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Simplification of the diagnostic work-up of primary aldosteronism and investigation of immunology mechanisms

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ABSTRACT

Background

Primary aldosteronism (PA) is the most common cause of arterial hypertension characterized by high levels of aldosterone, resulting in excessive mineralocorticoid receptor (MR) stimulation, and extensive hypertensive-mediated organ damage (HMOD). The diagnostic algorithm for PA is sequentially based on hormonal tests (screening and exclusion tests), followed by lateralization procedures (adrenal CT scanning and adrenal venous sampling [AVS]) to distinguish between unilateral and bilateral disease. Current guidelines recommend one or more exclusion tests in patients who performed the screening test with the measurement of aldosterone-to-renin ratio (ARR) to avoid further lateralization procedures in those who tested false-positive. To date the diagnostic gain provided by these exclusion tests over the ARR was examined only in few studies, and, therefore, stands on a weak level of evidence.

Moreover, growing experimental evidence has shown that immune system, especially T cells is involved in aldosterone-induced HOMD through MR activation. MR activation in animal models of hyperaldosteronism promoted T cells differentiation to the pro-inflammatory T helper 17 (Th17) subsets while decreasing the number of anti-inflammatory T regulatory (Tregs). Alteration of the balance between Th17 and Tregs contributed to the pathogenesis of hypertension and the associated complications. Furthermore, our previous work provided proof on the MR gene expression and protein expression in both human $CD4^+$ and $CD8^+$ T cells by Droplet Digital PCR and immunoblotting, respectively, and in vitro exposure to aldosterone promoted T cell

clonal proliferation and activation. However, up to now, there was no relevant research focused on the function of Th17 and Tregs in PA patients, and evaluate the effect of MR antagonists and surgery on these cells in patients with PA.

Aims

- To meta-analyze available studies of exclusion tests to provide a more accurate picture of their diagnostic accuracy and gain in the work-up of PA with a higher level of confidence.
- To investigate the levels of circulating Th17 and Tregs in PA patients and evaluate the effect of MR antagonists and surgery on these cells in PA patients.

Materials and methods

- Eligible studies reported on the diagnostic performance of the ARR and the exclusion tests for identifying unilateral PA (uPA) were selected using the "gold" standard (biochemical cure after adrenalectomy), or, whenever unavailable, a "golden" standard (adrenal imaging and/or AVS**)** as reference. Then, pooled sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), diagnostic odds ratios (DOR), the summary receiver operating characteristic (sROC) curve, and corresponding area under the curve (sAUC) with 95% confidence interval (CI) were examined.
- Blood samples from PA patients were obtained at 3-time points: before surgery, when patients had high PAC and were not treated with MR antagonists (T0); before surgery when patients had high PAC and were treated with MR antagonists (T1); one month after surgery when patients had normal PAC (T2). Immunologic

markers on Th17 ($CD4^+IL17^+$), pathogenic IL-23-dependent Th17 ($CD4^+IL17^+IL23R^+$), and Tregs ($CD4^+CD25^+FoxP3^+$) were analyzed by multicolor flow cytometry.

Results

- By increasing the overall sample size of the patients studied by these tests and comprising the experience gained in multiple centers, we found that two most popluar exclusion tests, captopril challenge test (CCT) and saline infusion test (SIT), had no diagnostic gain over ARR for diagnosing uPA. Hence, their systematic use is not justified after a carefully performed ARR.
- The percentage of circulating Th17 in PA patients was significantly lower after treatment of MR antagonists and post-surgery cure of the hyperaldosteronism; meanwhile, there was a decrease in pathogenic Th17 one month after surgery. Although there were no differences in the percentage of Tregs at these 3-time points, Th17/Tregs ratio was markedly decreased after treatment with MR antagonists and post-surgically cure of the hyperaldosteronism.

Conclusions

This meta-analysis revealed that the use of exclusion tests in patients with a high post-test probability of uPA, as identified by ARR values, could be unnecessary, if not confounding. Meanwhile, our current study for the first time showed that treatment with MR antagonists and post-surgically cure of the hyperaldosteronism can decrease the percentage of circulating Th17 and the ratio of Th17/Tregs in PA patients.

INTRODUCTION

1. What is primary aldosteronism?

Despite the awareness, treatment, and control of hypertension have improved substantially during the past several decades, there are still half of hypertensive patients on medication whose BP values are above the normal range (Chow et al., 2013; Olsen et al., 2016). The Lancet Hypertension Committee emphasized that one of the main reasons for poor BP control is the missing or delayed diagnosis of secondary hypertension (Olsen et al., 2016). Primary aldosteronism (PA) is the most common curable cause of arterial hypertension characterized by low levels of plasma renin and by high plasma levels of aldosterone (Conn & Louis, 1956), which are inappropriately high for the volume and BP status. Indeed, PA patients have more altered cardiovascular structure, such as left ventricular wall and carotid intima-media thickness (Bernini, Galetta, et al., 2008; Rossi et al., 2005), and cardiac fibrosis (Lin et al., 2012). PA is also associated with a higher incidence of atrial fibrillation (Burgess et al., 2016; Seccia et al., 2020), and negative cardiovascular outcomes (De Faria et al., 2013) than BP-matched essential hypertensive (EH) patients.

The diagnostic algorithm for PA is sequentially based on hormonal tests (screening and exclusion tests), followed by lateralization studies (adrenal CT scanning and AVS) to distinguish between unilateral and bilateral disease. Clinically, unilateral forms of PA (uPA) can be surgically curable. Adrenalectomy, especially when guided by AVS, cures the hyperaldosteronism and resolves or improves arterial hypertension, with markedly better outcomes when the diagnosis is made early (Rossi et al., 2018). In

the case of the bilateral forms where surgery is not indicated, the diagnosis allows a targeted drug treatment based on mineralocorticoid receptor (MR) antagonists and thus prevention of cardiovascular events (Colussi et al., 2013). Thus, an early identification of PA followed by the diagnosis of its subtypes is of paramount importance.

2. Prevalence of primary aldosteronism

The prevalence rate of PA varies according to the population being examined and the criteria used to diagnose it. The PAPY (Primary Aldosteronism Prevalence in hYpertension) study, the first large prospective survey that used a rigorous methodology to diagnose and subtype PA, reported a rate of 11.2% in consecutive newly diagnosed hypertensive patients (Rossi et al., 2006), albeit it increased along with the severity of BP elevation, from 3.9% to 9.7%, and up to 11.8% in hypertension stage 1, 2, and 3, respectively (Monticone et al., 2017), and up to 20% in patients with drug-resistant hypertension (RH) (Douma et al., 2008). In fact, RH is a common presentation of PA (Rossi et al., 2021). PA was also reported to involve about 2–3% of patients with an asymptomatic incidentally discovered adrenal mass (Mantero et al., 2000). Notwithstanding the convincing evidence for a high prevalence, there is still a quite popular misconception that PA is a rare disease. A large retrospective survey of general practitioners on hypertension and PA management in Germany and Italy with a high level of health care reported that doctors still regarded PA as very rare and therefore screened only 1% to 2% of their hypertensive patients (Mulatero et al., 2016). This practice was in striking conflict with the high (5.9%) prevalence of PA in 1672

unselected hypertensive patients from primary care centers, when a complete diagnostic work-up was performed (Maiolino, Calò, et al., 2017).

Another reason why PA is under-detected is that hypokalemia (<3.5 mEq/L), either spontaneous or diuretic-induced, was traditionally held to be the presenting sign of PA. The fact is that hypokalemia occurs only in a small proportion (9–37%) of PA patients (Baguet et al., 2016). In the PAPY study, more than 50% of the patients with an APA and 82% of those with a bilateral form were normokalemic (Rossi et al., 2006). Hence, sticking to the wrong strategy that hypokalemia is the essential performance of PA, many normokalemic PA patients would never be screened and diagnosed.

Overall, these findings call for actions to increase knowledge of the high prevalence of PA, and implementation of simplified strategies for screening and subtyping.

3. Diagnostic work-up for primary aldosteronism

3.1 Screening test

3.1.1 Target population

Recognizing the high prevalence of PA, the 2016 Endocrine Society Practical Guidelines widened the categories of hypertensive patients to be screened (Funder et al., 2016). This strategy is based on the consideration that the screening of PA is more cost-effective when undertaken in cohorts of patients with an enriched prevalence of PA. An observational study, however, suggested that PA is an evolving condition starting with a normotensive phase characterized by low renin and minimally elevated aldosterone levels, progressing to a clear biochemical phenotype, and ultimately evolving in stage 2–3 and/or drug-resistant hypertension (Jenifer M. Brown et al., 2020).

In accordance with the documented feasibility of normalizing BP and preventing cardiovascular complications with an early diagnosis and a specific treatment of PA (Rossi et al., 2013), it would justify the implementation of broader screening strategies, i.e. screening all newly diagnosed hypertensive patients. In addition, a cost-effectiveness analysis of the diagnosis and treatment of PA in Japan demonstrated a significant economic advantage as the screening test for PA is relatively inexpensive while further diagnostic steps have higher costs (Sato et al., 2015).

3.1.2 Measurement of aldosterone and renin

Plasma renin can be assessed as activity (PRA, ng/ml/h) which measures angiotensin I produced by the catalytic activity of renin using a radioimmunoassay (RIA), or as active renin (DRC, mIU/L or pg/mL) measured directly by a sandwich immunoradiometric assay or, more recently, by automated chemiluminescence immunometric assay (CLIA). Head-to-head studies comparing PRA with DRC have shown that with either assay the aldosterone-to-renin ratio (ARR) performed similarly well for PA screening (Burrello et al., 2016; Rossi et al., 2010). Notably, all available assays for both PRA and DRC become inaccurate in the low range of renin values (Rossi et al., 2010). Hence, to avoid overinflating the ARR value when renin is low, it is common practice to fix the renin value at a minimum (0.2 ng/mL/h for PRA, or 2 mIU/L for DRC). This avoids false-positive results and is particularly important in patients with low-renin essential hypertension, such as the elderly and people of African origin.

Plasma aldosterone concentration (PAC, ng/dL or pmol/L) is usually measured with RIA, or more recently by ultra-high performance liquid chromatography and tandem

mass spectrometry (LC-MS/MS) (Guo et al., 2018). PAC values measured with RIA varied widely across laboratories, likely depending on plasma extraction and cross-reactivity of aldosterone antibodies (Schirpenbach et al., 2006). Aldosterone LC-MS/MS assay displays higher accuracy, but the reference cut-off values need to be lowered since median aldosterone values measured by RIA are around 28% higher than those measured by LC-MS/MS (Guo et al., 2018).

3.1.3 Use of aldosterone-to-renin ratio

The use of ARR for the diagnosis of PA was firstly reported by the study of Hiramatsu *et al.* Numerous studies have demonstrated its superiority over the isolated measurement of plasma potassium or aldosterone or renin (Douillard et al., 2016), although valid estimates of test characteristics of the ARR are lacking (Montori & Young, 2002). Considering its high sensitivity and accuracy in the identification of PA, and its within-patient reproducibility when performed under standardized conditions, the ARR is recommended to detect possible cases of PA.

3.1.4 Conditions for the screening test

The PAC and renin values and, thereby, the ARR are influenced by multiple factors, which means that careful preparation of the patient and standardization of the conditions for testing are key steps when screening for PA (Table 1).

Factors Affecting ARR	Suggestion/Warning
Serum levels of potassium	Hypokalemia lowers aldosterone secretion. To avoid false-negative ARR values, correct hypokalemia, if present, before performing the test.
Plasma aldosterone concentration (PAC)	Physiologically a low salt intake and/or diuretic agents can lead to a high PAC. Measure 24-h urinary sodium excretion to estimate salt intake. Withdraw diuretic agents at least 4 weeks before testing as they raise renin and, if they cause hypokalemia, PAC can be factitiously low.
Renin	Fix the lowest level of renin to be used in the ARR at 0.2 ng/ml/h for PRA; at 2 mUI/l for DRC. This is common practice and makes good sense, although not supported by specific studies.
Patient position at blood sampling	Keep the patient resting supine or sitting for 60 min before sampling. Given the plasma half-life or plasma renin and aldosterone (roughly 15 min), this time will bring their values to baseline values.
Handling of the blood samples	Be aware that handling and storage of plasma samples differ for PRA and DRC assays: for the DRC plasma can be handled at room temperature. For PRA, blood tubes must be put immediately in salty iced water to block angiotensin-I generation and angiotensinogen consumption.
Drugs	α1-receptor blocker doxazosin and long-acting calcium channel blockers are allowed. MRAs can also be allowed if necessary to control BP and hypokalemia.
ARR calculation	Make use of the ARR-App to calculate the ARR in the correct unit of measure.
ARR accuracy	Identify the cut-off value that provides the best combination of sensitivity and specificity by receiver operating characteristic curves and Youden index analysis. Be aware that multivariate logistic discriminant analysis might provide better diagnostic accuracy. One such strategies is provided in the ARR-App.

Table 1. Suggestions for Correct Use of the ARR as a Screening Test for PA. Adapted from G.P. Rossi: Primary Aldosteronism: JACC State-of-the-Art Review. *J Am Coll Cardiol*. 2019 Dec 3;74(22):2799-2811.

3.2 Exclusion tests

3.2.1 Performance of exclusion tests

To maximize the sensitivity of the screening for PA, the low cut-off values of ARR for diagnosing PA, usually around 26 ng/dL/ng/mL/h (corresponding to 20.6 ng/mIU), are chosen (Rossi, 2019). Therefore, this strategy produces a high percentage of false positives. For example, in the PAPY study, by using a cutoff of 30 ng/dL/ng/mL/h, the false positive (FP) rate was 18% (Rossi et al., 2006). The latter must be excluded from the further expensive subtyping, which requires invasive AVS, a costly and minimally invasive procedure available only in highly specialized referral centers. Hence, to the end of selecting the patients for AVS, multiple tests, including the saline infusion test (SIT), the captopril challenge test (CCT), the oral sodium loading test (OLT), the fludrocortisone suppression test (FST), and the furosemide upright test (FUT) have been proposed according to both the Endocrine Society and the Japanese Guidelines (Funder et al., 2016; Nishikawa et al., 2011). They are generally referred to as "confirmatory" tests; however, this is an incomplete definition because what they serve for is to exclude patients from AVS. In fact, at the rate of prevalence of PA, ranging from 10% to 30%, usually seen at referral centers (Funder et al., 2016; Rossi et al., 2006), the negative predictive value of these tests is much higher than their positive predictive value (Maiolino, Rossitto, et al., 2017), which provided compelling evidence that they function as "exclusion", rather than as "confirmatory" tests for the diagnosis of PA. Therefore, throughout this paper, the term "exclusion" will be used to indicate these tests.

Exclusion test		Description	End point	Cut-off for PA diagnosis	Other requirements	Remarks
Saline infusion test (SIT)	**Recumbent**	4h infusion of 2L of 0.9% NaCl Recumbent position 1h before and during test	Post-infusion PAC	> 10 ng/dL PA highly likely 5-10 ng/dL PA intermediate likely < 5 ng/dL PA unlikely	Antihypertensive treatment adjustment Potassium supplementation	Contraindicated in patients with severe uncontrolled hypertension, renal insufficiency, cardiac arrhythmia, heart failure, severe uncorrected hypokalemia. Seated SIT is preferred.
	Seated	4h infusion of 2L of 0.9% NaCl Seated position 30 min before and during test	Post-infusion PAC	> 6 ng/dL (Australia) > 16 ng/dL (Taiwan) PA confirmed	Antihypertensive treatment adjustment Potassium supplementation Plasma cortisol is lower at the end than at baseline	
Captopril challenge test (CCT)		25–50 mg of captopril orally after sitting for at least 1 h.	PAC and PRA 2h after captopril	PAC > 11 ng/dl and PRA remaining suppressed Or ARR > 20 ng/dL/ng/ml/h: PA confirmed	Antihypertensive treatment adjustment Potassium supplementation	It avoids potential fluid overload in patients at risk (renal insufficiency, heart failure). Potential angioedema.
Oral sodium loading test (OLT)		Sodium intake >200 mmol (6g/24h) for 3 consecutive days	Urinary aldosterone excretion 24h from morning of day 3 to morning of day 4	> 12 or 14 ug/24h – PA highly likely < 10 ug/24h – PA unlikely	Antihypertensive treatment adjustment Potassium supplementation	Contraindicated in patients with severe uncontrolled hypertension, renal insufficiency, cardiac arrhythmia, heart failure, severe uncorrected hypokaliemia. 24h-urine collection inconvenient for patients and aldosterone measurement by HPLC-MS advisable.

Fludrocortisone suppression test (FST)	Every 6h for 4 days: - oral fludrocortisone 0.1 mg - slow-release KCl supplements Three times daily with meals: - slow-release NaCl supplements (30 mmol); Sufficient dietary salt	On day 4, PAC and PRA are measured at 10 a.m. (seated posture)	PAC > 6 ng/dL PRA < 1 ng/ml/h	Antihypertensive treatment adjustment Plasma cortisol at 10 a.m. is lower than 7 a.m. measurement	Requires hospital admission, blood test several times daily
Furosemide upright test (FUT)	Intravenous injection of 40 mg furosemide 2h standing (walking is permitted) before blood sampling in the sitting position	Post-infusion PRA	PRA < 2 ng/mL/h or PRC <8.0 pg/mL	Antihypertensive treatment adjustment	This test must be avoided in patients with advanced atherosclerosis at high risk for cerebrovascular events, and in those in whom arrhythmia may be induced by the test

Table 2. Exclusion tests for PA diagnosis. ARR, aldosterone-to-renin ratio; HPLC-MS, high performance liquid chromatography-mass spectrometry; PA, primary aldosteronism; PAC, plasma aldosterone concentration; PRA, plasma renin activity. Adapted from P. Mulatero et al: Genetics, prevalence, screening and confirmation of primary aldosteronism: a position statement and consensus of the Working Group on Endocrine Hypertension of The European Society of Hypertension. *J Hypertens*. 2020 Oct;38(10):1919-1928, and T. Nishikawa et al: Guidelines for the diagnosis and treatment of primary aldosteronism--the Japan Endocrine Society 2009. *Endocr J*. 2011;58(9):711-21

3.2.2 Rationale of exclusion tests

All these exclusion tests stand on the premise that in PA excessive aldosterone production is autonomous from renin-angiotensin system, a contention that is not evidence-based as aldosterone secretion was found to be affected by angiotensin (Ang) II in many PA patients (Holland et al., 1984; Irony et al., 1990). In the seminal study that introduced the SIT by currently accepted criteria, only five patients had an APA and two of them showed an increase of PAC with standing up indicating responsiveness to Ang II (Holland et al., 1984; Streeten et al., 1979). The existence of Ang II-responsive APA in up to 70% of the cases has been thereafter documented by multiple independent studies (Irony et al., 1990) (Gordon et al., 1987). Furthermore, the two most popular exclusion tests, CCT and SIT, when assessed prospectively in a large cohort of consecutive hypertensive patients, showed such an overlap of PAC responses between patients with and without APA that abated their value for individual patients discrimination and disproved the basic assumption of Ang II (Rossi et al., 2007). In addition, the type 1 angiotensin receptor, mediating aldosterone secretion in response to Ang II, was found to be expressed even in patients with the most florid PA phenotype due to an APA (Caroccia et al., 2021; Vanderriele et al., 2018).

Besides, according to the Brisbane group, the FST, which requires hospitalization and close monitoring of potassium levels and BP, would be the most reliable test and should represent the reference to be used for PA diagnosis. However, the performance of FST has never been validated according to the STARD methodology, according to which diagnostic approaches/tests should be evaluated against a clinical reference

standard or a gold standard (Bossuyt et al., 2015). Recognizing that there is a continuum between low-renin EH and PA (J.M. Brown et al., 2017; Monticone et al., 2018), thus validation of these exclusion tests should instead rely on a conclusive diagnosis of PA, which can be made retrospectively only in case of uPA when the patients were biochemically cured, i.e. when they showed normalization of PAC, ARR, and serum K^+ levels after surgical removal of an APA or an adrenal with unilateral adrenal hyperplasia. Accordingly, the methodology to be exploited in these studies should use biochemical cure after adrenalectomy as the "gold" standard and reference for validation of the exclusion tests.

3.2.3 Can exclusion tests be avoided?

Corresponding that systematic use of exclusion tests in clinical decision-making involves the risk of overlooking the PA patients who are Ang II-responsive, and that relying on these exclusion tests increase the complexity and costs of the diagnostic work-up, the 2016 Endocrine Society Clinical Practice Guidelines suggested the feasibility of skipping these tests in PA patients, who showed a florid phenotype, defined as spontaneous hypokalemia together with PRA or DRC below assay detection limits, and a PAC > 20 ng/dL (Funder et al., 2016). However, this strategy was not evidence-based until the largest study, entailing an exploratory cohort and a validation cohort, both recruiting over a thousand referred hypertensive patients prospectively, demonstrated that CCT provided no diagnostic gain over a carefully performed and interpreted ARR, the latter carrying essential quantitative information for PA diagnosis.

The ARR cutoff value exponentially decreases the rate of false positives to almost zero, therefore reducing the need for performing an exclusion test.

4. Pathophysiology of aldosterone-induced cardiovascular damage

4.1 Signals for T cell activation

Classically, T cells activation is a tightly organized process which requires 2 signals. Signal 1 involves T cell receptor (TCR) recognition of specific antigenic peptides presented by antigen-presenting cells (APCs). Signal 2 is co-stimulation, involving the interaction between B7 ligands on APCs and CD28 on T cells. Signal 1 and 2, established at the close interface between the T cell and the APCs, are called "immunologic synapse" and are sufficient to promote T cell proliferation, cytokine production, and often mobilization from secondary lymphoid organs. Once the immunological synapse is established, naive T cells become responsive to a further modulation by third signals, like hormones and cytokines (such as interleukin 12 (IL-12), IL-4 IL-1β, IL-23 and transforming growth factor-β [TGF-β]) produced and delivered by APCs and nearby stromal cells that activate key lineage-specific transcription factors that control their differentiation ('polarization') into various effector (Th1, Th2, and Th17) or regulatory (Treg) phenotypes.

4.2 T cell activation in aldosterone-induced cardiovascular damage

Animal models of hyperaldosteronism, such as deoxycorticosterone-high salt (DOCA-salt) or the chronic infusion of aldosterone in spontaneously hypertensive rats (SHR) drinking 1% NaCl solution, have provided evidence for the involvement of the immune

system in aldosterone-induced HMOD. The chronic administration of aldosterone and a high sodium diet resulted in coronary angiopathy in rats, characterized by the invasion of multiple immune cells in intramural coronary arteries (Sun et al., 2006). Such an invasion of inflammatory cells in the vasculature took place even before organ injury, suggesting that aldosterone-salt could induce an immunostimulatory state. Accordingly, the overexpression of oxidative stress products in circulating peripheral blood mononuclear cells (PBMCs) was observed as early as 1 week after being fed with aldosterone-salt (Ahokas et al., 2003). Chronic aldosterone-salt treatment was also accompanied by a time-dependent sustained activation of oxidative stress on endothelial cells and inflammatory cells, leading to a proinflammatory/fibrogenic phenotype involved in both vascular and nonvascular sites of injury (Sun et al., 2002). All these effects were inhibited by treatment with pharmacological MR antagonists (spironolactone or eplerenone), implicating that MR activation is essential for the development of aldosterone-induced inflammation (Y. Sun et al., 2002).

Proof of this concept further came from studies in recombination activating gene 1 deficient ($Rag1^{-/-}$) mice (Guzik et al., 2007), which were thereafter confirmed in Dahl salt-sensitive (SS) rats with Rag1 mutants (Mattson et al., 2013). Those animals lack mature T and B cells in both the circulation and in the spleen, thus creating a unique opportunity to address the role of these cells in hypertension. $Rag1^{-/-}$ mice were resistant to the hypertensive response induced by either Ang II or DOCA-salt. The adoptive transfer of T cells, but not B cells to these mice, restored the susceptibility to develop HMOD (Guzik et al., 2007). Meanwhile, renal infiltration of T cells,

following high-salt intake, was blunted in Rag1$^{-/-}$ Dahl SS rats as compared to controls (Mattson et al., 2013). Recently, experimental studies have focused upon the pathophysiological role of individual T-cell subsets in aldosterone-induced HMOD.

4.2.1 Th17

Th17 cells are a recently described subset of T-cells characterized by the expression of the master transcription factor retinoic acid-related orphan receptor (ROR) γt and by the production of IL-17 (Ivanov et al., 2006). Th17 cells are pro-inflammatory and exacerbate tissue damage and disease in conditions of chronic inflammation and autoimmunity (Louten et al., 2009). It shows that Th17 also plays a role in hypertensive pathology, and blunting Th17 signaling may alleviate the inflammation associated with HMOD. DOCA-salt increased the polarization of Th17, contributing to cardiac and renal damage in rats, independently of BP elevation (Amador et al., 2014). Treatment with an IL-17 neutralizing antibody (anti-IL-17) and IL-17 deficiency (IL-17$^{-/-}$) significantly reduced arterial hypertension, preserved vascular function, decreased superoxide production, and reduced T-cell infiltration in response to DOCA-salt (Amador et al., 2014). Meanwhile, there is a relation between salt intake and IL-17. Two independent studies showed that high-salt concentrations exaggerate Th17 production due to activation of the serum/glucocorticoid-regulated kinase 1 (SGK1), which stabilizes IL-23 receptor and reinforces the pathogenic function of Th17 (Kleinewietfeld et al., 2013; C. Wu et al., 2013).

4.2.2 Tregs

Tregs can provide a brake to the inflammatory process. They are a distinct subset of $CD4^+$ cells characterized by the expression of the forkhead transcription factor FoxP3 and the surface marker CD25. Aldosterone tended to decrease Treg in the renal cortex of C57BL/6 mice; on the contrary, adoptive transfer of Tregs lowered BP and ameliorated cardiac and renal injury in aldosterone-induced HMOD (Kasal et al., 2012). Additional evidence regarding the role of Tregs in hypertension comes from studies with normotensive (Brown Norway), hypertensive (Dahl SS), and consomic rats (SSBN2; in which chromosome 2 has been transferred from Brown Norway to Dahl SS rats) (Viel et al., 2010). Compared to Dahl SS rats, these SSBN2 rats had blunted hypertension, less vascular hypertrophy, and reduced aortic inflammation. These SSBN2 rats also had more aortic Tregs infiltration as evidenced by increased protein expression of Foxp3, TGF- β1, and IL-10, the three markers associated with the lineage of Tregs (Viel et al., 2010).

Notably, the balance between Th17 and Tregs in DOCA-salt induced hypertension was also altered. A higher ratio of Th17/Treg was seen in PBMCs, kidney, and heart of hypertensive rats; while spironolactone treatment ameliorated Th17 imbalance and fostered Treg count in DOCA-salt mice (Amador et al., 2014).

4.3 MR can act as a 3rd signal for T cell activation

In the late 80s, it has been observed that human splenic T cells contain proteins capable of binding aldosterone, supporting the presence of MR in T lymphocytes

(Armanini et al., 1988). Consistent with this, our previous work provided proof on the MR gene expression and protein expression in both human $CD4^+$ and $CD8^+$ T cells, by Droplet Digital PCR and immunoblotting respectively, and exposure to aldosterone in vitro promoted T cell clonal proliferation and activation. Recently, the study by Sun *et al.* provided a new signal 3 that has relevance to T cell activation in hypertension. The authors provided evidence that MR in T cells modulates their production of interferon gamma (IFN-γ) and ultimately promotes hypertension. T cell-specific MR knockout (TMRKO) in mice strikingly decreased both systolic and diastolic BP and attenuated aldosterone-induced HMOD, while T-cell MR overexpressing mice manifested more elevated BP compared with control mice, which was abolished by IFN-γ-neutralizing antibodies (Sun et al., 2017) (Figure 1).

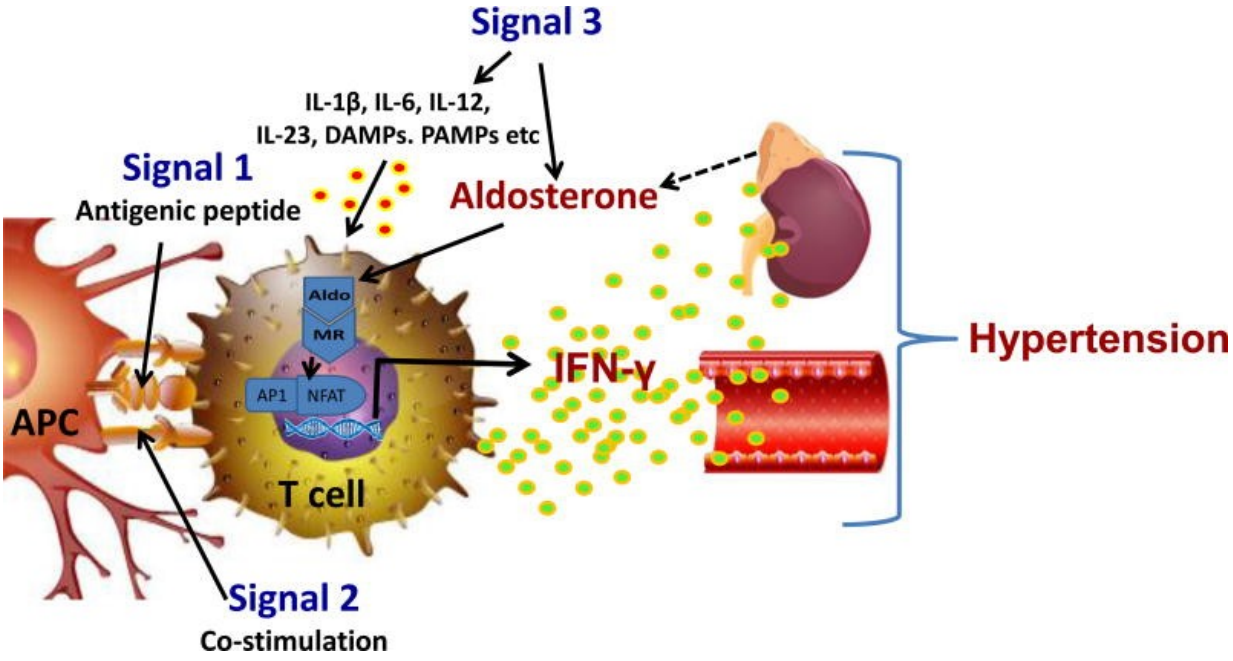

Figure 1. Aldosterone serves as a novel signal 3 for T cell activation. Antigen-presenting cells (APCs), including dendritic cells, B cells, macrophages, and others present antigenic peptides to the T cell receptor in the context of major histocompatibility complexes (Signal 1). This is accompanied by co-stimulatory signals, including interactions between B7 ligands on the APC and CD28 on T cells (Signal 2). T cells also possess numerous receptors for cytokines, Danger Associated Molecular Patterns (DAMPs) and Pattern Associated Molecular Patterns (PAMPs) that influence the T cell phenotype (Signal 3). Sun *et al.* show that aldosterone acting through the mineralocorticoid receptor (MR) acts as a previously unrecognized signal 3 by interacting with nuclear factor of activated T-cells 1 (NFAT1) and activator protein-1 (AP-1). This promotes the production of IFN-γ and ultimately vascular dysfunction and kidney damage leading to hypertension. Adapted from N.R. Barbaro *et al*. A new role of Mister (MR) T in Hypertension: Mineralocorticoid Receptor, immune system and hypertension. *Circ Res.* 2017 May 12; 120(10): 1527–1529.

4.4 Evidence of immune activation in PA patients

PA is a condition that has been found associated not only with extensive organ damage but also with increased systemic inflammation/inflammatory changes. To date, however, studies of the proinflammatory effects of aldosterone or MR activation in humans have relied on the measurement of circulating biomarkers of inflammation. Patients with PA displayed elevated levels of malondialdehyde (MDA), a lipoperoxidation marker of endothelial inflammation related to oxidative stress, and the amino terminal pro-peptide type I (PINP), a marker of myocardial collagen synthesis, as compared to those with EH (Stehr et al., 2010). Both markers decreased after specific treatment of PA with MR antagonists, reflecting the direct effect of aldosterone possibly mediated by MR activation. Importantly, there was a positive correlation between MDA, PINP, and ARR in PA patients (Stehr et al., 2010). Moreover, there were higher serum levels of IL-10, and TNF-α, and lower serum levels of TGF-β1 in patients with PA as compared to those with EH. Levels of TGF-β1 and TNF-α showed a remarkable correlation with ARR in the total group (PA+EH) (Carvajal et al., 2009). Furthermore, Krysiak and Okopien found that compared with BP-matched ET patients, PA patients showed increased monocyte release of TNF-α, IL-1β, and IL -6 and lymphocyte release of TNF-α, IFN-γ, and IL-2; Monocyte and lymphocyte cytokine release was higher in patients with EH than in normotensive subjects. Moreover, both treatment with MR antagonists or adrenalectomy in PA patients and anti-hypertensive drugs (not including MR antagonists) in ET patients can result in a decrease in monocyte and lymphocyte cytokine release. But this research just recruited 4 PA

patients, two underwent adrenalectomy and two received MR antagonists (Krysiak & Okopien, 2012).

AIMS OF MY WORK

Aim 1. To meta-analyze the diagnostic accuracy of exclusion tests in the diagnostic work-up of PA

Up to now, several studies have attempted to compare the performances of two or more exclusion tests in the diagnosis of PA; however, they suffered from several limitations, including the retrospective nature, the different cut-offs adopted, and most importantly, the fact that often one test was arbitrarily chosen as a reference standard over the others (Rossi et al., 2011), except for the AQUARR Study. Moreover, published studies that compared the screening test and the exclusion test(s) are few to date, and, therefore, the diagnostic gain of the exclusion test(s) over baseline ARR remains uncertain.

Hence, we performed a meta-analysis using biochemical cure after adrenalectomy as the "gold" standard, and if unavailable, a "golden" standard based on adrenal imaging and/or AVS to assess the overall diagnostic accuracy of the ARR and different exclusion tests for uPA diagnosis. By increasing the overall sample size of the patients studied by these tests and comprising the experience gained in multiple centers, we aimed to provide a more accurate picture of the diagnostic performance of these tests with a higher level of confidence.

Aim 2. To investigate circulating Th17 and Tregs in PA patients

Our previous work provided proof on the MR gene expression and protein expression in human T cells, by Droplet Digital PCR and immunoblotting, respectively. Existing findings have suggested that exposure to aldosterone (at concentration commonly detected in peripheral blood of PA patients) promoted T cells clonal proliferation and

activation. Thus, we further aimed to investigate the immunological consequences of chronical exposure to high PAC on Th17 and Tregs, and evaluate the effect of MR antagonists and surgery on these cells in PA patients.

META-ANALYSIS: METHODS, RESULTS, AND DISCUSSION

1. Methods

1.1 Search strategy and selection criteria

The study followed the preferred reporting items for systematic review and meta-analysis (PRISMA) statement (Liberati et al., 2009). A systematic search of relevant articles from PubMed, EMBASE, Web of Science, and Cochrane library databases on the diagnostic performance of the screening test and exclusion test on PA in human subjects, published between January 1st, 1970 and March 31st, 2021, was conducted, by using the following terms in combination, as Medical Subject Headings (MeSH) or Entree terms and test words, “hyperaldosteronism”, “sensitivity and specificity”, and “predictive value of tests”.

After the removal of duplicates, the eligibility of articles was assessed on the title and abstract by two reviewers independently, with divergences resolved after consensus by senior reviewers. The related bibliographies were also screened for relevant additional studies. Studies were considered eligible for inclusion if they fulfill the following criteria: (1) uPA diagnosis was established according to the gold or a golden standard; (2) the diagnostic accuracy of baseline ARR or at least one exclusion test was reported; (3) the studies reported sufficient data to construct a 2x2 table based on uPA. Studies were excluded if they comprised: (1) reviews, case reports, case-control studies; (2) duplicated data from the same or overlapping patients’ cohort.

1.2 Data extraction

Data extraction from the eligible articles was performed by two independent reviewers using a predefined standardized form. The form included the following characteristics of each study: authors, year of publication, country, period, setting (single or multiple; primary care or referral center), study design (prospective or retrospective), population (patients with suspected PA, or with positive ARR), number of uPA, number of controls, ratio of uPA, wash-out period, PA diagnosis, uPA diagnosis, serum K^+ level, potassium supplement, salt intake (liberal or restricted), index test(s), procedure of index test(s), cut-off value of index test(s), and a 2x2 table of true positives (TP), FP, false negatives (FN), and true negatives (TN). When a study offered the accuracy of baseline ARR and/or exclusion test(s) at different cut-off values, the cut-off value that provided the best combination of sensitivity and specificity, as assessed by the receiver-operating characteristic (ROC) curve and Youden Index (YI) analysis, was selected. Differences between reviewers were resolved by discussion and consensus when necessary.

1.3 Quality assessment

The methodological quality of the identified studies was assessed by two independent reviewers using the modified Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) criteria by RevMan 5. Risk of bias in these four domains: patient selection, index test, reference standard, flow and timing, was assessed by eight signaling questions, rating as yes/no/unclear. Risk of bias was judged as "low" if all the relevant questions in one domain were rated as "yes", otherwise as "high" or

"unclear" if one or more relevant questions were rated as "no" or "unclear". Concerns about applicability in the first three domains were judged as "low" if they were performed according to the ES/JES guidelines.

1.4 Data analysis and synthesis

The threshold effect was assessed by the Spearman correlation coefficient between Logit(sensitivity) and Logit (1-specificity), and a corresponding P-value over 0.05 suggested a non-threshold effect. I^2 was used to evaluate the influence of heterogeneity between studies caused by inconsistencies instead of chance. The random effects model was used when I^2 was more than 30%. The summary receiving operation characteristic (sROC) curves and the corresponding summary area under the curve (sAUC), pooled sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), diagnostic odds ratio (DOR), and the corresponding 95% confidence intervals (CI) were computed. Meta-regression was performed to investigate the potential covariates that might influence between-study inconsistency. Subgroup analysis was further conducted to explore heterogeneity. More than four studies in a subgroup were selected to achieve more robust results. Sensitivity analysis was performed to evaluate the quality and consistency of results by sequentially excluding a single study at a time. Potential publication bias was evaluated by visual inspection and the P-value of Deeks' funnel plot.

All analyses were performed with Meta-Disc version 1.4 and STATA version 12.0 (Stata Corp, College Station, TX). All statistical tests were two-sided, with a P-value of 0.05 denoting statistical significance.

2. Results

2.1 Literature search and study selection

The literature search in PubMed, EMBASE, Web of Science, and the Cochrane library provided 2698 articles, of which 1623 remained after the removal of duplicate entries. After review of the title and abstract, 1531 articles were excluded with 92 potentially relevant articles remaining. After full-text reading, 73 articles were excluded with the following reasons: other exclusion tests (n=5), case-control studies (n=13), exclusion test(s) aimed at PA subtyping (n=12), data unable to construct a 2x2 table by using uPA as the classification factor (n=33), duplicated data from the same or overlapping patients' cohorts (n=9), wrong calculation of plasma renin concentration (n=1). Finally, 19 articles entailing 28 separate datasets, fulfilling uPA diagnosis by the gold or a golden standard with reported sensitivity and specificity of the diagnostic test(s), were deemed eligible and analyzed in our meta-analysis (Figure 2).

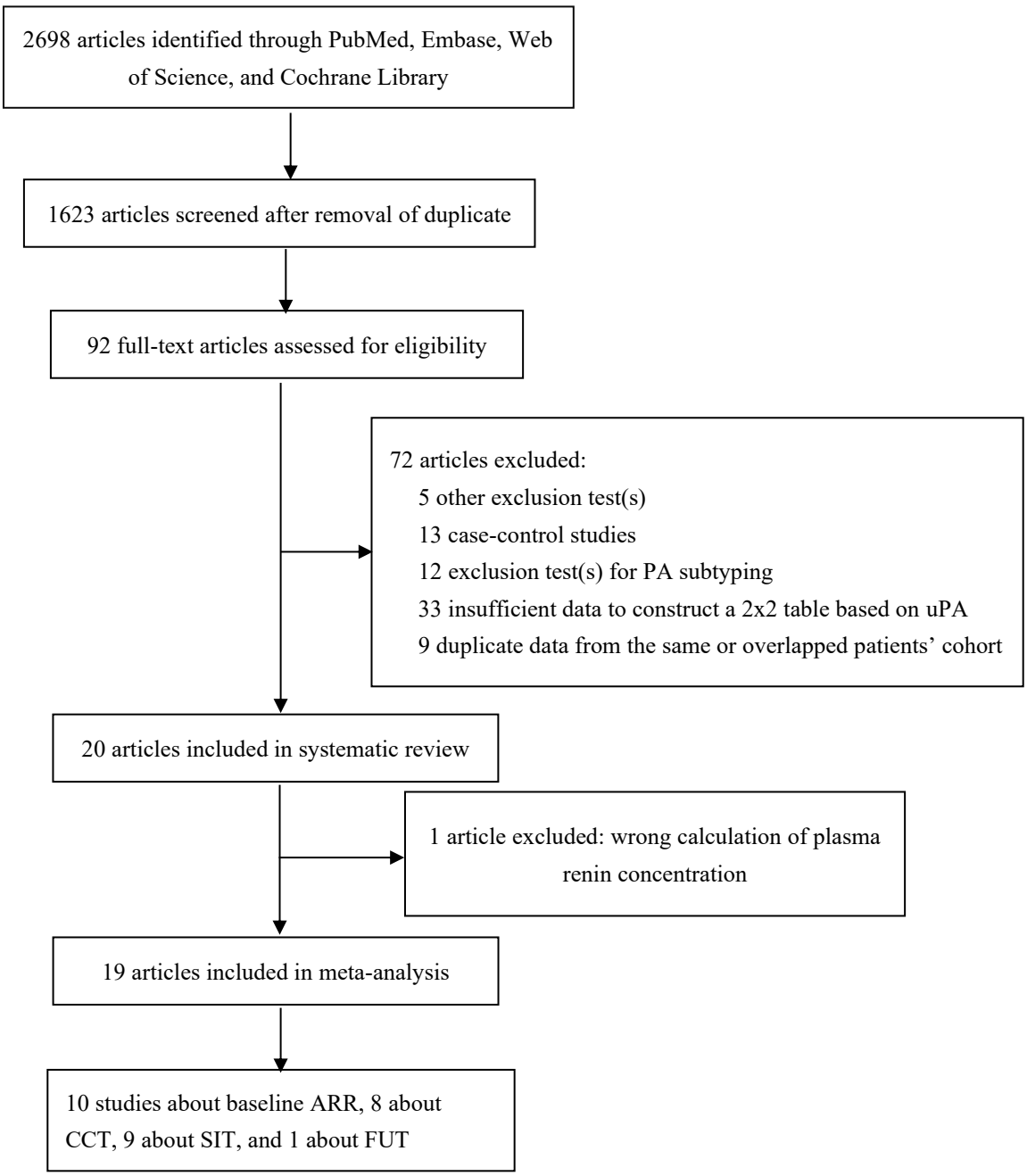

Figure 2. Flow chart of literature search and study selection. ARR, aldosterone-to-renin ratio; CCT, captopril challenge test; FUT, furosemide upright test; PA, primary aldosteronism; SIT, saline infusion test; uPA, unilateral primary aldosteronism.

2.1 Study characteristics

The characteristics of included studies are detailed in Table 3. All these 19 studies were conducted in hypertension referral centers in several European/Oceanian and Asian countries from 1998 till 2019. Of these, six assessed the diagnostic accuracy of baseline ARR (Bernini, Moretti, et al., 2008; Burrello et al., 2016; Ducher et al., 2012; Giacchetti et al., 2006; Vorselaars et al., 2018; Weickert et al., 2009), three assessed CCT (Kim et al., 2016; V. C. Wu et al., 2009, 2010), five assessed SIT (Mulatero et al., 2006; Rossi et al., 2007; Stowasser et al., 2018; Vivien et al., 2019; Zhang et al., 2020), and five assessed multiple tests using a head-to-head comparison (Fries et al., 2020; Maiolino, Rossitto, et al., 2017; Meng et al., 2018; Okamoto et al., 2019; Song et al., 2018). Among these, the study by Maiolino assessed both baseline ARR and CCT in an exploratory cohort and a validation cohort (Maiolino, Rossitto, et al., 2017); the study by Fries assessed both baseline ARR and SIT (Fries et al., 2020); the study by Okamoto assessed baseline ARR, CCT, SIT, and FUT (Okamoto et al., 2019); the studies by Song (Song et al., 2018) and Meng (Meng et al., 2018) assessed both CCT and SIT. None assessed the diagnostic accuracy of OLT nor FST.

Of them, 16 were prospective and three were retrospective. The target population was patients with newly diagnosed hypertension (n=2), patients with a high risk of PA according to the ES Guidelines (n=8), patients with positive ARR (n=6), patients with positive ARR plus one-third/fourth negative ARR (n=2), and patients with positive recumbent SIT (n=1). Eventually, 4130 subjects were included, of whom 618 were patients with uPA.

Antihypertensive medication was withheld for at least two weeks, and only calcium channel antagonists and/or α-blockers were allowed for uncontrolled blood pressure according to the ES Guidelines, except for Burrello's where the withdrawal was only conducted in 20% of eligible patients (Burrello et al., 2016).

The diagnosis of uPA was confirmed by the gold standard (n=11), or a golden standard (n=8). To construct the 2x2 table, the control group was chosen as patients with EH (n=16), patients with EH plus BAH (n=1) (Okamoto et al., 2019), patients with EH plus UAH plus BAH (n=1) (Ducher et al., 2012), and patients with non-PA including biochemically cured PA (n=1) (Stowasser et al., 2018).

	Period	Population	Index test(s)	uPA (n)	Controls (n)	Ratio of uPA	PA diagnosis	uPA diagnosis
Bernini et al (2008)	1998-2003	New HT	ARR	30	100	23·08%	Baseline PAC> 35 ng/dl with PRA< 0·5 ng/ml/h	Biochemical cure after surgery
Burrello et al (2016)	2014	Suspected PA	ARR	5	75	6·25%	Post-saline PAC> 5 ng/dl (138·7pmol/l); post-captopril ARR> 30 (ng/dl/ng/ml/h) (832·2pmol/l/ng/ml/h) and ADRR > 3.7 (ng/dl/mU/l) (102·6 pmol/l/mU/l)	Biochemical cure after surgery
Ducher et al (2012)	2006-2007	Suspected PA	ARR	12†	167‡	6·70%	An outcome committee	Pathology after surgery
Fries et al (2020)	2016-2019	Pos ARR (cutoff N.A.)	ARR, SIT	9	67	11·84%	Elevated ARR with PAC> 20 ng/dL, spontaneous hypokalemia, and suppressed PRA and/or positive exclusion testing	Biochemical cure after surgery
Giacchetti et al (2006)	1996-2000	Suspected PA	ARR	26	96	21·31%	(1) (a) baseline upright PAC and UAC above upper normal limits; plus (b) low upright PRA (1·0 ng/ml /h); plus (c) post-saline PAC≥ 10 ng/dl; (2) (a) plus (c) plus normal upright PRA; (3) (b) plus (c) plus normal baseline upright PAC and UAC; (4) (a) plus (b) plus post-saline PAC < 10 ng/dl) plus evidence of an adrenal mass.	Pathology after surgery
Kim et al (2016)	2011-2014	Pos ARR (>20 ng/dl/ng/ml/h)	CCT	36	13	73·47%	Post-saline PAC≥ 10 ng/dl	Biochemical cure after surgery

Maiolino et al (2017)¶	2000-2005	New HT	ARR, CCT	51	991	4·89%	Baseline ARR≥ 40 ng/dl/ng/ml/h or post-captoril ARR≥ 30 ng/dl/ng/ml/h or a logistic discriminant function≥ 0·50	Biochemical cure after surgery
Maiolino et al (2017)‖	2012-2015	New HT	ARR, CCT	30	1028	2·84%	Baseline ARR≥ 40 ng/dl/ng/ml/h or post-captoril ARR≥ 30 ng/dl/ng/ml/h or a logistic discriminant function≥ 0·50	Biochemical cure after surgery
Meng et al (2017)	2011-2016	Suspected PA	CCT, SIT	70	49	58·82%	Baseline ARR> 30 ng/dl/ng/ml/h	Biochemical cure after surgery
Mulatero et al (2006)	2004	Pos ARR (cutoff chosen by each center)	SIT	18	31	36·73%	Post-fludrocortisone PAC> 5 ng/dl	Biochemical cure after surgery
Okamoto et al (2018)	2012-2018	Pos ARR (>20 ng/dl/ng/ml/h)	ARR, CCT, SIT, FUT	16	86*	15·69%	At least two of three exclusion tests (Post-captopril ARR> 20 ng/dl/ng/ml/h; Post-saline PAC> 6·0 ng/dl; Post-furosemide PRA< 2·0 ng/mL/h)	AVS, LI ≥ 4
Rossi et al (2007)	2000-2005	Pos ARR (≥40 ng/dl/ng/ml/h) or post-CCT (ARR≥ 30ng/dl/ng/ml/h) or a logistic discriminant function ≥ 0.50 + 1/4 not fulling the above criteria	SIT	46	197	18·93%	Baseline ARR≥ 40 ng/dl/ng/ml/h or post-captoril ARR≥ 30 ng/dl/ng/ml/h or a logistic discriminant function≥ 0·50	Biochemical cure after surgery
Song et al (2017)	2013-2016	Pos ARR (>37 ng/mIU + 1/3 neg ARR	CCT, SIT	71	101	41·28%	Post-fludrocortisone PAC≥ 8.0 ng/dl	Biochemical cure after surgery
Stowasser et al (2018)	2012-2017	Pos ARR (>70 pmol/mIU and >55 pmol/mIU, when	SIT	25	17§	59·52%	Post-fludrocortisone PAC≥ 133 pmol/L	Biochemical cure after surgery

		aldosterone was measured by RIA and HPLC-MS/MS, respectively)						
Vivien et al (2019)	2010-2015	Pos ARR (>64 pmol/mIU)	SIT	24	76	24·00%	Post-saline PAC> 5 ng/dl or post-captopril PAC suppressed< 30%	AVS /CT/MRI
Vorselaars et al (2018)	2015-2017	Suspected PA	ARR	10	217	4·41%	Post-saline PAC >280 pmol/l and PRA >100 fmol/l /s	AVS/pathology
Weickert et al (2009)	2005-2006	Suspected PA	ARR	6	22	24·14%	High ARR, elevated PAC and UAC, low or suppressed PRA, and hypokalemia	Pathology after surgery
Wu et al (2009)	2003-2006	Suspected PA	CCT	47	64	42·34%	Post-saline PAC> 10 ng/dl or UAC≧12μg/24 h	Biochemical cure after surgery
Wu et al (2010)	2008	Suspected PA	CCT	39	63	38·24%	Post-saline PAC> 10 ng/dl	AVS, LI ≥ 4 or scintigraphy
Zhang et al (2020)	2018-2019	Pos-recumbent SIT (PAC >11.2 ng/dl)	SIT	46	20	69·70%	Post-saline PAC> 11·2 ng/dl	Biochemical cure after surgery

Table 3. Characteristics of included studies. ARR, aldosterone/plasma renin activity ratio; ADRR, aldosterone/direct renin concentration ratio; AVS, adrenal vein sampling; BP, blood pressure; CCT, captopril challenge test; CT, computed tomography; FUT, furosemide upright test; HT, hypertensives; LI, lateralization index; MRI, magnetic resonance tomography; Neg, negative; P, prospective; PA, primary aldosteronism; Pos, positive; R, retrospective; SIT, saline infusion test; UAC, urinary aldosterone concentration. *Control group as patients with bilateral hyperplasia or essential hypertension; † Patients with aldosterone-producing adenoma confirmed by pathology; ‡Control group as patients with suspected aldosterone-producing adenoma or unilateral hyperplasia or bilateral hyperplasia or essential hypertension; §Control group as patients with non-PA, among whom 14 were biochemically cured after surgery; ¶Exploratory cohort of Maiolino's study; ||Validation cohort of Maiolino's study.

2.3 Construction of 2X2 table

Of these ten studies about ARR, four were performed according to the ES Guidelines, i.e. in the morning after patients have been up for at least 2 hours and seated for 5-15 minutes, one after 30 minutes of exercise at walking pace, three after a rest in the sitting position, and two after a rest in a supine position. The diagnostic cut-off of ARR values ranged from 19.1 to 96.4 ng/mIU throughout studies. Among them, five were chosen according to the ROC curve and YI analysis for uPA diagnosis (Table 4).

Of these eight studies about CCT, all were performed according to the ES/JES Guidelines. The dosage of captopril was 50mg in seven studies and 25mg in one study. Patients were in a sitting position for 1-2 hours. Post-captopril ARR and PAC were chosen as the cutoff values in five and three studies, ranged from 15.6 to 51.5 ng/mIU and from 13 to 19 ng/dl, respectively. Among them, six were chosen according to the ROC curve and YI analysis for uPA diagnosis (Table 4).

Of these nine studies about SIT, all were performed with two liters of sodium chloride of 0.9% for two hours in a supine position (n=7) or a seated position (n=2). The cutoff values of post-saline PAC ranged from 3.0 to 15.2 ng/dl. Among them, four were chosen according to the ROC curve and YI analysis for uPA diagnosis (Table 4).

There was only one eligible study about the accuracy of FUT for UPA diagnosis, which was performed according to the JES Guideline. The cutoff value of post-furosemide PRA was chosen as 0.55 ng/ml/h according to the ROC curve and YI analysis for uPA diagnosis (Table 4).

Author, Year	Procedure	Cut-off value (original)	Cut-off value (converted)	Value by ROC curve	TP	FP	FN	TN
Screening test								
Bernini et al (2008)	Morning, after upright for at least 2h and seated for 5-15min	ARR > 96·4 ng/mIU	ARR > 96·4 ng/mIU	Yes	29	13	1	87
Burrello et al (2016)	Morning, after upright for at least 2h and seated for at least 15min	ARR ≥ 37 ng/mU and PAC ≥ 10ng/dl	ADRR ≥ 37 ng/mU and PAC ≥ 10 ng/dl	No	5	1	0	74
Ducher et al (2012)	Morning, after supine for 1h	ARR ≥ 32 ng/ng	ARR ≥ 20·2 ng/mIU	Yes	11	16	1	181
Fries et al (2020)	Morning, after seated for 15min	ARR ≥ 53 pmol/mU	ARR ≥ 19·1 ng/mIU	No	9	27	0	40
Giacchetti et al (2006)	Morning, after upright for 2h and seated for 5-15min	ARR ≥ 40 ng/dl/ng/ml/h	ARR ≥ 48·8 ng/mIU	No	26	15	0	81
Maiolino et al (2017)*	Morning, after seated for 1h	ARR ≥ 33·3 ng/dl/ng/ml/h	ARR ≥ 40·6 ng/mIU	Yes	40	117	11	874
Maiolino et al (2017)†	Morning, after supine for 1h	ARR ≥ 30·9 ng/dl/ng/ml/h	ARR ≥ 37·3 ng/mIU	Yes	29	64	1	964
Okamoto et al (2018)	Morning, after seated for 15min	ARR ≥ 52·8 ng/dl/ng/ml/h	ARR ≥ 64·4 ng/mIU	Yes	12	22	4	64
Vorselaars et al (2018)	Morning, after upright for at least 2h and seated for 5-15min	ARR > 7 pmol/fmol	ARR > 65·6 ng/mIU	No	10	23	0	194
Weickert et al (2009)	Morning, after upright for 30min	ARR ≥ 425 pg/ml/ ng/ml/h	ARR ≥ 51·8 ng/mIU	No	6	4	0	18

CCT								
Kim et al (2016)	Captopril, 50mg 1.5h seated	PAC ≥ 19 ng/dl	PAC ≥ 19·0 ng/dl	No	27	0	9	13
Maiolino et al (2017)*	Captopril , 50mg 2h seated	ARR ≥ 13. 9ng/dl/nd/ml/h	ARR ≥ 17·0 ng/mIU	Yes	40	120	51	871
Maiolino et al (2017)†	Captopril, 50mg 2h seated	ARR ≥ 12·8 ng/dl/nd/ml/h	ARR ≥ 15·6 ng/mIU	Yes	28	227	2	801
Meng et al (2018)	Captopril, 25mg 2h seated	PAC ≥ 15 ng/dl	PAC ≥ 15·0 ng/dl	No	68	9	2	40
Okamoto et al (2018)	Captopril, 50mg 1.5h seated	ARR ≥ 42·2 ng/dl/nd/ml/h	ARR ≥ 51·5 ng/mIU	Yes	12	16	4	70
Song et al (2017)	Captopril, 50mg 2h seated	PAC ≥ 13 ng/dl	PAC ≥ 13·0 ng/dl	Yes	68	5	3	96
Wu et al (2009)	Captopril, 50mg 1.5h seated	ARR ≥ 23·9 ng/dl/ng/ml/h	ARR ≥ 29·2 ng/mIU	Yes	28	6	11	57
Wu et al (2010)	Captopril, 50mg 1h seated	ARR ≥ 39·6 pmol/ng	ARR ≥ 9·0 ng/mIU	Yes	39	8	8	56
SIT								
Fries et al (2020)	Saline, 2l 4h supine	PAC ≥ 83 pmol/l	PAC ≥ 3·0 ng/dl	No	9	5	0	62
Meng et al (2017)	Saline, 2l 4h supine	PAC ≥ 10 ng/dl	PAC ≥ 10·0 ng/dl	No	69	10	1	39
Mulatero et al (2006)	Saline, 2l 4h supine	PAC ≥ 5 ng/dl	PAC ≥ 5·0 ng/dl	No	18	5	0	26
Okamoto et al (2018)	Saline, 2l 4h supine	PAC ≥ 15·2 ng/dl	PAC ≥ 15·2 ng/dl	Yes	14	9	2	77
Rossi et al (2007)	Saline, 2l 4h supine	PAC ≥ 6.8 ng/dl	PAC ≥ 6.8 ng/dl	Yes	38	49	8	148
Song et al (2017)	Saline, 2l 4h supine	PAC ≥ 10 ng/dl	PAC ≥ 10·0 ng/dl	Yes	68	4	3	97
Stowasser et al (2018)	Saline, 2l 4h seated	PAC ≥ 162 pmol/l	PA C≥ 5·8 ng/dl	Yes	24	1	1	16

Vivien et al (2019)	Saline, 2l 4h supine	PAC ≥ 5·7 ng/dl	PAC ≥ 5·7 ng/d	No	22	4	2	74
Zhang et al (2020)	Saline, 2l 4h seated	PAC ≥ 12·9 ng/dl	PAC ≥ 12·9 ng/dl	No	41	2	5	18
FUT								
Okamoto et al (2018)	Furosemide, 40mg 2h upright	PRA ≤ 0·55 ng/ml/h	PRA ≤0·55 ng/ml/h	Yes	13	23	3	63

Table 4. 2x2 table. ARR, aldosterone-to-renin ratio; CCT, captopril challenge test; FN, false negatives; FP, false positives; PAC, plasma aldosterone concentration; PRA, plasma renin activity; SIT, saline infusion test; ROC, receiver-operating characteristic curve; TN, true negatives; TP, true positives. * Exploratory cohort of Maiolino's study; †Validation cohort of Maiolino's study. For uniformity between the studies, all units were converted to ng/mIU for ARR, ng/dl for PAC, ng/ml/h for PRA.

2.4 Quality of eligible studies

The quality of included studies, assessed by the QUADAS-2 criteria, was summarized in Figure 3 and broken down by each study in Figure 4. In domain of patient selection, four were at a high risk of bias, as three (Ducher et al., 2012; Fries et al., 2020; Zhang et al., 2020) did not avoid inappropriate exclusions and one (Stowasser et al., 2018) compared data about UPA before and after surgery; seven (Burrello et al., 2016; Giacchetti et al., 2006; Kim et al., 2016; Meng et al., 2018; Weickert et al., 2009; V. C. Wu et al., 2010) had un unclear risk due to the lack of details. In domain of index test, there was no risk of bias in all studies as the results of baseline ARR and exclusion tests did not interpret with the knowledge of the final diagnosis of uPA. In domain of reference standard, 18 studies (four about baseline ARR, six about CCT, six about SIT), where uPA was diagnosed according to the gold standard, had a low risk; while other studies using a golden standard were at high risk. In domain of flow and timing, all studies were at a high risk of bias as a "positive" ARR and/or exclusion test result was typically followed by AVS, surgery, and biochemical/clinical assessment after surgery (reference standard) whereas a "negative" result was not. There were low concerns of applicability in all domains, except that in Burrello's (Burrello et al., 2016), only 20% of patients underwent pharmacological wash-out.

Figure 3. Quality assessment of included studies by QUADAS-2 criteria.
Stacked bars represent the proportion of studies with a high (red), or unclear (yellow) or low (green) risk of bias and applicability concerns. ARR, aldosterone-to-renin ratio; CCT, captopril challenge test; FUT, furosemide upright test; QUADAS-2, Quality Assessment of Diagnostic Accuracy Studies-2 criteria; SIT, saline infusion test.

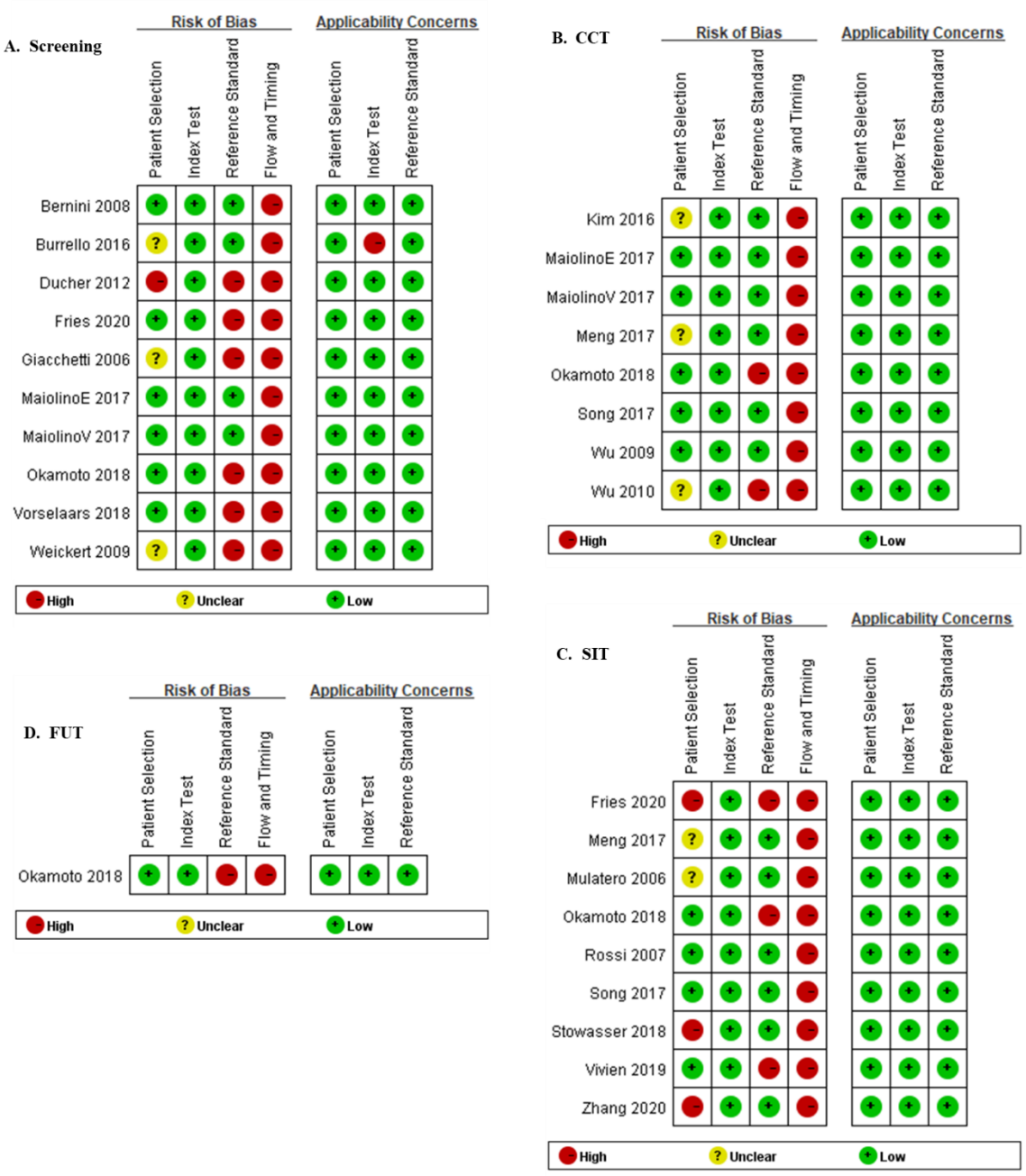

Figure 4. Quality assessment of each study by QUADAS-2 criteria. CCT, captopril challenge test; FUT, furosemide upright test; QUADAS-2, Quality Assessment of Diagnostic Accuracy Studies-2 criteria; SIT, saline infusion test.

2.5 Meta-analysis

2.5.1 Diagnostic threshold

Spearman correlation coefficient and P-value were -0.04 and 0.91 for baseline ARR, 0.36 and 0.39 for CCT, -0.10 and 0.80 for SIT, indicating no diagnostic thresholds. Thus, heterogeneity judgment for baseline ARR, CCT, and SIT was performed by non-threshold effects. I^2 values of pooled sensitivity, specificity, PLR, NLR, DOR for baseline ARR, CCT, and SIT were all greater than 30%, indicating the presence of heterogeneous non-threshold effects for all tests. Thus, the random-effect model was used for overall analysis.

2.5.2 Overall meta-analysis

For ARR, the pooled sensitivity and specificity were 0.91 (95% CI: 0.86-0.94, I^2 = 63.5%) and 0.90 (95% CI: 0.88-0.91, I^2 = 90.7%), respectively; the pooled PLR and NLR were 6.98 (95% CI: 4.44-10.98, I^2 = 91.9%) and 0.11 (95% CI: 0.05-0.23, I^2 = 48.2%), respectively; the pooled DOR was 76.68 (95% CI: 29.02-202.56, I^2 = 58.9%). In addition, the sAUC of these ten studies was 0.952 (Figure 5).

For CCT, the pooled sensitivity and specificity were 0.86 (95% CI: 0.82-0.90, I^2= 77.7%) and 0.84 (95% CI: 0.82-0.85, I^2 = 87.9%), respectively; the pooled PLR and NLR were 6.38 (95% CI: 4.21-9.66, I^2 = 85.7%) and 0.17 (95% CI: 0.10-0.28, I^2 = 71.2%), respectively; the pooled DOR was 46.85 (95% CI: 22.50-97.56, I^2 = 62.7%). In addition, the sAUC of these eight studies was 0.938 (Figure 6).

For SIT, the pooled sensitivity and specificity were 0.93 (95% CI: 0.90-0.96, I^2 = 53.7%) and 0.86 (95% CI: 0.83-0.89, I^2 = 80.4%), respectively; the pooled PLR and

NLR were 8.52 (95% CI: 4.84-14.99, I^2 = 81.6%) and 0.09 (95% CI: 0.05-0.16, I^2 = 46.1%), respectively; the pooled DOR was 123.76 (95% CI: 41.59-368.28, I^2 = 69.5%). In addition, the sAUC of these nine studies was 0.966 (Figure 7).

Since only one study on FUT was eligible, the primary data was used as pooling results: the pooled sensitivity and specificity were 0.80 and 0.73, respectively; AUC was 0.842.

These results indicated a good level of overall accuracy for baseline ARR at 19.1 to 96.4 ng/mIU, CCT with post-captopril ARR at 15.6 - 29.2 ng/mIU or PAC at 13.0 - 19.0 ng/dl, SIT with post-saline PAC at 3.0 -15.2 ng/dl, FUT with post-furosemide PRA at 0.55 ng/ml/h. Overall, there were no significant differences in the diagnostic accuracy of baseline ARR, CCT, and SIT (p = 0.569 for baseline ARR vs CCT; p = 0.318 for baseline ARR vs SIT; p = 0.178 for CCT vs SIT), while no solid conclusion could be drawn for FUT due to the very limited data.

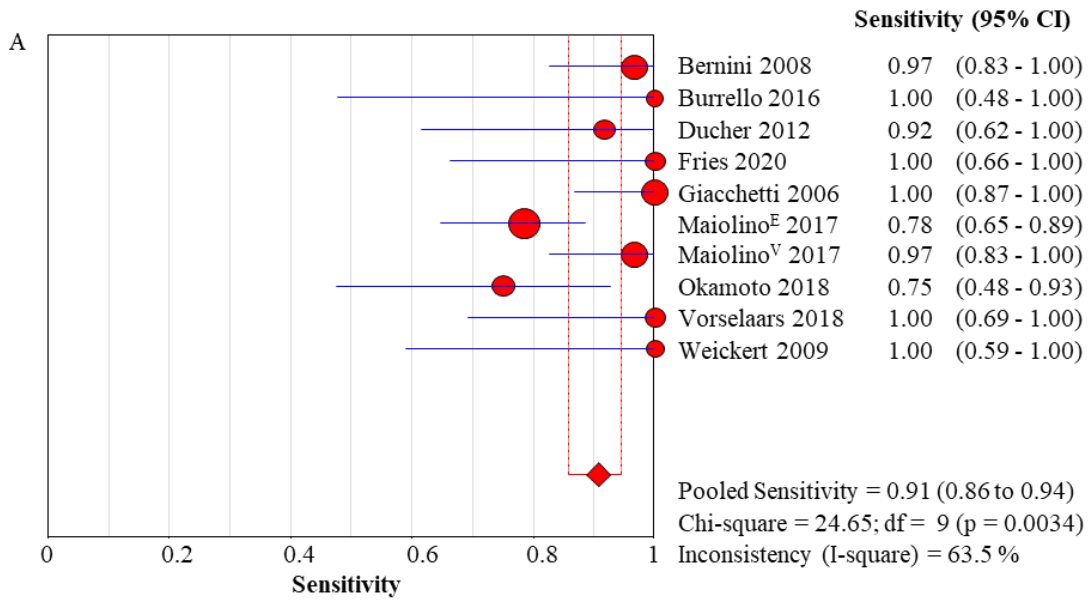

Figure 5A. Sensitivity of baseline ARR for unilateral primary aldosteronism. Error bars on the plots of sensitivity represent the 95% confidence intervals. Circle size is proportional to the weight of the study.

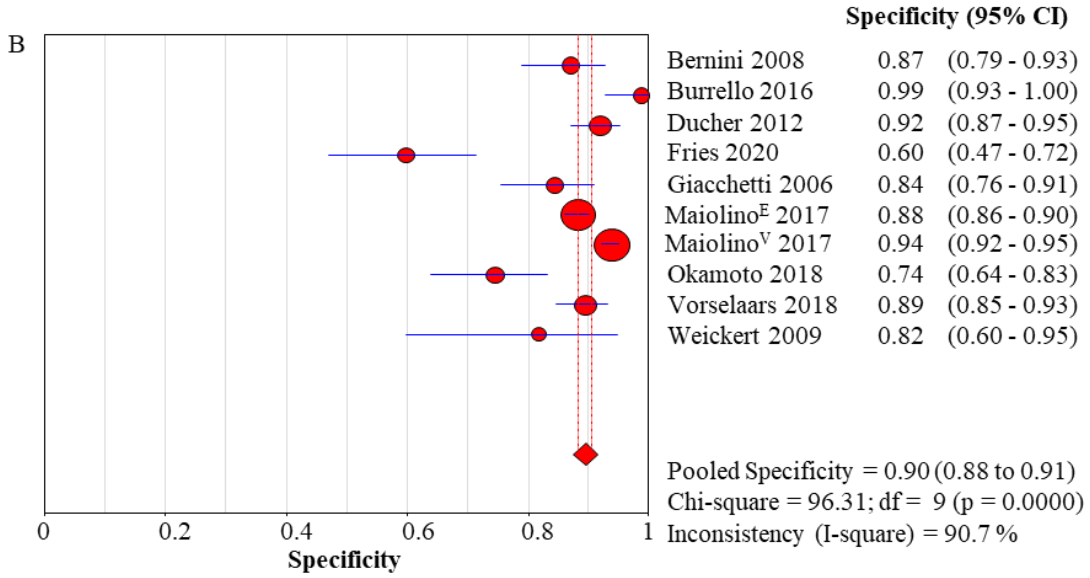

Figure 5B. Specificity of baseline ARR for unilateral primary aldosteronism. Error bars on the plots of sensitivity represent the 95% confidence intervals. Circle size is proportional to the weight of the study.

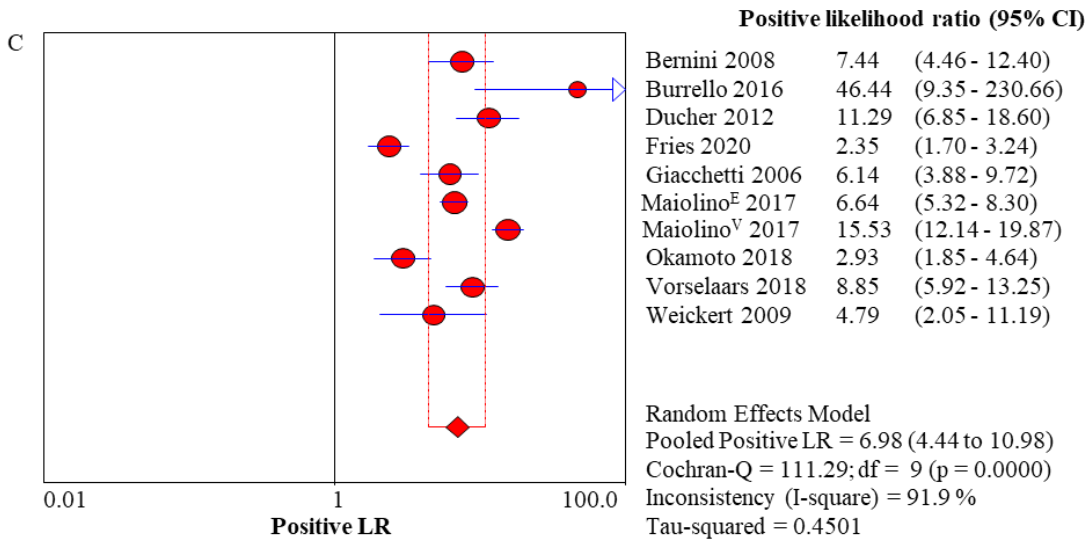

Figure 5C. Positive likelihood ratio of baseline ARR for unilateral primary aldosteronism. Error bars on the plots of sensitivity represent the 95% confidence intervals. Circle size is proportional to the weight of the study.

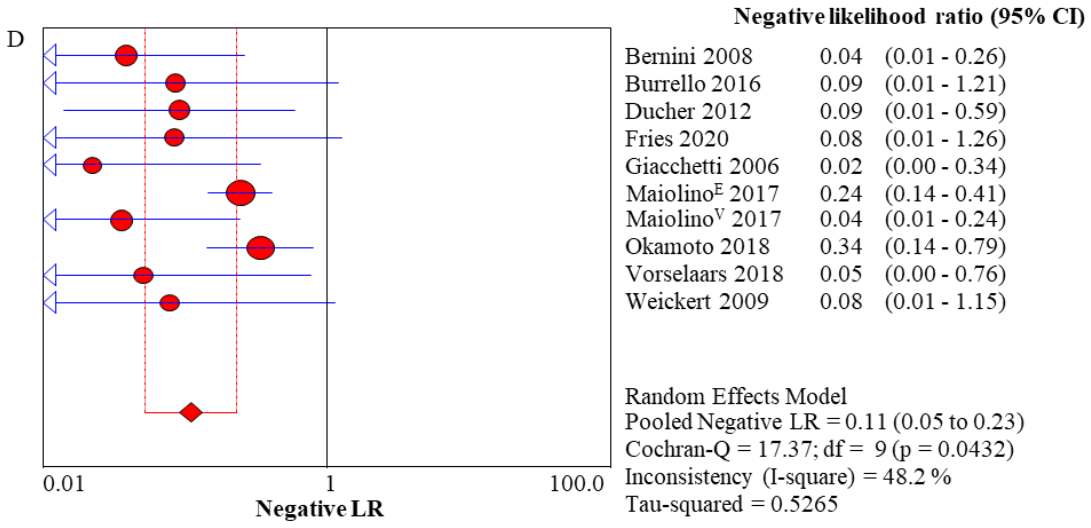

Figure 5D. Negative likelihood ratio of baseline ARR for unilateral primary aldosteronism. Error bars on the plots of sensitivity represent the 95% confidence intervals. Circle size is proportional to the weight of the study.

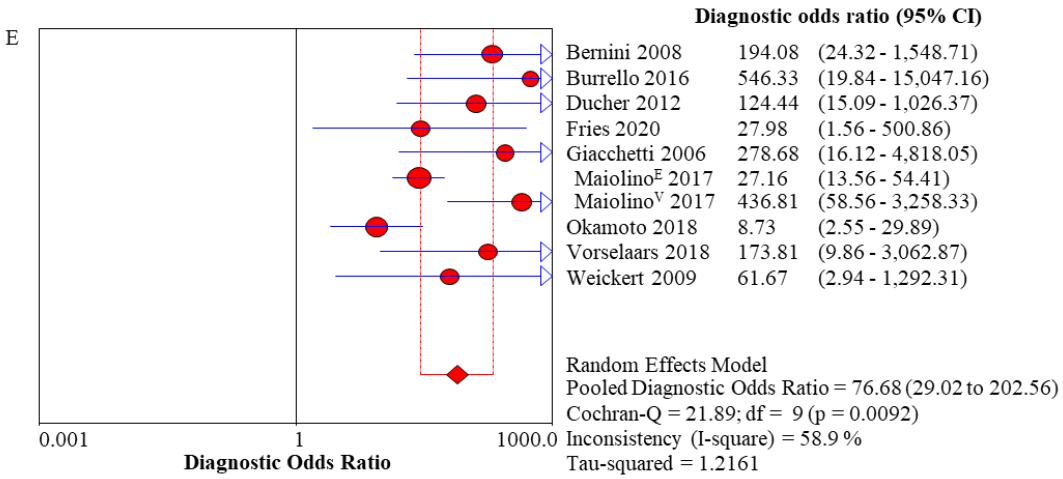

Figure 5E. Diagnostic odds ratio of baseline ARR for unilateral primary aldosteronism. Error bars on the plots of sensitivity represent the 95% confidence intervals. Circle size is proportional to the weight of the study.

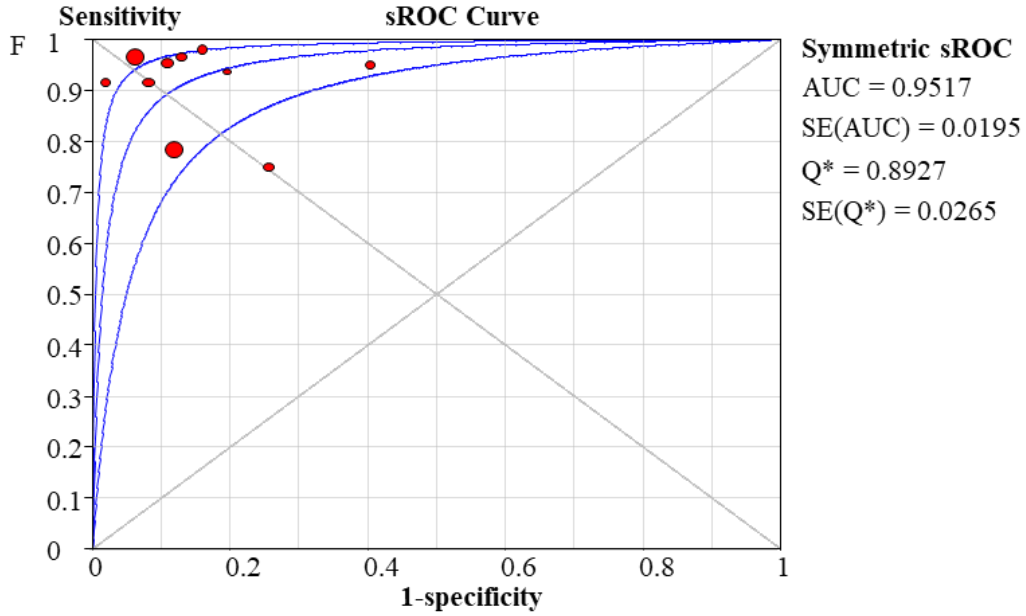

Figure 5F. sROC curve of baseline ARR for unilateral primary aldosteronism. Circles in the sROC curve are observed estimates from individual studies, each representing an independent sample of patients. Circle size is proportional to the weight of the study. sROC, summary receiving operation characteristic.

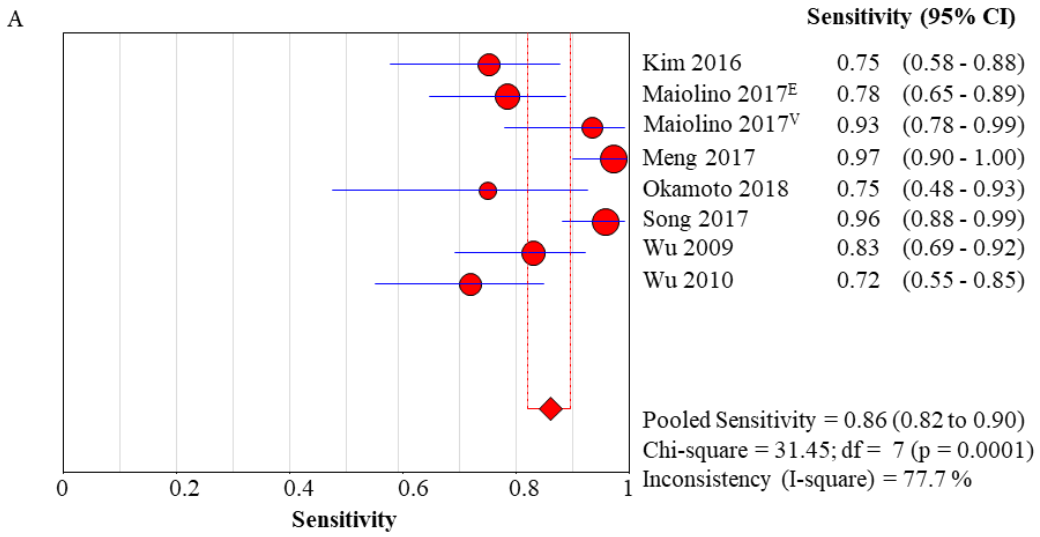

Figure 6A. Sensitivity of captopril challenge test for unilateral primary aldosteronism. Error bars on the plots of sensitivity represent the 95% confidence intervals. Circle size is proportional to the weight of the study.

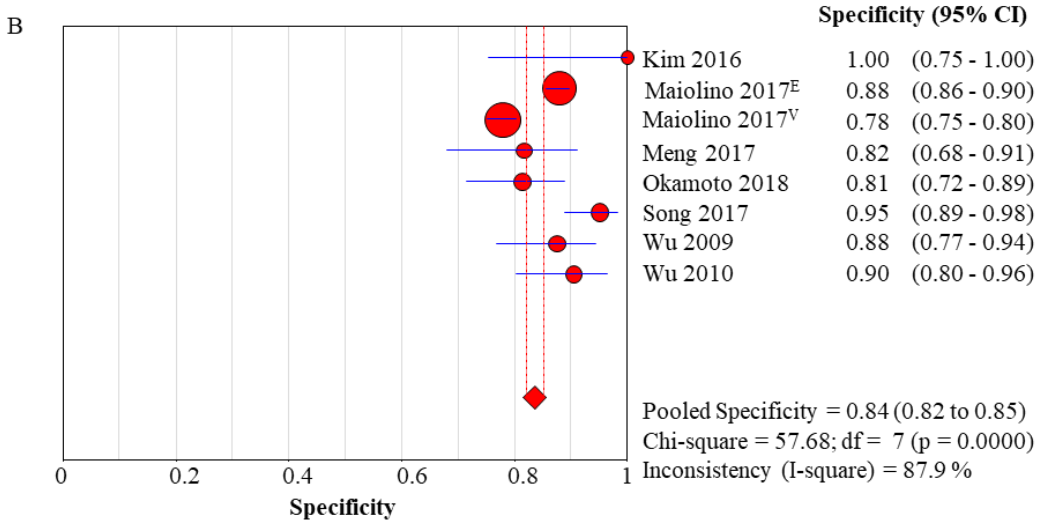

Figure 6B. Specificity of captopril challenge test for unilateral primary aldosteronism. Error bars on the plots of sensitivity represent the 95% confidence intervals. Circle size is proportional to the weight of the study.

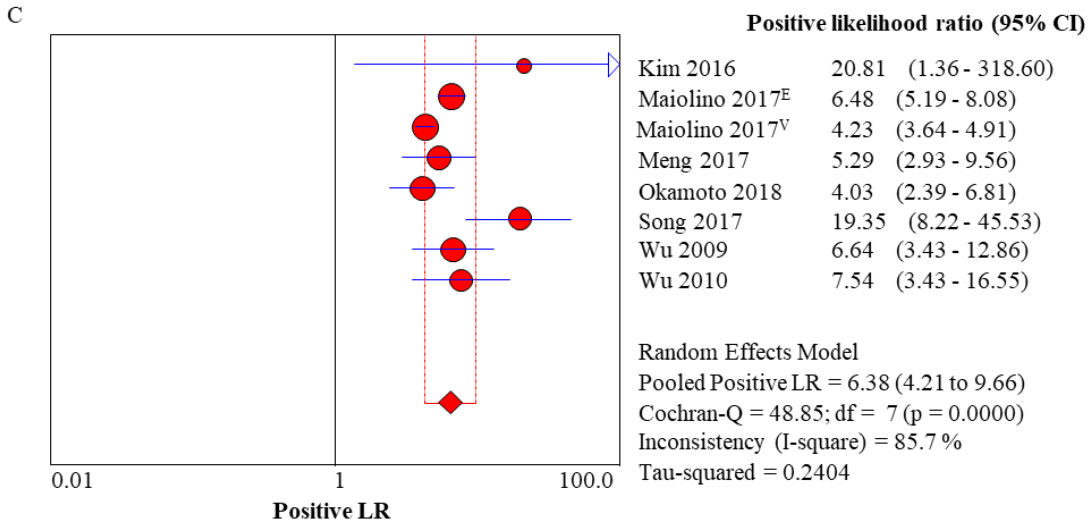

Figure 6C. Positive likelihood ratio of captopril challenge test for unilateral primary aldosteronism. Error bars on the plots of sensitivity represent the 95% confidence intervals. Circle size is proportional to the weight of the study.

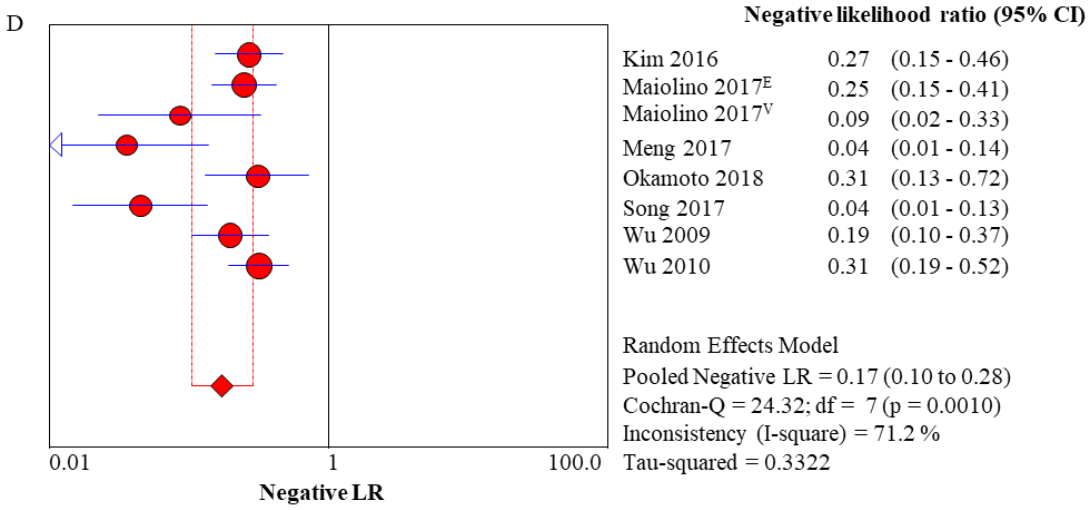

Figure 6D. Negative likelihood ratio of captopril challenge test for unilateral primary aldosteronism. Error bars on the plots of sensitivity represent the 95% confidence intervals. Circle size is proportional to the weight of the study.

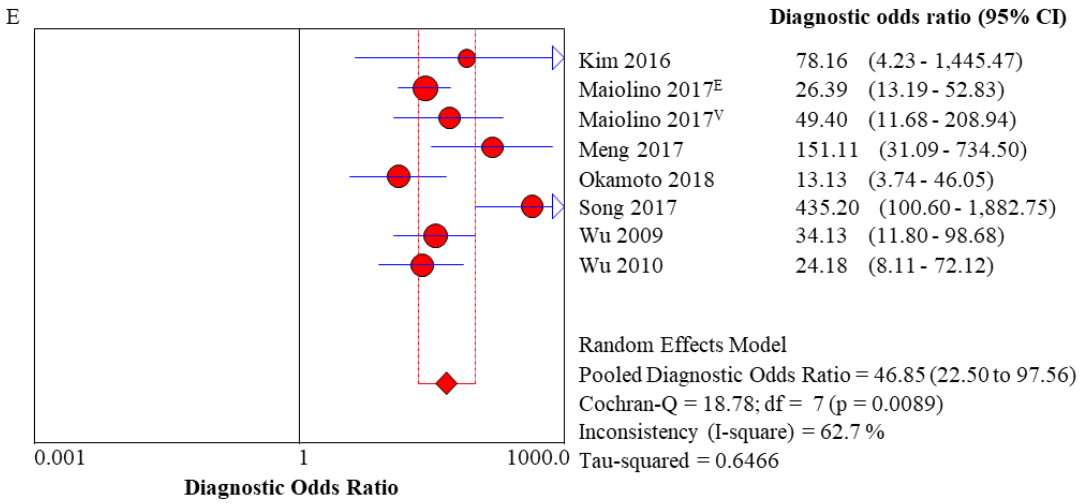

Figure 6E. Diagnostic odds ratio of captopril challenge test for unilateral primary aldosteronism. Error bars on the plots of sensitivity represent the 95% confidence intervals. Circle size is proportional to the weight of the study.

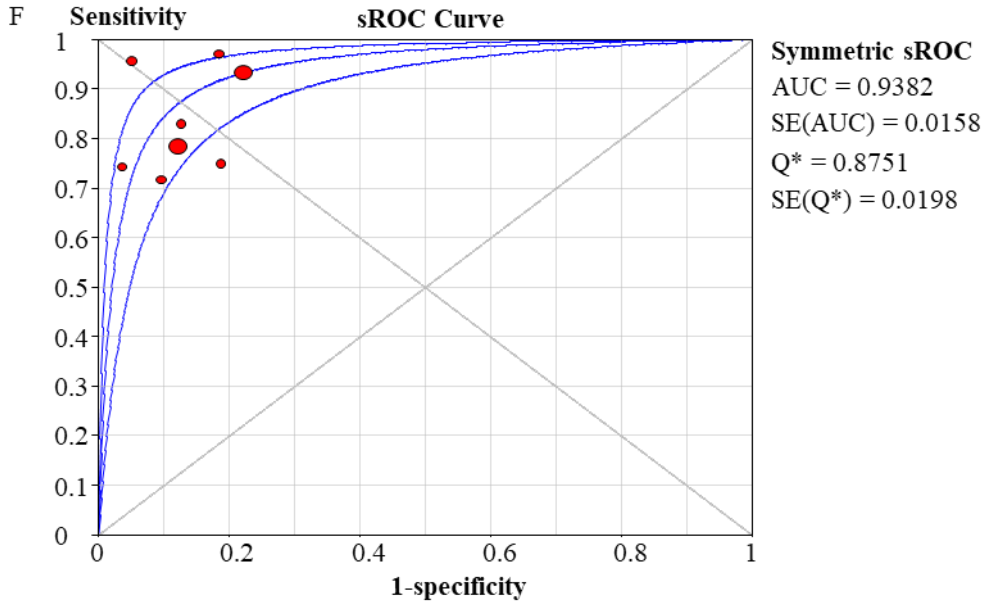

Figure 6F. sROC curve of captopril challenge test for unilateral primary aldosteronism. Circles in the sROC curve are observed estimates from individual studies, each representing an independent sample of patients. Circle size is proportional to the weight of the study. sROC, summary receiving operation characteristic.

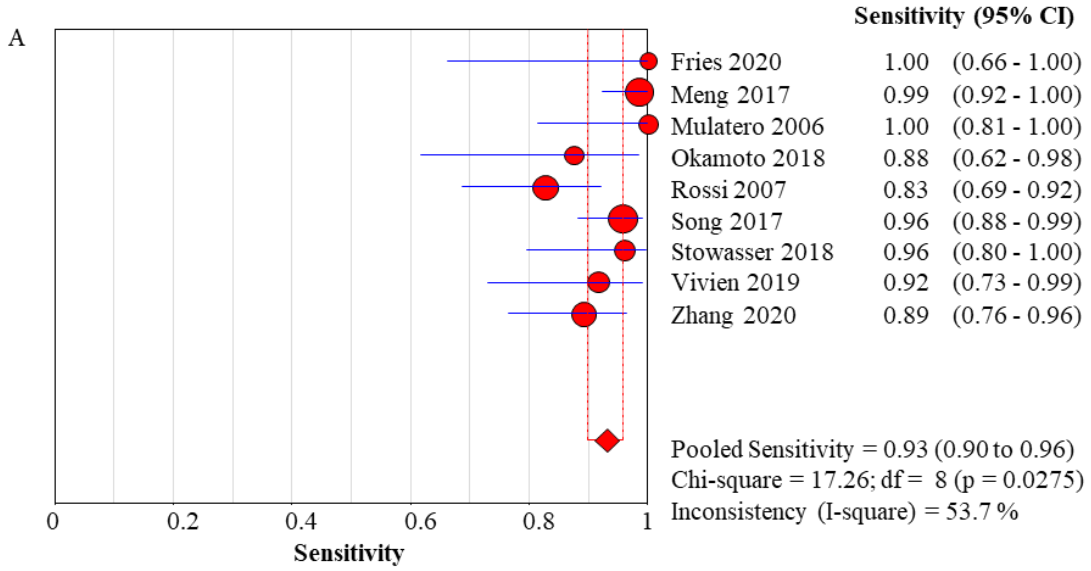

Figure 7A. Sensitivity of saline infusion test for unilateral primary aldosteronism. Error bars on the plots of sensitivity represent the 95% confidence intervals. Circle size is proportional to the weight of the study.

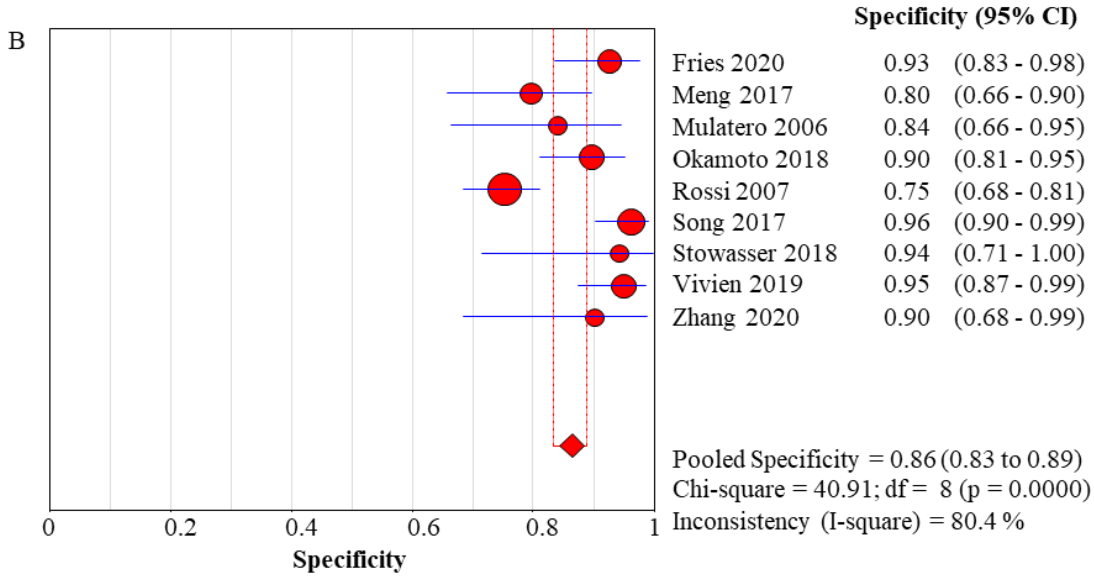

Figure 7B. Specificity of saline infusion test for unilateral primary aldosteronism. Error bars on the plots of sensitivity represent the 95% confidence intervals. Circle size is proportional to the weight of the study.

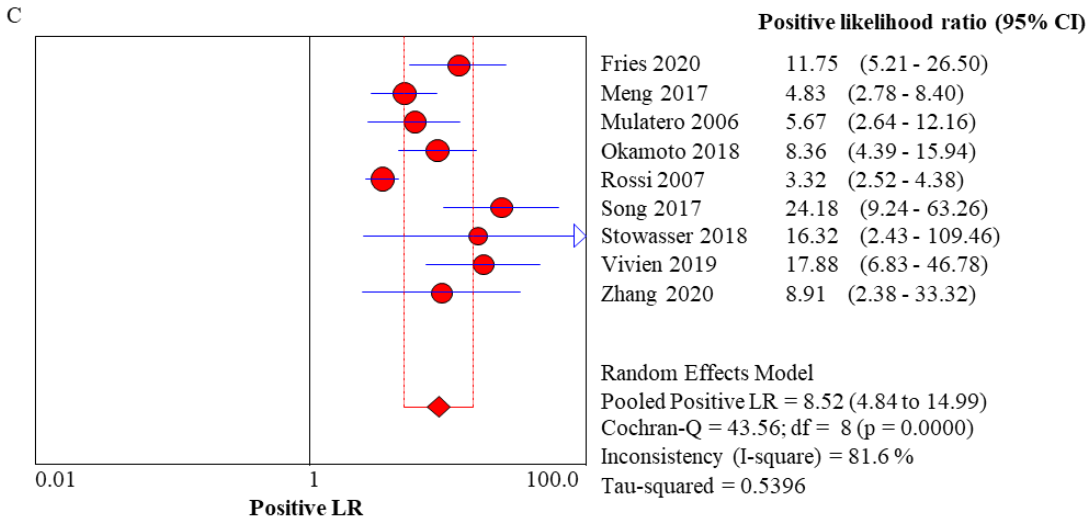

Figure 7C. Positive likelihood ratio of saline infusion test for unilateral primary aldosteronism. Error bars on the plots of sensitivity represent the 95% confidence intervals. Circle size is proportional to the weight of the study.

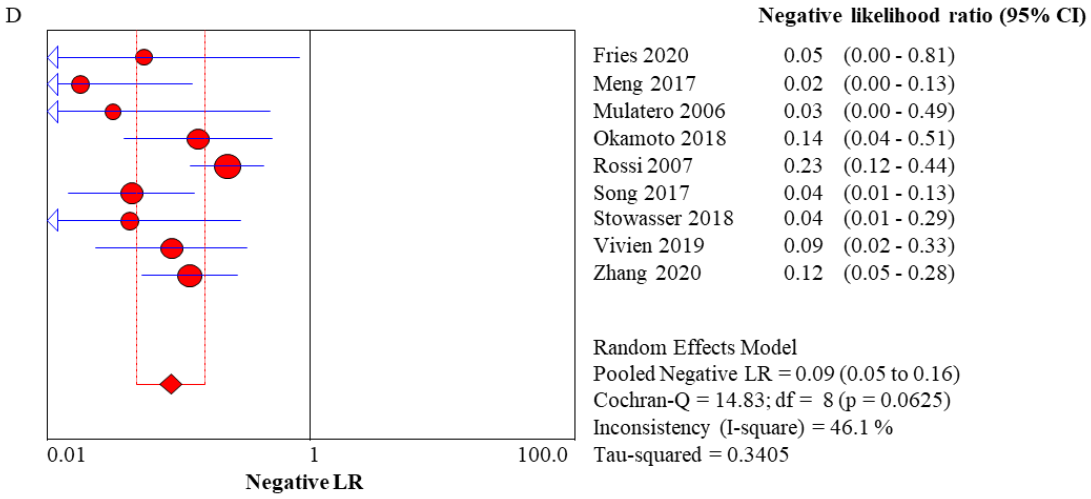

Figure 7D. Negative likelihood ratio of saline infusion test for unilateral primary aldosteronism. Error bars on the plots of sensitivity represent the 95% confidence intervals. Circle size is proportional to the weight of the study.

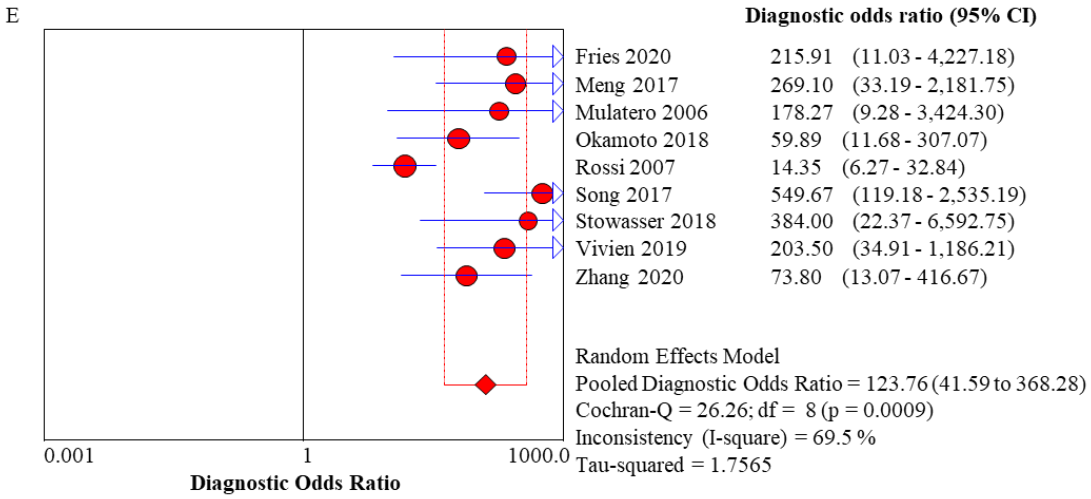

Figure 7E. Diagnostic odds ratio of saline infusion test for unilateral primary aldosteronism. Error bars on the plots of sensitivity represent the 95% confidence intervals. Circle size is proportional to the weight of the study.

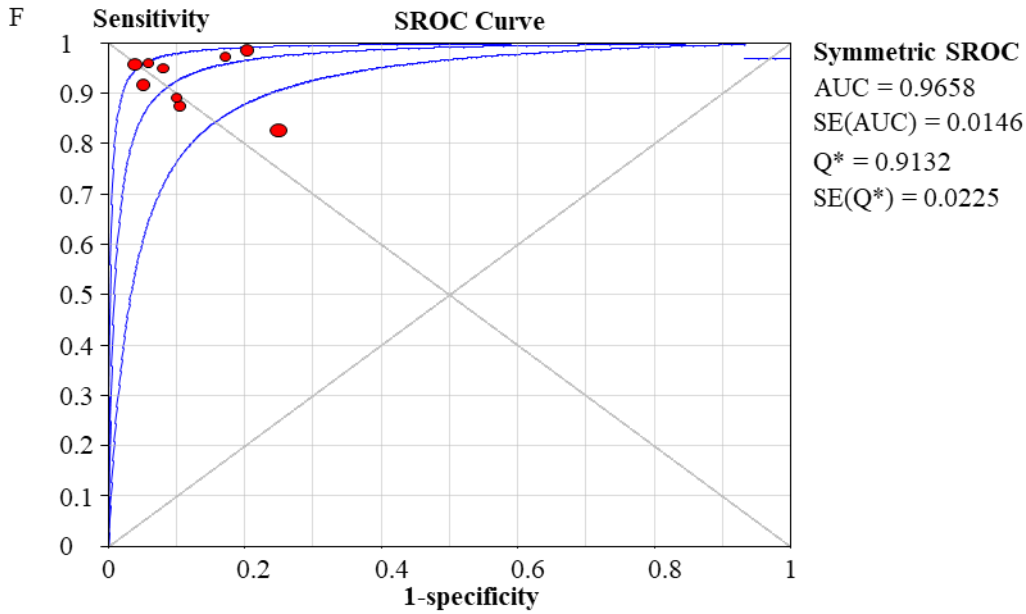

Figure 7F. sROC curve of saline infusion test for unilateral primary aldosteronism. Circles in the sROC curve are observed estimates from individual studies, each representing an independent sample of patients. Circle size is proportional to the weight of the study. sROC, summary receiving operation characteristic.

2.5.3 Meta-regression

Factors including regions (Europe/Oceania or Asia), populations (patients with suspected PA or with positive ARR), cut-off value (predefined or chosen by ROC curve), reference standard for UPA diagnosis (gold or golden) were analyzed in the meta-regression. Post-CCT variable (PAC or ARR) and posture (seated or supine) were also taken into account to explore the heterogeneity in CCT and SIT, respectively. For baseline ARR, none of these factors could explain the heterogeneity (Table 5). For CCT, post-CCT variable (PAC or ARR) might have a significant influence on the heterogeneity ($p= 0.0245$) (Table 6). For SIT, none of these factors accounted for the heterogeneity (Table 7).

Meta-Regression (1)			
	Coeff.	Std.Err	P-value
Region	-0.483	2.0764	0.8254
Population	-2.071	1.8159	0.3057
Cutoff	1.062	1.2241	0.4252
Reference	0.199	1.2733	0.8821
Meta-Regression (2)			
	Coeff.	Std.Err	P-value
Region	-0.406	1.9854	0.8446
Population	-1.943	1.5818	0.2654
Cutoff	1.138	1.166	0.3668
Meta-Regression (3)			
	Coeff.	Std.Err	P-value
Population	-2.172	0.9057	0.0476
Cutoff	1.315	0.9084	0.1911
Meta-Regression (4)			
	Coeff.	Std.Err	P-value
Population	-1.998	0.97	0.0734
Meta-Regression (5)			
	Coeff.	Std.Err	P-value
Cutoff	0.814	1.0451	0.4582

Table 5. Meta-regression of baseline ARR.

Meta-Regression (1)			
	Coeff.	Std.Err	P-value
Region	-0.761	0.7272	0.4857
Population	-0.745	0.9194	0.5664
Cutoff	-0.613	1.3114	0.7216
ARR or PAC	2.435	0.8912	0.2233
Reference	-0.230	0.7860	0.8190
Meta-Regression (2)			
	Coeff.	Std.Err	P-value
Region	-0.798	0.7158	0.3808
Population	-0.897	0.7584	0.3584
Cutoff	-0.529	1.2794	0.7194
ARR or PAC	2.472	0.8822	0.1072
Meta-Regression (3)			
	Coeff.	Std.Err	P-value
Study region	-0.931	0.6390	0.2410
Population	-1.074	0.6264	0.1850
ARR or PAC	2.253	0.7052	0.0495
Meta-Regression (4)			
	Coeff.	Std.Err	P-value
Population	-0.458	0.4626	0.3780
ARR or PAC	1.851	0.6492	0.0463
Meta-Regression (5)			
	Coeff.	Std.Err	P-value
ARR or PAC	2.005	0.630	0.0245
Meta-Regression (6)			
	Coeff.	Std.Err	P-value
Population	-0.869	0.7024	0.2709

Table 6. Meta-regression of captopril challenge test.

Meta-Regression (1)			
	Coeff.	Std.Err	P-value
Region	0.307	0.8838	0.7514
Population	1.49	1.5171	0.3983
Cutoff	-0.81	1.3515	0.591
Posture	1.958	0.9612	0.1344
Reference	-0.308	1.0919	0.7962
Meta-Regression (2)			
	Coeff.	Std.Err	P-value
Region	0.599	0.6488	0.4078
Population	1.239	0.9162	0.2478
Cutoff	-0.636	1.0026	0.5601
Posture	2.158	0.7027	0.0373
Meta-Regression (3)			
	Coeff.	Std.Err	P-value
Region	0.724	0.6182	0.2941
Population	0.794	0.5908	0.2367
Posture	1.947	0.6188	0.0255
Meta-Regression (4)			
	Coeff.	Std.Err	P-value
Population	0.925	0.6634	0.2129
Posture	1.955	0.6818	0.0285
Meta-Regression (5)			
	Coeff.	Std.Err	P-value
Posture	1.839	0.783	0.0512
Meta-Regression (6)			
	Coeff.	Std.Err	P-value
Population	0.771	1.0601	0.4906

Table 7. Meta-regression of saline infusion test.

2.5.4 Sensitivity analysis and subgroup analysis

Sensitivity analysis identified one outlier for baseline ARR, one outlier for CCT, and two outliers for SIT (Figure 8-10). After excluded, the overall results did not change significantly, suggesting the pooled results of baseline ARR, CCT, and SIT were statistically reliable (Table 8-10).

Subgroup analysis showed that the sAUCs of baseline ARR were not significant differences between each pair of subgroups (0.9382 vs 0.9811, p=0.166 between cut-off values "chosen by ROC curve" and "predefined"; and 0.9678 vs 0.9085, p=0.305 between "gold" and "golden" standard for UPA diagnosis) (Table 8). For CCT, there were fewer than four studies in each pair of subgroups, leading to the failure of subgroup analysis (Table 9). For SIT, the sAUCs were not significant differences between each pair of subgroups (0.9642 vs 0.9716, p=0.802 between "Europe/Oceania" and "Asia"; 0.9697 vs 0.9712, p=0.956 between cut-off values "chosen by ROC curve" and "predefined"; 0.9486 vs 0.9854, p=0.203 between "seated" and "supine" posture) (Table 10).

2.5.5 Publication bias

Neither Deek's funnel plot nor Deeks test showed evidence of publication bias (p= 0.73 for baseline ARR, p= 0.53 for CCT, p= 0.85 for SIT) (Figure 11-13).

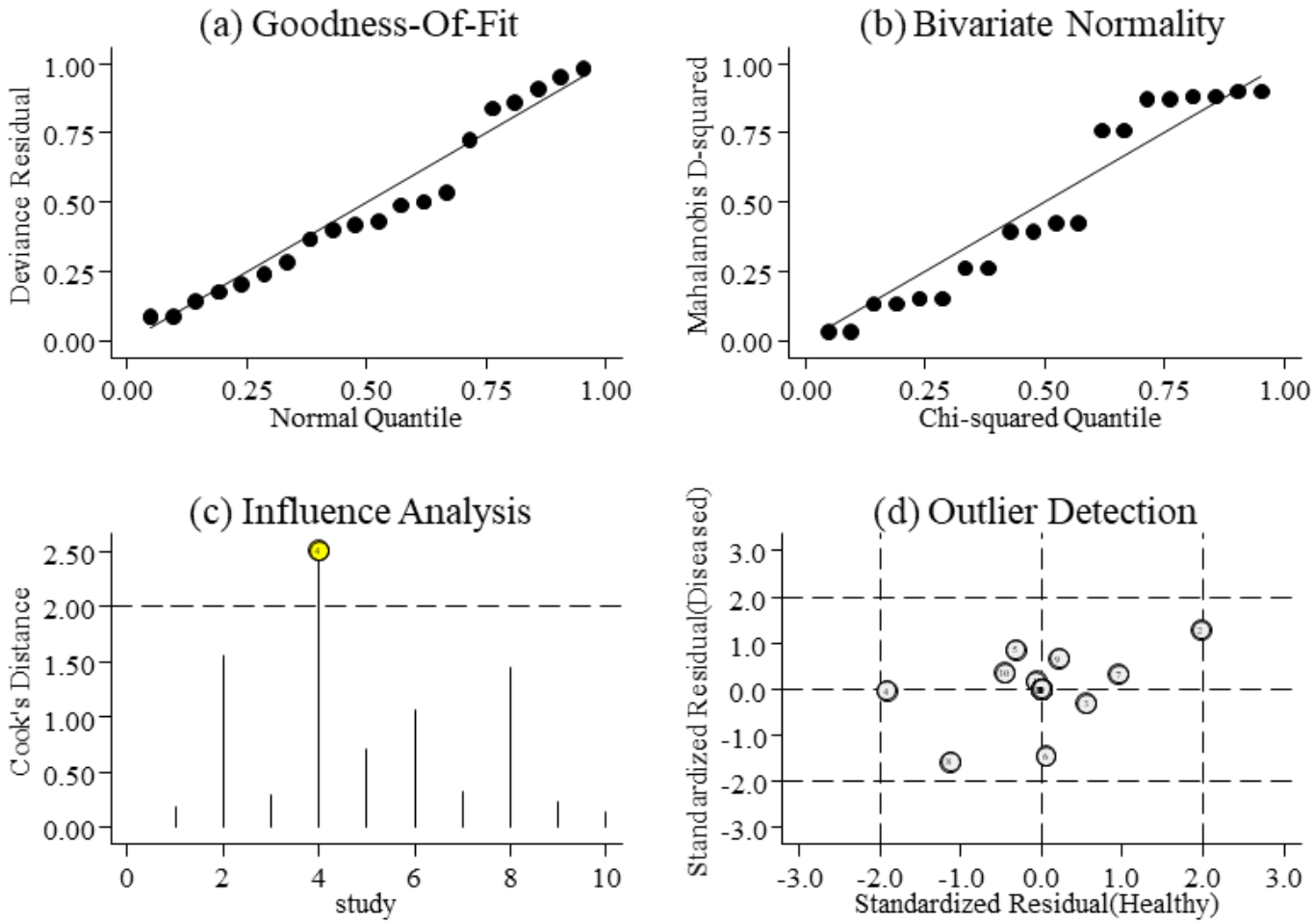

Figure 8. Sensitivity analysis of baseline ARR.

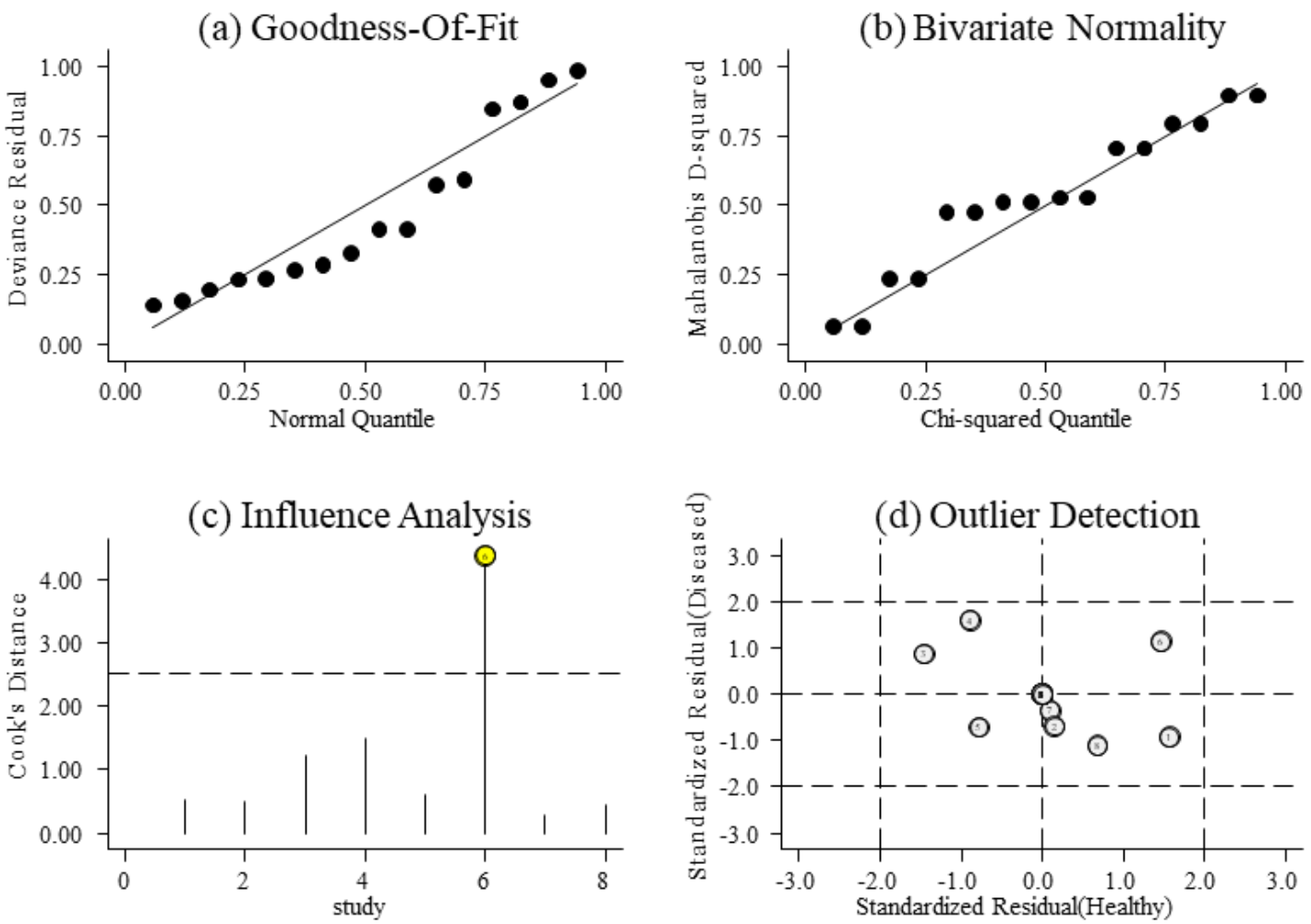

Figure 9. Sensitivity analysis of captopril challenge test.

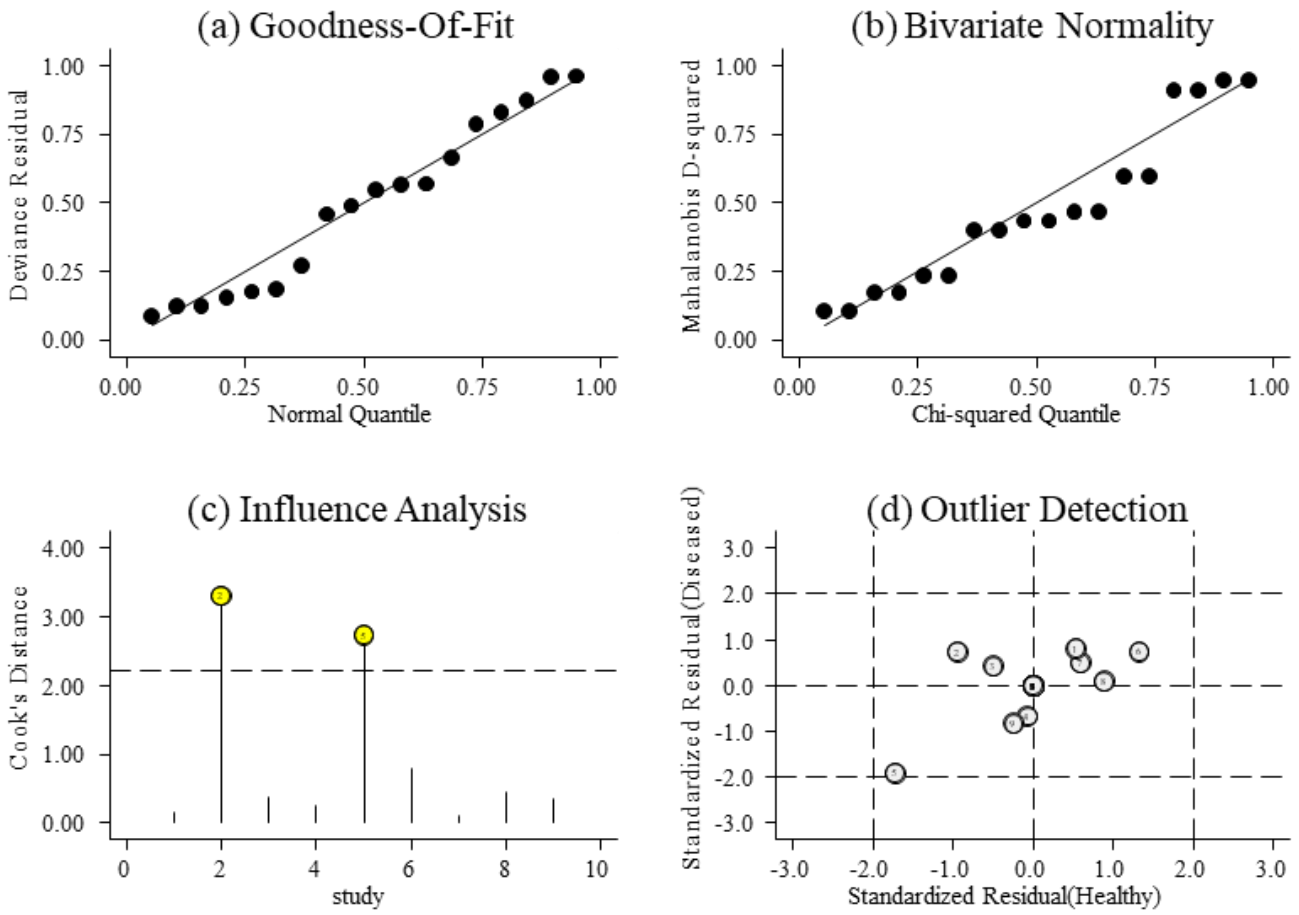

Figure 10. Sensitivity analysis of saline infusion test

Subgroups	Studies (n)	Sensitivity	Specificity	Positive likelihood ratio	Negative likelihood ratio	Diagnostic odds ratio	Summary area under the curve
Total	10	0.91 (0.81-0.94, I^2=63.5%)	0.90 (0.88-0.91, I^2=90.7%)	6.89 (4.44-10.98, I^2=91.9%)	0.11 (0.05-0.23, I^2= 48.2%)	76.68 (29.02-202.56, I^2=58.9%)	0.9517 (0.9135-0.9899, Q=0.8927)
Cut-off value							
Chosen by the ROC curve	5	0.87 (0.80-0.92, I^2=65.4%)	0.90 (0.89-0.92, I^2=90.1%)	7.67 (4.33-13.60, I^2=92.7%)	0.14 (0.06-0.34, I^2=64.1%)	60.21 (16.24-223.30, I^2=75.0%)	0.9382 (0.8810-0.9954, Q=0.8751)
Predefined	5	1.00 (0.94-1.00, I^2=0.0%)	0.85 (0.82-0.88, I^2=91.3%)	6.42 (3.04-13.55, I^2=89.6%)	0.06 (0.02-0.19, I^2=0.0%)	128.76 (33.99-487.76, I^2=0.0%)	0.9811 (0.9542-1.0000, Q=0.9393)
P-value		0.000*	0.004*	0.710	0.243	0.425	0.166
Reference standard							
Gold	5	0.90 (0.83-0.94, I^2=69.1%)	0.91 (0.90-0.92, I^2=86.7%)	10.21 (6.32-16.51, I^2=87.1%)	0.08 (0.02-0.28, I^2=59.5%)	142.25 (30.40-665.67, I^2=68.1%)	0.9678 (0.9357-0.9999, Q=0.9163)
Golden	5	0.93 (0.84-0.98, I^2=64.1%)	0.82 (0.78-0.85, I^2=89.3%)	4.63 (2.50-8.58, I^2=88.1%)	0.12 (0.03-0.40, I^2=47.6%)	43.99 (9.99-193.72, I^2=52.8%)	0.9085(0.79719-1.0000, Q=0.8405)
P-value		0.516	0.000*	0.047*	0.667	0.282	0.305
Outliner excluded	9	0.90 (0.85-0.94, I^2=65.0%)	0.90 (0.89-0.91, I^2=85.3%)	7.90 (5.30-11.78, I^2=87.2%)	0.11 (0.05-0.24, I^2=53.1%)	85.39 (29.88-244.07, I^2=63.5%)	0.9523 (0.9151-0.9895, Q=0.8937)
P-value		0.809	1.000	0.657	1.000	0.883	0.982

Table 8. Subgroup analysis for diagnostic effect estimates of baseline ARR.

Subgroups	Studies (n)	Sensitivity	Specificity	Positive likelihood ratio	Negative likelihood ratio	Diagnostic odds ratio	Summary area under the curve
Total	8	0.86 (0.82-0.90, I^2=77.7%)	0.84 (0.82-0.85, I^2=87.9%)	6.38 (4.21-9.66, I^2=85.7%)	0.17 (0. 10-0.28, I^2=71.2%)	46.85 (22.50-97.56, I^2=62.7%)	0.9382 (0.9072-0.9692, Q=0.8751)
Outliner excluded	7	0.84 (0.79-0.88, I^2=73.7%)	0.83 (0.82-0.85, I^2=86.6%)	5.44 (4.01-7.37, I^2=71.3%)	0.21 (0.14-0.32, I^2=55.6%)	31.66 (19.47-51.49, I^2=15.1%)	0.9222 (0.8979-0.9465, Q=0.8559)
P-value		0.517	0.163	0.544	0.530	0.383	0.392

Table 9. Subgroup analysis for diagnostic effect estimates of captopril challenge test

Subgroups	Studies (n)	Sensitivity	Specificity	Positive likelihood ratio	Negative likelihood ratio	Diagnostic odds ratio	Summary area under the curve
Total	9	0.93 (0.90-0.96, I^2=53.7%)	0.86 (0.83-0.89, I^2=80.4%)	8.52 (4.84-14.99, I^2=81.6%)	0.09 (0.05-0.16, I^2=46.1%)	123.76 (41.59-368.28, I^2=69.5%)	0.9658 (0.9372-0.9944, Q=0.9132)
Region							
Europe	5	0.91 (0.84-0.95, I^2=56.7%)	0.84 (0.80-0.87, I^2=84.0%)	8.15 (3.51-18.90, I^2=83.5%)	0.11 (0.04-0.25, I^2=37.4%)	103.73 (19.39-554.94, I^2=70.7%)	0.9642 (0.9127-1.0000, Q=0.9108)
Asia	4	0.95 (0.91-0.97, I^2=53.9%)	0.90 (0.86-0.94, I^2=69.6%)	9.02 (4.51-18.05, I^2=66.4%)	0.07 (0.03-0.17, I^2=49.2%)	160.21 (53.05-483.82,I^2=38.9%)	0.9716 (0.9414-1.0000, Q=0.9224)
P-value		0.177	0.027*	0.855	0.483	0.671	0.802
Cut-off value							
Chosen by ROC curve	4	0.91 (0.86-0.95, I^2=55.6%)	0.84 (0.80-0.88, I^2=89.6%)	9.40 (2.66-33.19, I^2=91.8%)	0.10 (0.04-0.27, I^2=65.3%)	99.67 (13.91-713.97, I^2=84.9%)	0.9697 (0.9205-1.0000, Q=0.9194)
Predefined	5	0.95 (0.91-0.98, I^2=52.1%)	0.89 (0.85-0.93, I^2=53.9%)	8.03 (4.87-13.26, I^2=45.4%)	0.07 (0.04-0.16, I^2=15.3%)	157.72 (61.09-407.24, I^2=0.0%)	0.9712 (0.9479-0.9945, Q=0.9717)
P-value		0.174	0.084	0.820	0.554	0.681	0.956
Posture							
Seated	5	0.89 (0.82-0.93, I^2=25.9%)	0.83 (0.79-0.87, I^2=78.6%)	7.51 (3.52-16.04, I^2=79.3%)	0.15 (0.10-0.25, I^2=6.0%)	57.87 (17.06-195.27, I^2=58.1%)	0.9486 (0.8914-1.0000,Q=0.8886)
Supine	5	0.97 (0.93-0.99, I^2=19.1%)	0.91 (0.87-0.94, I^2=77%)	9.72 (4.36-21.79, I^2=75.9%)	0.05 (0.02-0.10, I^2=0.0%)	313.99 (120.62-817.38, I^2= 0.0%)	0.9854 (0.9731-0.9977,Q=0.9480)
P-value		0.016*	0.004*	0.647	0.020*	0.032*	0.203
Outliner excluded	7	0.94 (0.90-0.97, I^2=10.2%)	0.93 (0.90-0.95, I^2=9.9%)	10.98 (7.22-16.70, I^2=28.8%)	0.08 (0.05-0.14, I^2=0.0%)	172.30 (81.92-362.40, I^2=0.0%)	0.9754(-,Q=0.9290)
P-value		0.672	0.001*	0.480	0.766	0.623	0.630

Table 10. Subgroup analysis for diagnostic effect estimates of saline infusion test.

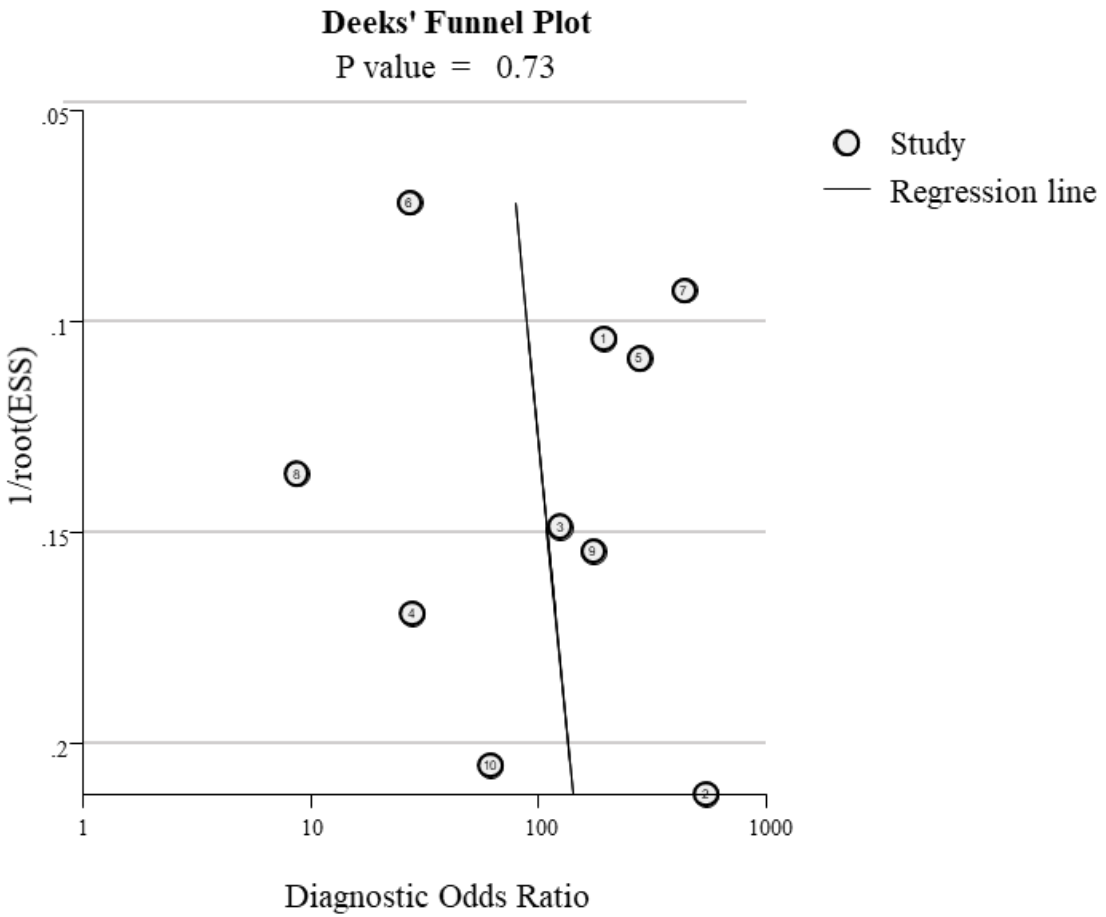

Figure 11. Publication bias of baseline aldosterone-to-renin ratio.

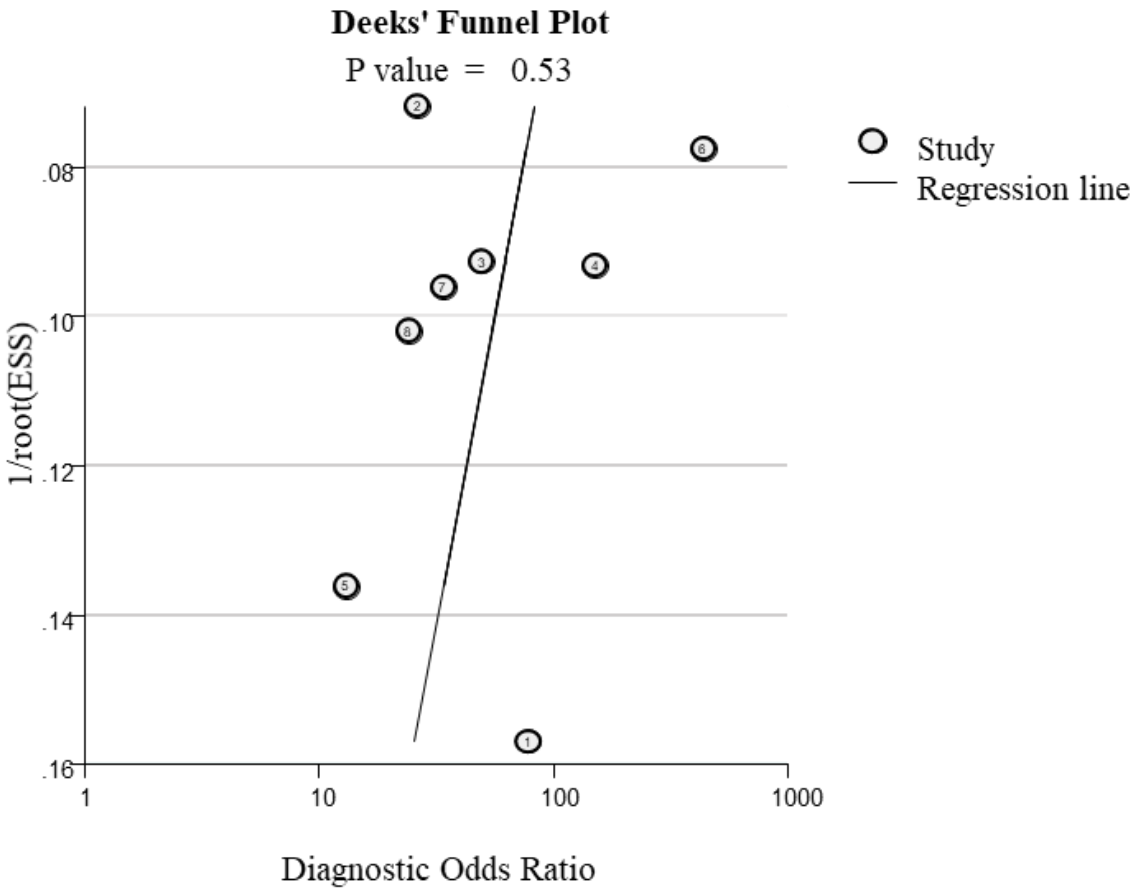

Figure 12. Publication bias of captopril challenge test.

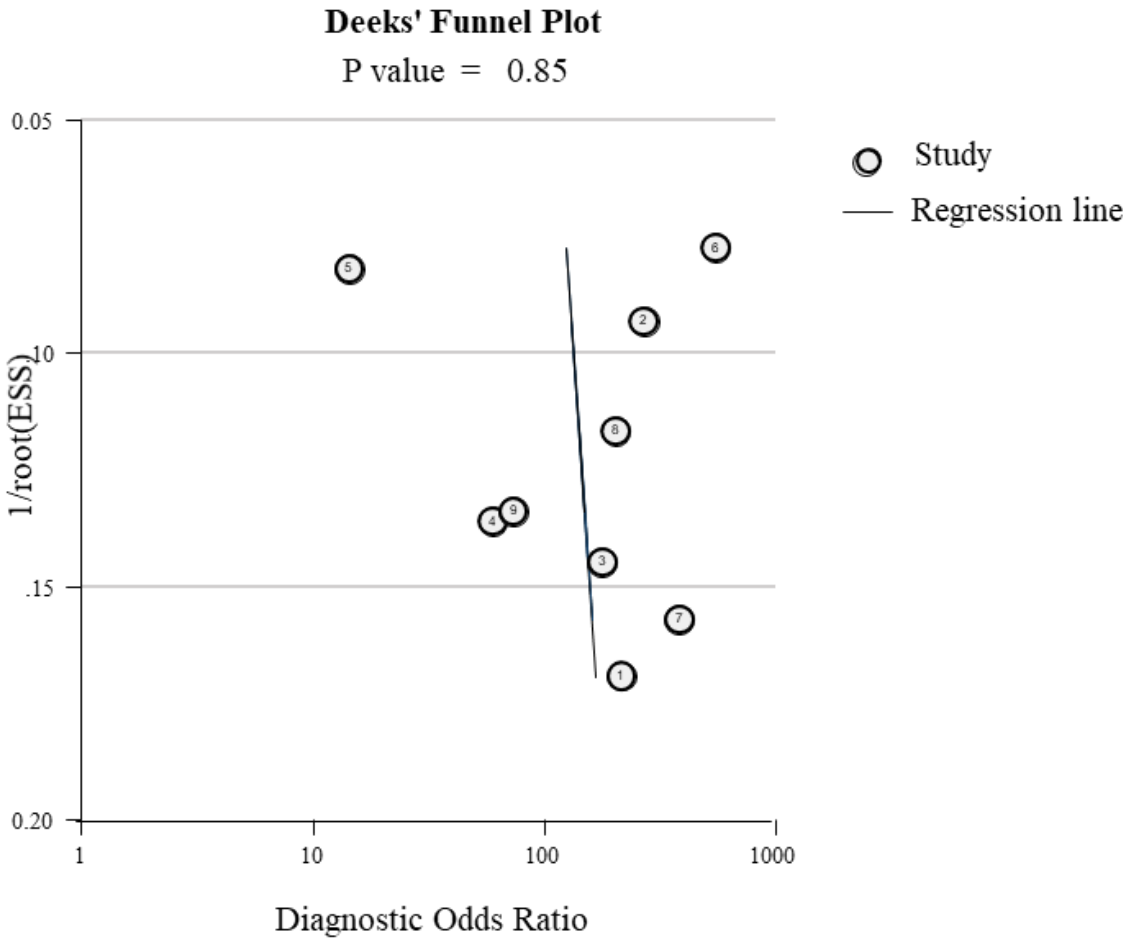

Figure 13. Publication bias of saline infusion test.

3. Discussion

This is the first systematic review and meta-analysis that used a strong methodology to assess the overall diagnostic accuracy of the baseline ARR and of the different exclusion tests for uPA diagnosis to the best of our knowledge. Strengths of the methodology used for this meta-analysis are 1) inclusion of only cohort studies; 2) accurate definition of the criteria for patient selection; 3) use of the gold standard, or when unavailable, a golden standard as the reference for identifying uPA; 4) pre-specification of the index test and reference standard.

We found that when the study results were pooled together, baseline ARR, CCT, and SIT provide high accuracy for identifying uPA. However, CCT and SIT furnished no diagnostic gain over baseline ARR.

According to the largest study by Maiolino (Maiolino, Rossitto, et al., 2017), which comprised over a thousand referred newly-diagnosed hypertensive patients prospectively both in an exploratory cohort and a validation cohort, both recruiting, with the final diagnosis of uPA by "four corners" criteria, the CCT provided no diagnostic gain over a carefully performed and interpreted ARR. The study by Okamato prospectively recruited 102 consecutive hypertensive patients with ARR greater than 20, and evaluated the performance accuracy of ARR, CCT, SIT, and FUT (Okamoto et al., 2019). The ROC curve analysis showed that PAC values at screening and after SIT had the highest diagnostic abilities of distinguishing APA from patients with EH and IHA. Multivariate regression analyses showed that PAC at screening was a strong predictor of the saline and captopril test results, and PRA at screening was

an independent predictor for CCT. Another study by Fries (Fries et al., 2020) recruited 104 consecutive patients suspected of PA, and evaluated the performance accuracy of screening test and SIT. They found that the highest accurate post-SIT PAC cut-off value measured by LC–MS/MS was 83 pmol/L (3.0 ng/dL), yielding a sensitivity and specificity of 96.9% (95% CI: 83.8–99.9) and 92.5% (95% CI: 83.4–97.5), respectively. Overlap of post-SIT $PAC_{LC\text{-}MS/MS}$ values was found between patients with EH and IHA, while all patients with APA were correctly classified. However, the fact is that $PAC_{LC\text{-}MS/MS} < 83$ pmol/L marked the lower limit of quantification (LLOQ) for reliable PAC measurement by their LC-MS/MS assay, whereas this value was still below the predicted LC-MS/MS cutoff values.

Moreover, the QUADAS-2 criteria cannot generate a summary "quality score", we developed a novel quantitative scoring method to assess the risk of bias (Table 11). This novel scoring method consists of 10 tissues in 4 key domains: study design and patient selection, index test, reference standard, and flow and timing, which can receive a score from 0 to 10 each, with an overall score maximum of 100. We plan to apply this novel scoring system to the studies that we selected and to further improve the methodology of our meta-analysis. By comparing the results of studies stratified as "high" and "low" quality based on the novel quality scores, we expect to seek for the effect of quality on estimates of diagnostic accuracy of baseline ARR and exclusion tests.

Item	Tissue	Score
Study design & Patient selection	1. If patients were enrolled consecutively or randomly, or not	0 to 10
	2. If patients were pre-selected [patients with positive and negative ARR, or pre-selected patients (e.g. with positive ARR)]	0 to 10
Index test	3. If the cut-off values of ARR and/or exclusion tests were pre-specified, or not	0 to 10
	4. If the results were interpreted with no knowledge of the final diagnosis	0 to 10
Reference standard	5. The appropriateness of the reference standard used to classify uPA	0 to 10
	6. If the results were interpreted with no knowledge of the index test	0 to 10
Flow & Timing	7. If the time interval between the index and reference standard is appropriate	0 to 10
	8. If all patients received the reference standard	0 to 10
	9. If all patients received the same reference standard;	0 to 10
	10. If some patients were excluded, and why, from the analysis	0 to 10
	Total	**0 to 100**

Table 11. Quantitative scoring method for the assessment of bias risk. ARR, aldosterone-to-renin ratio; uPA, unilateral primary aldosteronism

The analysis also demonstrated a moderate-high heterogeneity, but the source could not be found using meta-regression. Firstly, different methods of biochemical measurements were used in the included studies. At present, there are three main ways of measuring aldosterone: RIA, CLIA, and LC-MS/MS, but these measurements exhibit significant interassay variability and produce different aldosterone levels. Moreover, to calculate ARR, the measurement of renin can be performed as PRA by RIA and DRC by CLIA or LC-MS/MS, respectively. Secondly, the diagnostic thresholds to determine a positive test differed between studies, some predefined and some chosen according to the ROC curve for the diagnosis of PA or uPA. For the CCT, the

interpretation of post-CCT values (PAC or ARR) might be a source of heterogeneity. Only three studies were using post-CCT PAC values, leading to the failure of subgroup analysis. Finally, regarding the study population, there were several inconsistent factors such as race, K^+ concentration, salt intake, and uPA ratio, which might also induce heterogeneity. The performance of baseline ARR was done mostly in patients with suspected PA, and the uPA ratio ranges from 2.84% to 24.14%, whereas CCT and SIT were performed mostly to patients with positive ARR even with another positive exclusion test, and uPA ratios were from 2.84% to 73.47%, from 11.84% to 69.74%, respectively.

Besides, the performance of each test was analyzed specifically for uPA diagnosis using the gold or a golden standard, our meta-analysis is significantly limited by the small number of studies available, especially concerning FUT, FST, and OLT. Surprisingly, there was no eligible study of FST which has been considered the most reliable test for PA diagnosis. In addition, there was a methodological flaw among the included studies in determining the accuracy as a "positive" ARR and/or exclusion test result was typically followed by AVS, surgery, and biochemical/clinical assessment after surgery (reference standard) whereas a "negative" result was not. This form of verification bias might result in a substantial overestimation of test performance.

Finally, this meta-analysis revealed that the use of exclusion tests in patients with a high post-test probability of uPA, as identified by ARR values, could be unnecessary, if not confounding. A markedly raised ARR value strongly indicates the high possibility of PA, thus in our institution when the ARR is high (i.e., >45 ng/mIU) or repeatedly

elevated, the patients who seek a surgical cure are offered AVS without further testing. This is a prominent simplification of the work-up that, without endangering the diagnostic accuracy and the clinical outcome, can save a good deal of time and money.

INVESTIGATING TH17 AND TREG IN PA PATIENTS: METHODS, RESULTS, AND DISCUSSION

1. Methods

1.1 Patients and biochemical testing

Patients with PA were recruited and blood samples were obtained at 3-time points: T0, before surgery, when patients had high PAC and were not treated with MR antagonists; T1, before surgery when patients had high PAC and were treated with MR antagonists; T2, one month after surgery when patients had normal PAC and were not treated with MR antagonists. Main clinical characteristics, including systolic BP, diastolic BP, s-K^+, PAC, DRC, and ARR were summarized. PAC and DRC were measured using the commercially available chemiluminescent assay LIAISON Direct Renin Kit (DiaSorin, Saluggia, Italy) and LIAISON XL Aldosterone kit (DiaSorin), respectively, of which the accuracy has been validated in a prospective study (Rossi et al., 2016). All participants provided written consent to the study, which was approved by the Institutional Review Board of Padova University.

1.2 Preparation of PBMCs

20 mL blood samples were collected in heparinized tubes in the morning between 8:00 and 10:00 am for PBMCs isolation. PBMCs were isolated by density-gradient centrifugation using Lymphoprep™ (Stemcell Technologies, Grenoble, France) for 30 min at 2000 rpm, and washed twice with phosphate-buffered saline (PBS) by centrifugation at 1500 rpm for 10 min. The number of living cells was counted by the TC20™ automated cell counter (Bio-Rad Laboratories, Segrate, Italy). 8 x10^6 cells

were re-suspended with 400 µl staining buffer (PBS + 5% fetal bovine serum) for flow cytometry.

1.3 Flow cytometry

For Th17, 100 µL cells (2 x 10^6) were added to the bottom of each 5m L 12 x 75 mm tube. Then, 2.5 µL anti-CD4 (APC-Cy^{TM7}, clone RPA-T4) and 15 µL anti-IL23R (APC, clone 218215) were added, vortexed gently, and incubated for 20 min at RT in the dark. After surface staining, cells were washed with 2 mL staining buffer by centrifugation at 1500 rpm for 10 min. Cells were fixed using 100 µL of the intracellular fixation buffer (eBioscience, San Diego, CA) for 10 min at RT in the dark. Then cells were permeabilized with 100 µL of the intracellular permeabilization buffer (eBioscience) and stained with 10 µL anti-IL-17/IL-17A (PE, clone 41802) for 30 min at RT in the dark.

For Tregs, surface staining was performed with 2.5 µL anti-CD4 (APC-Cy^{TM7}, clone RPA-T4), and 2.5 µL anti-CD25 (PE-Cy^{TM}7, clone M-A251) for 20 min at RT in the dark. After being washed with 2 mL staining buffer, cells were fixed with 2 mL of 1X working solution FoxP3 Buffer A (BD Biosciences, San Jose, CA) for 10 min at RT in the dark. Then cells were washed and permeabilized with 0.5 mL of 1X working solution FoxP3 Buffer C (BD Biosciences) for 30 min at RT in the dark. Cells were washed and re-suspended in the residual volume of staining buffer. 2.5 µL anti-FoxP3 (PerCP-Cy™5.5, clone 236A/E7) was added for 30 min at RT in the dark.

All antibodies used were supplied by BD Biosciences, except anti–IL23R (Bio-Techne, MN, USA) and anti-IL17A (Bio-Techne). Samples were assessed on a FACSCanto

II analyzer (BD Biosciences), and the data were processed by the BD FACS Diva software program (BD Biosciences).

2. Results

2.1 Clinical features of PA patients

For this study, 23 PA patients (59% men, 41% women) aged 52±10 years were enrolled. Among them, 10, 16, and 14 were thawed blood at T0 (high PAC, without MR antagonists), at T1 (high PAC, with MR antagonists), at T2 (normal PAC, without MR antagonists), respectively (Table 12). There were no significant differences in PAC, DRC, and ARR in patients with or without MR antagonists, which was consistent with previous experimental results (Rossitto et al., 2019). One month after surgery, 43% (n= 6) of these patients were cured of hypertension, as they needed no antihypertensive medications. The post-surgery systolic BP values dropped in a highly significant manner (on average by 19 mmHg and 23 mmHg compared to T0 and T1, respectively). The post-surgery PAC values fell into the normal range in a highly significant manner which, along with normalization of serum s-K^+, demonstrated biochemical cure in all patients, meanwhile DRC increased significantly.

	T0 (n = 10)	T1 (n = 16)	T2 (n = 14)	P-value (T0 vs T1)	P-value (T0 vs T2)	P-value (T1 vs T2)
SBP (mmHg)	150±19	154±17	131±12	n.s.	<0.05	<0.001
DBP (mmHg)	89±12	90±14	86±7	n.s.	n.s.	n.s.
HR	73±6	74±9	71±13	n.s.	n.s.	n.s.
s-K^+ (mmol/L)	3.9±0.4	3.8±0.3	4.2±0.2	n.s.	<0.05	<0.01
PAC (ng/dL)	23.3 (12.8-32.8)	22.5 (10.3-49.7)	6.1 (3.2-7.8)	n.s.	<0.001	<0.001
DRC (mIU/L)	2.5 (2.0-3.5)	2.9 (2.0-3.7)	9.4 (2.0-14.0)	n.s.	<0.05	<0.05
ARR (ng/mIU)	81 (29-116)	75 (50-147)	5 (5-15)	n.s.	<0.001	<0.0001

Table 12. Clinical characteristics of patients with uPA. Data are presented as mean±SD or median (interquartile range). T0, before surgery, when patients had high PAC and were not treated with MR antagonists; T1, before surgery when patients had high PAC and were treated with MR antagonists; T2, one month after surgery when patients had normal PAC and were not treated with MR antagonists. ARR, aldosterone to renin ratio; DBP, diastolic blood pressure; DRC, direct renin concentration; PAC, plasma aldosterone concentration; SBP, systolic blood pressure; uPA, unilateral primary aldosteronism. n.s., no significant difference.

2.2 Circulating Th17 and Tregs in PA patients

Representative flow cytometry dot plots showing the gating strategy used to identify circulating Th17 and Tregs are shown in Figure 14 and Figure 16. As shown in Figure 17, the percentage of circulating Th17 ($CD4^+IL17A^+$) in PA patients was significantly lower after treatment of MR antagonists and biochemical cure after surgery ($p< 0.05$, T0 vs T1; $P< 0.05$, T0 vs T2); meanwhile, there was a decrease in pathogenic Th17 ($CD4^+IL17A^+IL23R^+$) 1 month after surgery ($P< 0.05$, T0 vs T2). Although there were no differences in the percentage of Tregs ($CD4^+$ $CD25^+FoxP3^+$) at these 3 times points, the ratio of Th17 and Tregs were markedly decreased after treatment of MR antagonists and biochemical cure after surgery ($p< 0.01$, T0 vs T1; $P< 0.001$, T0 vs T2).

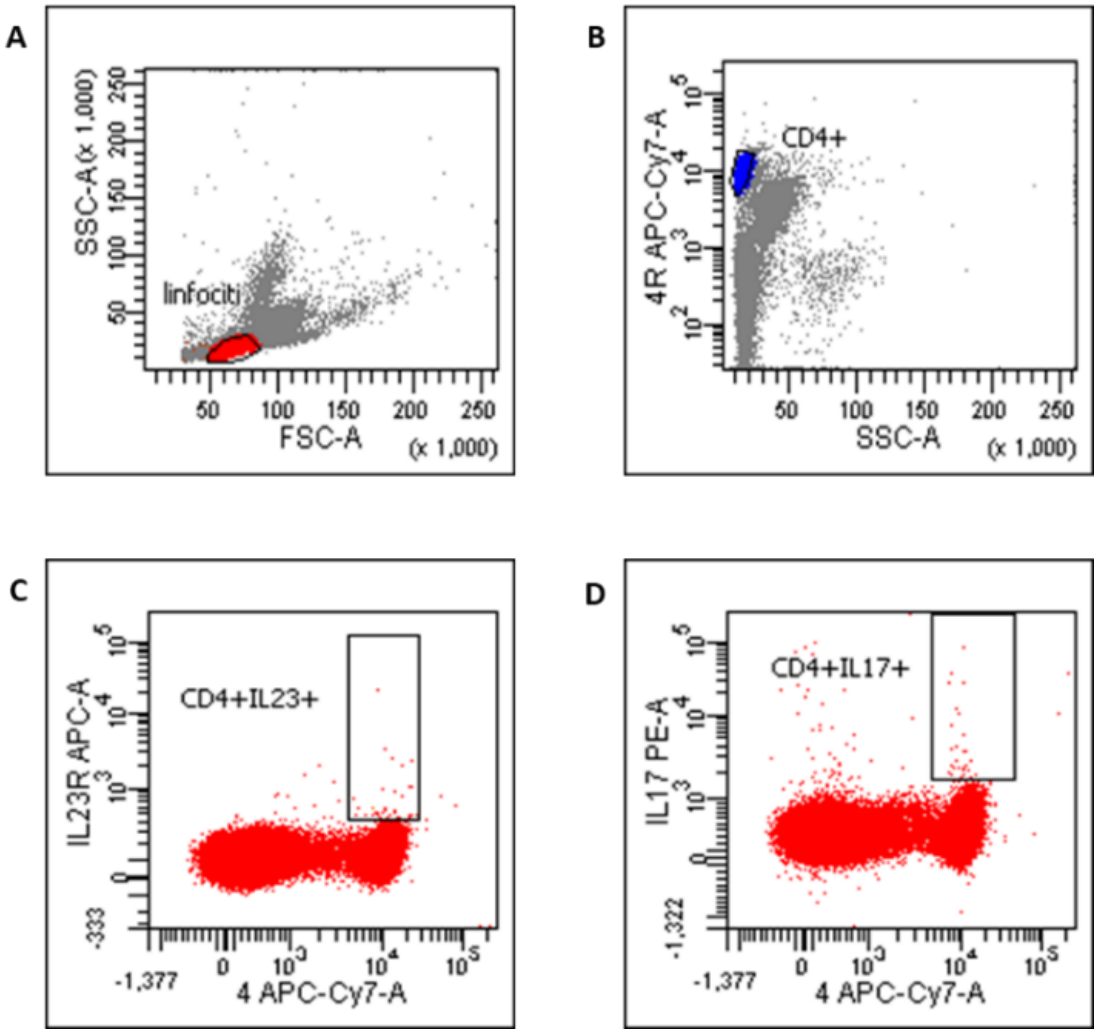

Figure 14. Representative flow cytometry dot plots showing the gating strategy used to identify circulating Th17 cells.

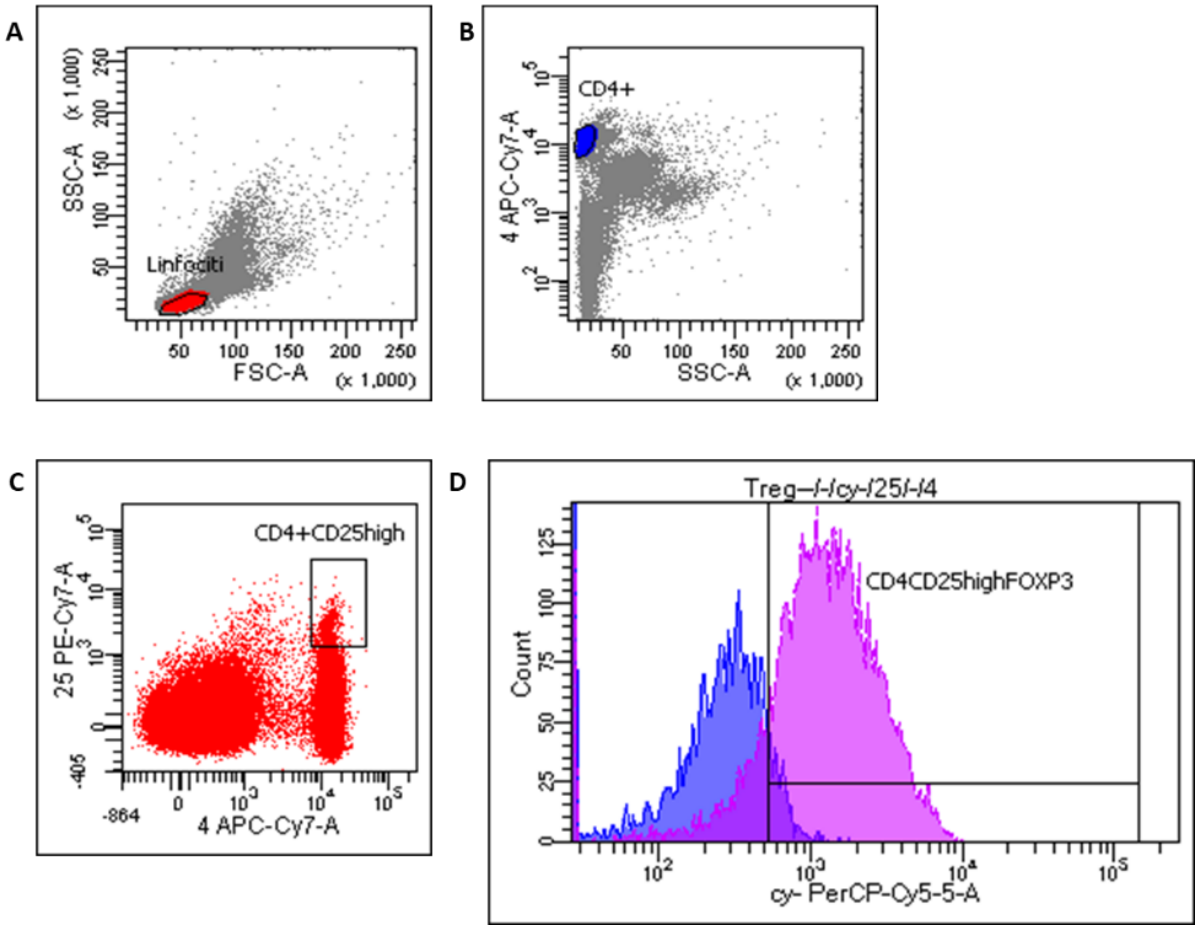

Figure 15. Representative flow cytometry dot plots showing the gating strategy used to identify circulating Tregs.

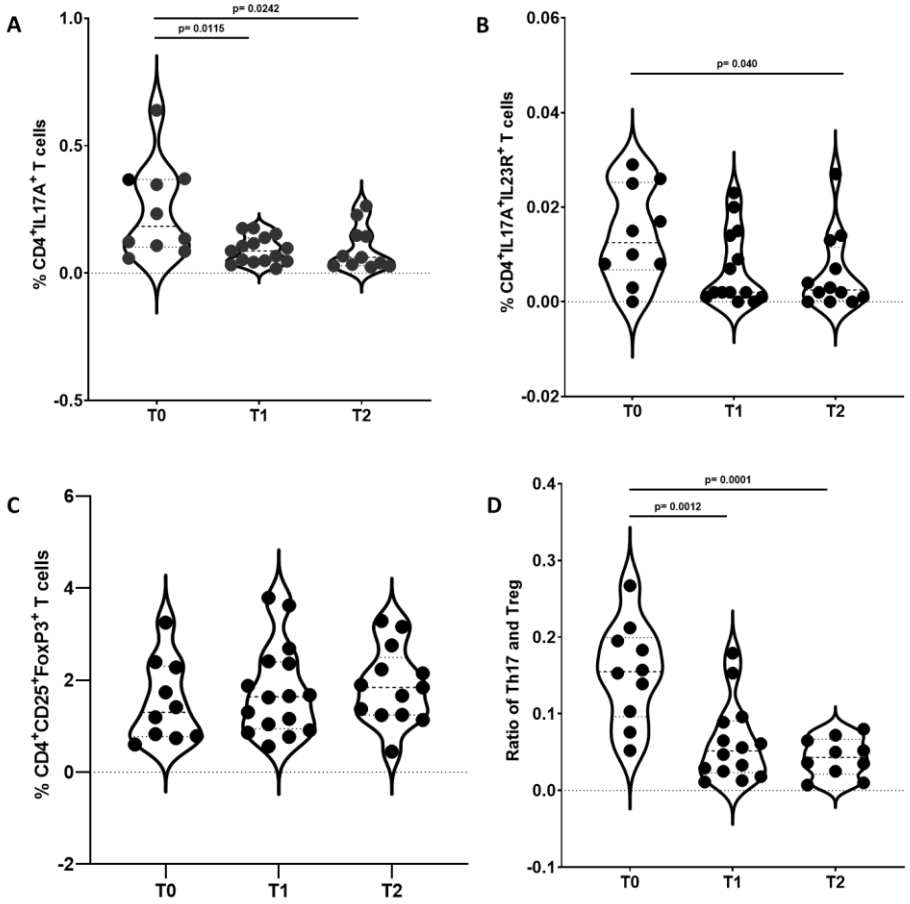

Figure 16. The violin plots show immunophenotyping of CD4$^+$ T cells from PA patients at 3 time points. T0, before surgery, when patients had high PAC and were not treated with MR antagonists; T1, before surgery when patients had high PAC and were treated with MR antagonists; T2, one month after surgery when patients had normal PAC and were not treated with MR antagonists. (A) The percentage of Th17, defined by IL17A$^+$/CD4$^+$ T cells, (B) The percentage of pathogenic Th17, defined by IL17A$^+$IL23R$^+$/CD4$^+$ T cells, (C) The percentage of Treg, defined by CD25$^+$FoxP3$^+$/CD4$^+$ T cells, (D) Ratio of Th17 and Tregs.

3. Discussion

Our current data demonstrate that treatment with MR antagonists and post-surgically cure of the hyperaldosteronism can decrease the percentage of circulating Th17 cells and the ratio of Th17/Tregs in PA patients.

To our knowledge, this is the first study evaluating circulating Th17 and Tregs, and the balance between Th17 and Tregs in the field of PA, a prototype condition of chronic MR activation. Imbalance between Th17 and Treg cells as two rival and intertwined factors has emerged as a prominent factor in the regulation of autoimmune and inflammatory disorders (Knochelmann et al., 2018).

Furthermore, our work provided evidence about the presence and functional relevance of the MR on human T cells and the effect of aldosterone exposure (at concentration commonly detected in peripheral blood of PA patients) on T cells clonal proliferation and activation. Now we are setting a protocol to investigate if and how aldosterone in vitro acts as a 3rd signal and modifies the activation and polarization of human Th17 and Tregs in the absence or presence of anti-CD3/28 mAbs.

The limitation of our current study is that a small number of PA patients were enrolled due to the inconvenience caused by the COVID-19 pandemic. Few patients were tested in all these 3 time points, especially T0 (before surgery when patients had high PAC and were not treated with MR antagonists), as the EMIRA study found that canrenone (50-100 mg/d, orally) neither lowered plasma aldosterone nor increased renin; thereby, the high ARR and true positive rate remained unaffected (Rossi et al., 2020). Given this, in our center MR antagonists have been commonly used especially

in the existence of RH and hypokalemia. In the future work, we will set up a wash-out period without MR antagonists before surgery for each PA patient. Thus, we can make within-patient comparison of Th17 and Tregs, which will offer strong evidence on the effect of MR antagonists and post-surgery chemical cure on the balance of Th17 and Tregs. Meanwhile, BP-matched EH patients with/without the treatment of MR antagonists will be enrolled to detect if these changes were due to aldosterone *per se*, independently of BP.

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