion of cortisone to cortisol by inhibiting 11B-HSD1 (hydroxysteroid 11-β dehydrogenase 1) reduces cognitive decline independent of amyloid beta (Aβ) plaque [83]. There are ongoing clinical trials investigating the therapeutic usefulness of lowering glucocorticoids in age-related neurodegenerative diseases [84, 85]. The production of terminal steroids by peripheral tissues in aging is equally important to consider. For example, 11B-HSD1 activity has been shown to increase in human skin, likely contributing to increased circulating cortisol [86]. Another mechanism that increases glucocorticoid levels is reduced steroid inactivation through steroid sulfation [87]. A recent study showed that increasing the percentage of sulfated (inactivated) steroid hormones alleviated symptoms of age-related disease (eg, AD) and extended lifespan [88]. While this study was carried out in *C elegans* and focused on neurobiology, these results highlight the need to investigate steroid sulfation in the aged adrenal. Finally, a study in mole rats demonstrated that breeders lived longer compared to nonbreeders [89]. Transcriptional analysis identified low expression of glucocorticoid genes and high expression of sex steroid gene signatures in the adrenal as features of long-lived breeders. This finding is also consistent with higher cortisol levels in nonbreeder mole rats, and is particularly interesting given that high levels of glucocorticoids in humans have been associated with higher mortality rates in certain clinical settings [90, 91]. Further mechanistic insights into age-associated changes in cortisol levels may help alleviate

age-related pathologies such as cardiovascular disease, immunosuppression, and neurodegeneration.

The most significant change in adrenal steroid hormones with aging is the linear reduction of DHEA-S, which occurs both in males and females and is independent of menopause [92, 93]. The biological activity of DHEA-S is largely attributed to its transformation into bioactive androgens or as neurosteroids [94]. The age-related reduction in adrenal androgen output is unsurprising given the decrease in zR size over time (discussed earlier). However, recent studies show that 11-oxygenated androgens that are also synthesized in the adrenal do not decline in aged humans [95-97]. The exact pathway that maintains this steroid pool during aging is unknown, but the spatial localization of some essential conversion enzymes changes with aging in the adrenal cortex [31, 97]. HSD3B2 (zF) and CYB5A (zR) exhibit colocalization in aged women, and the zF and zR boundary becomes less evident [33]. Additionally, changes in conversion rate may contribute to the redirection of steroid synthesis in aging. For example, in human mononuclear cells, the converted hormone rate increases for many sex steroid precursors from young (aged 23-28 years) to old (aged 52-66 years) samples [98]. It would be interesting to conduct a similar study using human adrenal lysates. Overall, while the exact mechanisms driving DHEA-S decline and sustained 11-oxygenated androgen levels are poorly understood, further investigation is needed to better assess the contribution of these factors to the aging process.

Senescence

A central hallmark of aging is the systemic accumulation of senescent cells [1]. Cellular senescence is a stable state of cell cycle arrest, and can be induced by a variety of stressors including severe DNA damage, telomere attrition, or oncogene activation [99, 100]. Classically, senescent cells are characterized by several distinct features, including changes in cell morphology, accumulation of lysosomal senescence-associated β-galactosidase, and upregulation of the cyclin-dependent kinase inhibitors (CDKi) p16 and p21, some of which have been noted in the adrenal [36, 54, 101]. Moreover, unique from other forms of cell cycle arrest, senescent cells activate a hypersecretory state known as the senescence-associated secretory phenotype (SASP) [99, 100]. The SASP comprises proinflammatory cytokines, chemokines, growth factors, and other molecules that can profoundly affect the tissue microenvironment [100, 102]. Senescent cells have pleiotropic roles during development and aging. From a beneficial standpoint, regulated senescence facilitates proper development of certain tissues [103, 104], promotes wound healing [105], and prevents the immediate outgrowth of damaged, preneoplastic cells [106, 107]. However, persistent senescent cells that accumulate with age [108, 109] are hypothesized to contribute to various age-related pathologies if left unresolved [110, 111].

Within the aging adrenal cortex, individual senescent markers have been evaluated using human cohorts. A Japanese study measured telomere length, which is commonly shortened in senescent cells [112]. This study reported an unexpected age-associated increase in telomere length in the zR compared with the zG and zF, and also showed a modest increase in zR telomere length with age in males and females [24, 113]. This is surprising given that cells of the zR are expected to be the longest-lived adrenal cortex cells and telomere lengthening is quite unusual. However, the analysis was conducted using hematoxylin-eosin staining as opposed to using zonal-specific protein markers, and the authors also note the zR exhibiting autofluorescent lipofusin [24]. Therefore, some cells contained within the histological zR could be newly recruited myeloid cells, given this distinct adrenal feature (discussed earlier). This study did however find that zF cells in older individuals exhibit shorter telomeres than cells of the zG, which is consistent with the centripetal migration model. Other studies have measured the senescent markers p16 and p21 in conditions associated with aldosterone overproduction. Here, it was concluded that while differentiated aldosterone-producing cells had the lowest levels of p16 and p21, there were no other significant differences across adrenocortical zones [114]. However, this study did not stratify samples by age so it is unclear whether CDKi levels may change in respective zones during aging. Notably, expression of *Cdkn2a*, which encodes p16, is significantly elevated in old (aged 27 months) compared to young (aged 6 months) mouse adrenals [54]. Given that ablation of p16-positive senescent cells alleviates numerous age-associated diseases [115, 116], it would be interesting to further explore the role of p16 in the aged adrenal.

In addition to markers related to its growth arrest phenotype, senescent cells can be distinguished by the SASP. The adrenal cortex SASP has not been well defined in an aged context; however, known SASP genes such as *Il1b*, *Il1a*, *Tnfsf10*, and *Il7r* are upregulated in aged mouse adrenal tissues [54]. These data are interesting in the context of senescence induction in SF1:*Znrf3* conditional knockout (cKO) mice, whereby males and females exhibit senescence-activated growth arrest following an initial phase of hyperplasia. However, the SASP response is of much greater magnitude in males and includes similar genes to that seen in the normal, aged adrenal [25]. Additional studies in fibroblasts have shown that glucocorticoids partially reduce the SASP in the context of radiation- or oncogene-induced senescence. In this study, Laberge et al [117] demonstrate that glucocorticoid receptor activation impairs IL-6 secretion and downstream IL-1α/NF-κB signaling, both of which are key SASP components. While glucocorticoids are known to induce senescence through increased DNA damage, these data suggest that SASP activation may be suppressed in the presence of glucocorticoids. In light of this data, it would be interesting to measure corticosterone in SF1:*Znrf3* cKO mice during senescence activation, which could contribute to the dampened SASP response in females compared with males given that females typically have higher levels of endogenous glucocorticoids [31, 118].

Adrenal Disease

The most evident age-associated pathology in the adrenal cortex is the increased prevalence of tumors, both benign and malignant [91, 119, 120]. In mouse studies, an increase in the number of adrenal nodules was associated with aging in particular strains. However, no age-dependent increase in adrenal nodules was noted in the commonly used C57BL/6J strain [34]. Given that differences in tumor susceptibility between some strains has been linked to polymorphisms in *Nr5a1* (encoding steroidogenic factor-1, SF-1) [121], it would be useful to compare genetic differences between various strains in an aged context. Large population studies in humans have also identified an age-associated increase in adrenal tumors.

A recent study by a group at the Mayo Clinic showed that more than 90% of adrenal tumors are found in patients older than 40 years, with a median age at diagnosis of 62 years (n = 1287, including adenomas, benign tumors, malignant masses, and pheochromocytoma) [119]. Additionally, a European study of non–aldosterone-producing adrenocortical adenomas (n = 3565) found that the median age at diagnosis was 61, yet mortality was higher for women younger than 65 [120]. In an unselected postmortem series with an average age of 80 years, cortical nodules were found in the adrenal of more than half of patients (n = 598) [122]. Moreover, a Japanese autopsy study found that 75% of adrenal adenomas detected were in patients older than 50 years, strongly supporting an important role for aging in adrenal tumor development [123].

Subcapsular hyperplasia is also often observed in aged mouse adrenals, details of which can be found in Table 1 [37, 38, 124]. This feature has been noted in the adrenals of *Ezh2* cKO mice (driven by SF-1-Cre) [125]. EZH2 is a histone methyltransferase that is highly deregulated in adrenocortical carcinoma (ACC), where high EZH2 expression is associated with worse prognosis [126-128]. Adrenal cortex–specific loss of EZH2 in mice results in dedifferentiation of cortical cells that activate GATA4 and accumulate at the periphery of the gland. This was demonstrated through lineage tracing and found to also occur normally during aging in 12-month-old female mice [125, 126]. These results suggest a potential age-induced reversal of centripetal migration to repopulate the adrenocortical stem cell compartment, which could result from progenitor cell exhaustion with advanced aging.

To better understand how aging influences adrenal tumorigenesis, researchers have turned to mouse models. Alterations in hallmark ACC pathways including WNT/β-catenin, p53/Rb, and insulin-like growth factor signaling have been the main focus to date given frequent dysregulation of these pathways in human ACC tumors [25, 30, 129-133]. While many of these models develop benign or malignant adrenal tumors, nearly all exhibit incomplete penetrance and require an aged setting. We previously established the SF1:*Znrf3* cKO model to study Wnt-driven adrenal tumorigenesis [134]. Despite robust adrenal hyperplasia in young animals, we found that adrenal tumors did not develop until after 1 year of age [25]. Moreover, tumors were sex dimorphic with a higher prevalence of metastatic ACC tumors in females compared with males. Thus, this model recapitulates the age- and sex-dependence of human ACC [135]. While the mechanism is incompletely understood, it is clear from our work and that of others that the myeloid immune response, which is at least partially regulated by male sex steroid hormones, plays a key role [25, 30].

Adrenal tumors also exhibit activation of the hallmark aging process, cellular senescence (discussed earlier). In benign adrenal hyperplasia, classical senescent markers, such as p16 and p21, are expressed. Notably, cortisol-producing adenomas displayed higher p16 and p21 levels compared to APAs. Cortisol has been shown to promote senescence-associated cell cycle arrest via increased DNA damage and oxylipin production. However, a mechanism for glucocorticoid-induced senescence in adrenal tumors has not been fully investigated [136-138]. In ACC, large-scale genomics studies have identified known cell cycle arrest genes, including *TP53* and *CDKN2A*, to be frequently inactivated [139, 140], suggesting that bypassing senescence may be one route to accelerated adrenal tumorigenesis. However, senescence activation and the associated SASP is known to remodel the microenvironment to support tumor growth and epigenetically reprogram premalignant cells in other cancer types [141, 142]. This is consistent with our SF1:*Znrf3* cKO model, in which tumors develop only after senescence-induced tissue remodeling. Thus, understanding the molecular mechanisms that underlie these pleiotropic roles of senescence in cancer may reveal new therapeutic targets.

Antiaging Interventions

As the global population continues to age, the search for effective strategies to combat age-associated diseases has become an increasing priority [143]. One such study examined the effects of vitamin E deficiency on mice, revealing a 2-fold increase in lipofusin accumulation in mouse adrenals over an 18-month period compared to controls. While reducing lipofusin levels may have physiological advantages, this study did not find any increase in lifespan [144]. Similarly, transferrin levels in human blood have been shown to dramatically decrease with aging. As a result, exogenous supplementation has been proposed as an antiaging strategy [145]. In this study, 18- to 20-month-old Balb/c mice were transfused with pooled human plasma transferrin for 20 days and compared to an aged matched saline group as well as a young (3-month-old) cohort. Transfusion significantly increased circulating lymphocyte profiles, thymus weight, and zinc plasma levels (carried in transferrin) compared to the saline-treated group. Additionally, adrenal weight in the transfused group was larger than the saline group and comparable to that of 3-month-old mice. The authors suggest a potential relationship between the altered immune response and adrenals representing a younger weight phenotype [146]. Functional experiments looking at inflammatory markers or steroid hormones were not carried out in either study, but would be useful parameters to include in the future.

The decline of DHEA-S with age has led to the exploration of DHEA supplementation as an antiaging strategy. While many studies have evaluated the efficacy of DHEA supplementation, the results are not overwhelmingly convincing. Some studies report an increase in overall well-being, but specific androgen-mediated effects, such as increased libido, have not been shown to differ between DHEA and placebo groups [147]. A Japanese study monitored DHEAS levels in men (n = 394) longitudinally for 27 years and found that those with high levels (>200 μg/dL) have increased survival compared to both mid (130-199 μg/dL) and low (<129 μg/dL) groups. DHEAS levels were also an independent predictor of death from any cause in men. However, this study found no significant differences in a similar cohort of women (n = 544) [148], which is consistent with other results from studies in females (for a detailed clinical review, see [149]).

While the exact mechanism for the potential benefits of DHEA-S has not been defined, there are some data to suggest an immune-mediated response. This is interesting given the age-associated increase in infection coupled with decreased immune function [150]. Although mice have endogenously low levels of DHEAS, a study using 15-month-old female Balb/c mice supplemented with DHEA (0.01%) found increased T- and B-cell proliferation and IL-2, interferon γ, and IL-6 production. They also showed a similar response in

retrovirally infected mice [151]. Translating these findings to humans, a study in age-advanced men (mean age 63; n = 9) with low DHEAS evaluated the effect of DHEA (50 mg daily, for 20 weeks) supplementation on immune function. Their findings showed a robust increase in immune cells such as B cells, monocytes, ILC2 (type 2 innate lymphoid cells), and natural killer cells as well as associated cytokines. Additionally, the ratio of cortisol to DHEA was dramatically decreased compared to the placebo group, suggesting that proinflammatory DHEA can balance the immunosuppressive action of cortisol [152]. This relationship is supported by other studies whereby a high cortisol-to-DHEA ratio is predictive of all-cause mortality in a Vietnamese cohort with metabolic syndrome [153]. Additionally, in an older-aged cohort of hip fracture patients, those with a high cortisol-to-DHEA ratio showed an increased infection rate, decreased neutrophil response, and lower overall survival [154]. Therefore, the beneficial effect of DHEA is likely more complex, and intertwined with glucocorticoids rather than androgen levels alone. It would be intriguing for future studies investigating the benefits of DHEA supplementation in healthy individuals to include measurements of all adrenal steroids. Nevertheless, it is still unclear if DHEA-S supplementation acts as a reservoir of androgen precursors or has an independent antiaging effect. There is compelling evidence suggesting that DHEA or downstream androgens have the ability to modulate the immune response in the context of disease. For example, women with PCOS (androgen excess) have an increased rate of COVID-19 infections, and conversely women with *CYB5A* polymorphisms (androgen excess) have a reduced risk of rheumatoid arthritis (autoimmune disease) [155, 156]. This observation is also apparent in our SF1:*Znrf3* cKO mouse model of adrenal cancer, whereby androgen deprivation reduces myeloid cell accumulation and DHT treatment promotes myeloid infiltration [25, 30]. While high levels of inflammation may sometimes be considered pathological, in this model it is associated with tumor protection. In translating these findings to a clinical context, we found that a myeloid response gene signature was higher in males compared to females and associated with better overall and progression-free survival in the TCGA-ACC cohort [25]. Additionally, regions of ACC tumors with high CD68-positive (myeloid marker) staining were associated with low levels of aggressiveness (per Ki67 staining) [25]. These data highlight how some specific inflammatory responses can be protective in the context of cancer, and the importance of the physiological context when designing strategies to implement "antiaging" drugs.

Rapamycin is a highly popularized antiaging drug that has shown some longevity-based effects in vivo [157]. As an inhibitor of mTOR, rapamycin modulates essential growth and survival pathways, which makes it an attractive antiaging therapy. A study in UM-HET3 mice demonstrated that rapamycin treatment reduced spontaneous adrenal tumor formation at age 22 months by 70% (control: 20% incidence, rapamycin: 6% incidence). However, rapamycin did not have any effect on adrenal hyperplasia [158]. Canagliflozin, a sodium-glucose cotransport 2 inhibitor used to treat diabetes, has also been proposed as an antiaging drug. While canagliflozin treatment reduced the incidence of tumors in 22-month-old aged male mice, it conversely increased the number of tumors in female mice [159]. It would be interesting to understand the mechanistic drivers behind these effects in the adrenal and evaluate the effect on hormone production.

A more recent approach to antiaging is the use of senolytics, which are drugs that selectively kill senescent cells. The most established senolytic regimen is dasatinib and quercetin (D + Q), which is currently in clinical trial for age-related diseases such as AD and head and neck cancer (NCT05724329 and NCT05422885) [111]. Although there are no extensive studies into the use of this combination in the adrenal, there are in vitro studies looking at the effect of quercetin alone in H295R human ACC tumor cells. These data show that quercetin (and other flavonoids) have significant aromatase activity and induce CYP19 expression to a level comparable of 8Br-cAMP [160]. This could lead to potential hormonal issues and detrimental effects in the context of hypersecreting tumors, given that estrogen excess is associated with very poor prognosis in ACC [161]. Nevertheless, senolytics using alternative mechanisms are an attractive therapeutic avenue that should be explored in the context of age-associated adrenal diseases.

Conclusion

Overall, there are many age-related changes in the adrenal cortex that can contribute to intra-adrenal disease and disruption of circulating steroid hormones. While there are many challenges within aging research such as costly in vivo timelines and difficulty obtaining clinical specimens, there are sufficient data to warrant more detailed, mechanistic studies. Specifically, further understanding immune changes in the context of hormonal state may provide greater insight into aged-associated immune diseases. Similarly, the increased prevalence of adrenal tumors with aging highlights the potential for environmental and genetic changes that occur within the aging adrenal microenvironment, and a better understanding of these factors will help facilitate the development of novel therapeutic interventions.

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Disclosures

The authors have nothing to disclose.

Data Availability

Data sharing is not applicable to this article as no data sets were generated or analyzed during the current study.

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