GENETICS IN ENDOCRINOLOGY

Impact of race and sex on genetic causes of aldosterone-producing adenomas

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Abstract

Primary aldosteronism (PA) is a common cause of secondary hypertension. Recent technological advances in genetic analysis have provided a better understanding of the molecular pathogenesis of this disease. The application of next-generation sequencing has resulted in the identification of somatic mutations in aldosterone-producing adenoma (APA), a major subtype of PA. Based on the recent findings using a sequencing method that selectively targets the tumor region where aldosterone synthase (CYP11B2) is expressed, the vast majority of APAs appear to harbor a somatic mutation in one of the aldosterone-driver genes, including *KCNJ5*, *ATP1A1*, *ATP2B3*, *CACNA1D*, *CACNA1H*, and *CLCN2*. Mutations in these genes alter intracellular ion homeostasis and enhance aldosterone production. In a small subset of APAs, somatic activating mutations in the *CTNNB1* gene, which encodes β -catenin, have also been detected. Accumulating evidence suggests that race and sex impact the somatic mutation spectrum of APA. Specifically, somatic mutations in the *KCNJ5* gene, encoding an inwardly rectifying K^+ channel, are common in APAs from Asian populations as well as women regardless of race. Associations between APA histology, genotype, and patient clinical characteristics have also been proposed, suggesting a potential need to consider race and sex for the management of PA patients. Herein, we review recent findings regarding somatic mutations in APA and discuss potential roles of race and sex on the pathophysiology of APA as well as possible clinical implications.

Invited Author's profile

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Drs Rainey and Nanba have led the use of immunohistochemistry-guided, gene-targeted, next-generation sequencing of DNA from formalin-fixed, paraffin-embedded adrenal tissue, including aldosterone-producing adenomas. This approach has expanded research studies to archival adrenal tumor tissue and helped to better define the impact of sex, age and race on the acquisition of disease-causing somatic gene mutations.

Introduction

Renin-independent autonomous aldosterone production from one or both adrenal glands causes hypertension and often hypokalemia. This condition is known as primary aldosteronism (PA) which was first reported by Jerome Conn in 1955 (1). PA is now recognized as a frequent cause of secondary hypertension, accounting for 6% of hypertensive patients in primary care practice (2). A recent study demonstrated a significant number of unrecognized yet biochemically confirmed PA even in normotensive subjects with a continuum of renin-independent aldosterone production that parallels hypertension severity (3). There are several subtypes of PA, including sporadic and rare familial forms. The vast majority of PA is classified as either sporadic aldosterone-producing adenoma (APA) or idiopathic hyperaldosteronism (IHA) (4). In general, patients with APA present with more severe phenotypes than those with IHA (4). To better understand this disease, significant efforts have been made to determine the pathogenesis of PA.

Recent advances in the genetic analysis using next-generation sequencing (NGS) have allowed the identification of the genetic causes of sporadic as well as familial PA. NGS has identified mutations in genes that control ion homeostasis of adrenocortical cells in PA patients. The affected genes include *KCNJ5* (5), *ATP1A1* (6), *ATP2B3* (6), *CACNA1D* (7, 8), *CACNA1H* (9), and *CLCN2* (10, 11). Mutations in these genes mostly stimulate aldosterone production via the activation of the calcium signaling pathway by increasing intracellular calcium levels (12). Aldosterone-driver somatic mutations in these genes have been identified in the majority of APAs (13, 14, 15, 16, 17). The identification of somatic mutations in APA has provided new avenues for improved patient care such as steroid biomarkers and potential new treatment options for patients with APA harboring somatic *KCNJ5* mutations, one of the most common genetic causes of APA (17, 18, 19, 20, 21). Emerging evidence indicates that there are racial and sex differences in the prevalence of aldosterone-driver somatic mutations. In this review, we summarize recent findings regarding the genetic causes of APA and discuss the impact of race and sex on the pathogenesis of APA as well as its clinical significance.

Identification of somatic mutations in APA

The genetic causes of sporadic APA remained poorly defined until the application of NGS. In 2011, the

laboratory of Dr Richard P Lifton identified two recurrent heterozygous somatic mutations in *KCNJ5* (p.G151R and p.L168R) in APAs using whole-exome sequencing (WES) (5). The group also identified a germline *KCNJ5* variant (p.T158A) in a family with a Mendelian form of severe hyperaldosteronism with massive adrenal hyperplasia (5), which is now defined as familial hyperaldosteronism type III (FH-III). The *KCNJ5* gene encodes an inwardly rectifying K⁺ channel (GIRK4) which is highly expressed in the zona glomerulosa (ZG) of the adrenal cortex (5, 22, 23). Mutations in or near the ion selectivity filter of GIRK4 lead to increased Na⁺ conductance and cell membrane depolarization, resulting in increased intracellular calcium levels which enhance aldosterone synthase (*CYP11B2*) expression and aldosterone production (5, 24, 25, 26) (Fig. 1).

Subsequently, somatic mutations in other genes, including *ATP1A1* (6, 8), *ATP2B3* (6), *CACNA1D* (7, 8), *CACNA1H* (27), and *CLCN2* (28), have been identified in APA. The *ATP1A1* gene encodes Na⁺/K⁺-ATPase α 1 subunit. *In vitro* studies suggest that the loss of Na⁺/K⁺ pump function as a result of *ATP1A1* mutation causes reduced

Figure 1

Proposed mechanisms leading to an autonomous aldosterone production in adrenal tumor cells harboring aldosterone-driver somatic mutations. Over 90% of aldosterone-producing adenomas (APAs) harbor somatic mutations in *KCNJ5*, *ATP1A1*, *ATP2B3*, *CACNA1D*, *CACNA1H*, and *CLCN2*. Most of these somatic mutations cause directly or indirectly (via cell membrane depolarization) increased intracellular calcium levels that activate calcium signaling pathways, enhance aldosterone synthase (*CYP11B2*) expression, and renin-independent aldosterone production. Notably, on a cell-based study of mutant *ATP1A1*, no pathologic increase in cytosolic calcium levels was observed despite the membrane depolarization. Alternative mechanisms including cellular acidification have been proposed for autonomous aldosterone production in *ATP1A1*-mutated APA.

affinity to K^+ , intracellular acidification due to abnormal H^+ leak, and cell membrane depolarization, leading to excess aldosterone production with no pathologic alteration in cytosolic Ca^{2+} activity (29) (Fig. 1). The *ATP2B3* gene encodes a plasma membrane calcium ATPase (PMCA3). Cell-based studies indicate that mutations in *ATP2B3* cause loss of its pump function and pathological cation permeability leading to increased intracellular calcium levels and enhanced aldosterone production (30) (Fig. 1).

The *CACNA1D* gene encodes a voltage-dependent L-type calcium channel subunit α -1D ($Ca_v1.3$). Unlike other aldosterone-driver genes, mutations in *CACNA1D* have been identified throughout the gene (31). Although few have been functionally characterized, mutations in *CACNA1D* are thought to be gain-of-function mutations that induce increased Ca^{2+} entry as a result of channel activation at less depolarized potentials, leading to excess aldosterone production (7, 8) (Fig. 1). Notably, *de novo* germline mutations in *CACNA1D* have also been identified in pediatric cases with PA and neurodevelopmental abnormalities (7). This condition is currently defined as PA with seizures and neurologic abnormalities (PASNA) syndrome.

The *CACNA1H* gene encodes voltage-dependent T-type calcium channel subunit α -1H ($Ca_v3.2$). The association between this gene and PA has initially been explored by the identification of recurrent germline *CACNA1H* mutation (p.M1549V) in early onset PA patients as the cause of FH-IV (9). Cell-based studies demonstrated that the *CACNA1H*^{M1549V} induces a shift of activation to less depolarizing potentials, allowing increased Ca^{2+} entry and stimulation of aldosterone production (9, 32) (Fig. 1). A recent study suggests that abnormal Na^+ permeability of mutant $Ca_v3.2$ could also contribute to the depolarization of the membrane potential (33). Daniil *et al.* (34) identified four different additional heterozygous germline *CACNA1H* mutations in PA patients with different clinical phenotypes. We recently identified a recurrent somatic *CACNA1H* mutation (p.I1430T) in APAs without known aldosterone-driver mutations (27). The *CACNA1H* p.I1430T mutation was functionally characterized as having the ability to increase *CYP11B2* mRNA expression and aldosterone production using a doxycycline-inducible HAC15 adrenal cell line (27).

Gain-of-function germline *CLCN2* mutations in patients with FH-II and early onset PA were identified by two groups in 2018 (10, 11). The *CLCN2* gene encodes chloride voltage-gated channel 2 (ClC-2). Mutations in *CLCN2* cause increased *CYP11B2* expression and aldosterone production by membrane depolarization via

enhanced chloride efflux (10, 11) (Fig. 1). Dutta *et al.* (28) screened 80 APAs and found a somatic *CLCN2* mutation, p.G24D, in one patient, which is identical to the one identified and functionally characterized as a disease-causing *de novo* germline mutation that causes in an early onset PA case (11). Besides the p.G24D mutation, we identified an additional *CLCN2* somatic mutation (c.62-2_74del) in an APA although the clinical significance of this mutation is unclear until its functional effect on aldosterone production is determined (35). Notably, two different mouse models for PA due to *CLCN2* mutations have recently been developed (36, 37). These mouse models will provide valuable tools to study PA pathogenesis and develop new therapeutics for PA.

Somatic activating mutations in *CTNNB1* (β -catenin) have been identified in adrenocortical tumors, including cortisol-producing adenoma (38, 39, 40, 41), adrenocortical carcinoma (38, 42), and non-functioning adrenocortical adenoma (38, 43), suggesting a role of *CTNNB1* mutation in adrenal tumorigenesis. Somatic *CTNNB1* mutations have also been found in APAs with the prevalence of 2–5% (44, 45, 46). β -catenin is a key protein of the Wnt/ β -catenin signaling pathway which plays an important role in adrenal cortex development and adrenal steroidogenesis (47, 48, 49, 50). Constitutive activation of β -catenin in the mouse adrenal causes adrenal hyperplasia and hyperaldosteronism (51). A recent study demonstrated that gain-of-function mutation targeted to the mouse adrenal ZG caused a progressive hyperplastic expansion of the ZG and elevated aldosterone production. Surprisingly no difference was observed in plasma renin activity between mice with and without β -catenin gain-of-function mutation (52). Further studies are needed to determine the molecular mechanisms of enhanced aldosterone production in *CTNNB1*-mutated APA.

Somatic mutation prevalence in APA using conventional approaches

Since the identification of aldosterone-driver somatic mutations in APA, significant efforts have been made to study the prevalence of these mutations. In the studies mainly conducted in European countries, the prevalence of *KCNJ5* mutations has been reported to be approximately 40% (44, 53, 54, 55, 56, 57, 58). The largest mutation prevalence study was conducted by Fernandes-Rosa *et al.* using a material obtained through the European Network for the Study of Adrenal Tumors (ENS@T) (56). In that study, somatic mutations were identified in 257 out of

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474 APAs (54.2%), including mutations in *KCNJ5* (38.0%), *CACNA1D* (9.3%), *ATP1A1* (5.3%), and *ATP2B3* (1.7%).

The prevalence of *KCNJ5* mutations appears to be much more common in East Asian and South East Asian countries, including China (59, 60), Taiwan (61), Japan (62, 63), South Korea (64), and Thailand (65), with the prevalence of approximately 70% although there are a few studies reporting a similar *KCNJ5* mutation prevalence to that in European cohorts (66, 67). In Asian populations, mutations other than in *KCNJ5* have only been rarely reported: *ATP1A1* in 0–2.4%, *ATP2B3* in 0–2.5%, and *CACNA1D* in 0–2.5% (46, 59, 60, 61, 64, 68). Overall, previous studies have identified somatic mutations in 54–80% of APAs, with a higher detection rate in Asian populations compared with that in European populations. Of note, these prevalence studies have isolated DNA from macroscopically identified adrenal tumor tissue or formalin-fixed, paraffin-embedded (FFPE) tissue without consideration of tumor CYP11B2 expression and majority performed mutation hotspot-based Sanger-based sequencing (conventional approaches).

CYP11B2 IHC-guided sequencing analysis

CYP11B2 and 11 β -hydroxylase (CYP11B1) are steroidogenic enzymes required for the final steps of aldosterone and cortisol production, respectively. CYP11B1 and CYP11B2 are encoded by two genes; each containing nine exons spread over approximately 7000 bp of DNA. The encoded proteins are 93% identical in the predicted amino acid sequence. Because of the high sequence similarity between CYP11B2 and CYP11B1, the generation of specific antibodies against these enzymes was only accomplished in the past 10 years (69). Subsequently, the development of highly specific monoclonal antibodies against CYP11B2 and CYP11B1 significantly improved our understanding of the histopathology of adrenals with PA (70). CYP11B2 immunohistochemistry (IHC) clearly demonstrated a positive expression in surgically resected adrenal tumors (APAs) from PA patients. At the same time, CYP11B2 IHC revealed minor histopathologic subgroups of PA adrenals, including cases with distinct intra-tumor CYP11B2 heterogeneity (71), with multiple APAs (13, 14, 72), as well as cases with a dominant CYP11B2-negative tumor with a satellite APA or aldosterone-producing micronodules (formerly known as aldosterone-producing cell clusters) in the adjacent adrenal tissue (13, 14, 73, 74, 75). The variations in PA adrenal presentation raised concerns regarding the accuracy of mutation prevalence

studies that did not assess the CYP11B2 expression of pathologic material prior to sequencing. To overcome this issue, we developed a CYP11B2-IHC-guided sequencing method using DNA from FFPE tumor material (13, 14). Using this approach, aldosterone-driver somatic mutations have been identified in approximately 90% of APAs (13, 14, 15, 16, 17), which is significantly higher than that seen using conventional approaches (44, 56, 76). As mentioned earlier, many of the NGS-identified mutations especially in the *CACNA1D* gene have not yet been functionally characterized. Therefore, current studies may be overestimating the prevalence of somatic mutations responsible for APA aldosterone overproduction.

CYP11B2 IHC-guided sequencing basically allows the initial definition of sources of inappropriate aldosterone production followed by its capture in consecutive FFPE sections under a dissecting microscope. The scraped material is used for DNA isolation after deparaffinization. FFPE DNA sequencing can be performed with whole-exome, gene-targeted next-generation sequencing (NGS) panels, or traditional Sanger sequencing. Targeted NGS is a preferable method because of its high sensitivity, ability to utilize small amounts of DNA, and especially for identifying mutations that span the *CACNA1D* gene which encodes more than 2000 amino acids (31). Although the CYP11B2 IHC-guided sequencing approach is time-consuming and labor-intensive, it has provided accurate and important findings as discussed later.

Impact of race on genetic causes of aldosterone-producing adenoma

There is considerable evidence that supports the concept of racial differences in the prevalence of somatic mutations in APA. However, there were some concerns regarding the accuracy of the mutational analysis with conventional approaches that may impact the prevalence estimates. Using a CYP11B2 IHC-guided sequencing method, we have investigated the APA-related somatic mutation spectrum in cohorts of different races, including Americans of European descent (whites) (13), Americans of African descent (blacks) (14), and Japanese (East Asians) (15) (Fig. 2). In the analysis of 75 APAs from whites, somatic *KCNJ5* mutations were the most frequent genetic cause of APA (43%), followed by *CACNA1D* (21%), *ATP1A1* (17%), *ATP2B3* (4%), and *CTNNB1* (3%) (13). The prevalence of *KCNJ5* mutations appears to be similar to the results of the European multicenter collaborative study (56), while the CYP11B2 IHC-guided approach combined with deep gene

Figure 2

Race and sex differences in the genes affected by aldosterone-driver somatic mutations in APA based on CYP11B2 IHC-guided sequencing analysis. (A, B and C) Distribution of somatic mutations in APA from white Americans (A), black Americans (B), and Japanese (East Asian) patients (C). Data were adopted from our previous studies (13, 14, 15, 27, 35). APAs from black patients were not assessed for mutations in *CACNA1H* and *CLCN2* genes due to limited sample availability.

sequencing improved the detection rate of mutations in genes such as *CACNA1D* and *ATP1A1*. Subsequent whole-exome sequencing identified somatic mutations in *CACNA1H* and *CLCN2* in small subsets of APA (27, 35). Large cohort studies will be needed to determine the prevalence of these rare APA mutations.

In agreement with most of the previous reports from East Asian countries (59, 60, 61, 62, 63), *KCNJ5* mutations were common in Japanese (73% of 106 APAs) when analyzed using the CYP11B2 IHC-guided method (15). While somatic mutations in other aldosterone-driver genes have been thought to be very rare events in the Asian population, the CYP11B2 IHC-guided capture combined with deep sequencing methods successfully identified mutations in *CACNA1D*, *ATP1A1*, *ATP2B3*, and *CACNA1H* with the prevalence of 14, 5, 4, and 1%, respectively (15). A meta-analysis of 1636 patients with APA from 13 studies indicates a possible association between the incidence of *KCNJ5* mutation and sodium intake (77) although dedicated studies are needed to assess its causal relationship. This topic was further discussed in depth by Williams *et al.* (78). Besides high sodium intake which may lead to more severe phenotype and/or early detection of the disease, the authors proposed a possible selection bias due to diagnostic procedures as a potential factor for a high incidence of *KCNJ5* mutations in East Asians based on the evidence of a more pronounced

phenotype of *KCNJ5*-mutated APA compared to that of non-*KCNJ5*-mutated APA (78). Other considerations would include the much higher circulating levels of phytoestrogens seen in East Asian men vs European men (79). Phytoestrogens can activate both traditional and cell surface estrogen receptors (80, 81). Differences in reproductive hormones and particularly circulating estrogens have been considered as a possible hormonal cause of the dominance of *KCNJ5* mutations in APA from women vs men of all races (77, 82).

The high levels of hybrid steroids (18-hydroxycortisol and 18-oxocortisol), which are metabolites of cortisol, have been reported in patients with APA (83, 84) as well as rare familial forms of PA (FH-1 due to a chimeric *CYP11B1/CYP11B2* gene and FH-III due to germline *KCNJ5* mutations) (85, 86). Since these hybrid steroid levels are lower in patients with IHA than those with APA, the role of hybrid steroid measurement for PA subtype prediction (APA vs IHA) has been studied (87, 88, 89). However, due to high variability in hybrid steroid values, a significant overlap between APA and IHA was observed (89). Recent steroid profiling studies using liquid chromatography-tandem mass spectrometry further revealed a higher hybrid steroid production in *KCNJ5*-mutated sporadic APAs compared with non-*KCNJ5*-mutated APAs (17, 18, 19). The somatic mutation distribution may partly explain the variability in hybrid steroid levels in APA patients. The high prevalence of somatic *KCNJ5* mutations in APAs from East Asian patients may also explain the better utility of hybrid steroid measurement for subtype prediction (APA vs IHA) in East Asians (90) compared with Europeans (89, 91). One goal for hybrid steroid measurement would be to decrease IHA patient adrenal venous sampling (AVS), which is an invasive procedure with a limited availability. However, for PA patients with high circulating hybrid steroids, AVS would likely still be needed to lateralize disease. While cautiously optimistic about the utility of hybrid steroids as a PA diagnostic tool, the current complexity of hybrid steroid measurement and data interpretation requires standardization and, therefore, will remain unavailable for most clinical programs for the foreseeable future.

In contrast to whites and East Asians, *CACNA1D* mutations appear to be the most common genetic cause in blacks with the prevalence of 42% based on the analysis of 73 APAs using CYP11B2 IHC-guided sequencing approach (14). The second most prevalent genetic alteration in blacks was *KCNJ5* mutations (34%), followed by *ATP1A1* (8%) and *ATP2B3* (4%) (14). In what appears to be a unique characteristic of black patients with PA, Zilbermint *et al.*

(92) reported an association with germline variants in *Armadillo repeat-containing protein 5* (*ARMC5*) gene, which is a putative tumor suppressor gene. Mutations in this gene were initially found as a genetic cause of primary bilateral macronodular adrenocortical hyperplasia, a rare form of Cushing syndrome (93). Although germline *ARMC5* variants may predispose to certain types of aldosterone-driver mutations, it remains an open area of research.

Although the mechanisms leading to variation in the APA somatic mutation spectrum remain unclear, the clarification of racial differences in the genetic causes of APA may help in the development of new diagnostics and personalized therapeutics for PA patients. To determine a more accurate somatic mutation prevalence, large multicenter prospective studies using a CYP11B2 IHC-guided sequencing approach would be desirable.

Impact of sex on genetic causes of aldosterone-producing adenoma

Sex differences in the type of aldosterone-driver mutations have been well documented particularly in the *KCNJ5* gene. A predominant occurrence of somatic *KCNJ5* mutations in APAs from women has been reported in many studies mainly conducted in European countries (44, 53, 54, 56, 57), while this sex difference has been debated in Asian populations where the prevalence of somatic *KCNJ5* mutations is very high in both men and women (59, 60, 61, 62, 63, 64, 65, 66, 67). Our recent studies using the CYP11B2 IHC-guided sequencing method with targeted NGS demonstrated significantly higher incidences of *KCNJ5* mutations in women than men in Japanese (15) as well as other races, including white (13) and black Americans (14) (Fig. 2). The molecular mechanisms underlying sex differences in the prevalence of somatic *KCNJ5* mutations in APA are largely unknown. Reproductive hormone differences between men and women represent a clear sex difference that could impact tumor growth and/or aldosterone production. The adrenal cortex expresses both conventional estrogen receptors (mainly ER β) as well as G protein-coupled estrogen receptor 1 (GPER1) (82). *In vitro* studies have demonstrated that estradiol can stimulate aldosterone production via GPER1, which is expressed predominantly in APA (82). Estrogens, therefore, could play a role in PA pathogenesis in women although its association with APA genotype remains to be elucidated.

Besides sex, other clinical characteristics of patients with *KCNJ5*-mutated APA include the young onset of

the disease, high plasma aldosterone concentration, and large tumor size (77). The florid PA phenotype in patients with *KCNJ5*-mutated APA may explain early detection of the disease, resulting in the frequent observation of somatic *KCNJ5* mutations in APA from young patients (54). Histologic analysis revealed that *KCNJ5*-mutated APAs contain a higher percentage of lipid-rich clear cells (zona fasciculata (ZF)-like cells) compared with non-*KCNJ5*-mutated APAs (17, 44, 94, 95, 96, 97). Abundant expression of CYP17A1, which is required for cortisol biosynthesis, has also been observed in *KCNJ5*-mutated APAs (17, 35, 94, 97). Co-expression of CYP11B2 and CYP17A1 explains the production of hybrid steroids in *KCNJ5*-mutated APAs. Considering the high prevalence of somatic *KCNJ5* mutations in women of all races examined to date, the measurement of hybrid steroids may provide an additional diagnostic tool for the prediction of PA subtype (APA vs IHA) in women although it does not provide any information regarding the laterality of the disease. In contrast to the *KCNJ5* gene, mutations in *CACNA1D* have been more often observed in men than women regardless of race (13, 14, 15, 56) and *CACNA1D*-mutated APAs tend to be smaller than *KCNJ5*-mutated APAs (8, 44, 56).

Somatic activating *CTNNB1* mutations in APA appear to occur more frequently in women than men (45, 46). There is a case series report suggesting the possible association between somatic *CTNNB1* mutations and enhanced tumor *LHCGR* and *GNRHR* expression in two pregnant women and one postmenopausal woman with PA (98). Aberrant G-protein-coupled receptors, including *LHCGR* and *GNRHR*, have been documented in a subset of PA patients (99, 100, 101, 102, 103, 104). *In vitro* studies using H295R cell models transfected with *LHCGR* or *GNRHR* demonstrated that the corresponding agonist treatments resulted in a dose-dependent increase in CYP11B2 reporter activity (99, 103). Interestingly, in a recent study by Gagnon *et al.* (105), no *CTNNB1* mutation was identified in 11 PA patients with positive or partial aldosterone response to GnRH. While the ectopic tumor expression of these receptors provides an intriguing explanation for hormonal activation of aldosterone production in a subgroup of APAs, the molecular mechanisms by which *CTNNB1* mutations cause inappropriate aldosterone production in APA but not in other adrenal tumors remain unclear.

Conclusions

Recent technological advances in APA genetic analysis have led to the detection of aldosterone-driver somatic

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mutations in the vast majority of these tumors. Accumulating data suggests that there are clear race and sex differences in the distribution of somatic mutations in APAs. Although the mechanisms underlying these differences remain unknown, consideration of race and sex could be important for future personalized medicines in the management of PA patients.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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