INTRODUCTION

Hypertension is a highly prevalent cardiovascular disease, which is associated with various morbidities and even mortality.\(^1\)-\(^4\) Although medications and treatment strategies are widely available, over 50\% of hypertensive patients are unable to control and normal blood pressure could be hard to maintain.\(^5\) Consequently, hypertensive adverse complications might greatly influence the quality of life and survival benefits. Nearly 90\% of hypertensive patients are primary hypertension, whereas secondary hypertension makes up an increasing...
position and is highly correlated with cardiovascular-related events, including renal parenchymal lesions, renovascular disease, Cushing’s syndrome, pheochromocytoma, and primary aldosteronism (PA).

PA is a common cause of secondary hypertension, which is characterized by significantly elevated blood pressure, autonomic, or diuretic-induced hypokalemia due to excessive mineralocorticoid receptor (MR) activation and intravascular volume expansion, with a prevalence of 5%–12% of all hypertensive patients and 17%–23% in resistant hypertension.6–8 Several confirmatory and subtype tests for PA have been proposed by the American Endocrine Society9 while interventional operations, discrepancies in decision criteria, susceptibility to subjective judgments, and risk–benefit ratios limit their clinical application. Therefore, plasma renin activity (PRA) has been recommended to be a more convenient and reliable biomarker for disease screening and is used to evaluate the necessity of further interventional confirmatory testing.

Moreover, previous studies indicated that PRA is associated with the efficacy evaluation of medications, as well as diseases other than PA, such as type 2 diabetes mellitus, Cushing’s syndrome, and atrial fibrillation.10–13 Despite its clinical value, the analytical process of PRA can be influenced by various factors, which leads to discrepant results that are difficult to assess or interpret.

In this review, we briefly clarified the relevance of PRA to PA and other common endocrine-related diseases, and we also assessed the utility of PRA as a clinical biomarker in the diagnosis and prognosis of these diseases. Meanwhile, common PRA measurement methods and factors affecting analytical accuracy were summarized, and we highlighted possible solutions for improving the standardization and consistency status of PRA measurement. This review aims to help clinicians and laboratory staff understand PRA assays comprehensively and enhance the clinical utility of PRA.

2 OVERVIEW OF PRA

PRA and direct renin concentration (DRC) play essential roles in the evaluation of circulating renin levels. DRC represents the renin concentration in plasma, while PRA reflects the renin enzyme-like activity that is required to cleave the endogenous substrate angiotensinogen (AGT) and generate angiotensin 1 (Ang1); this allows us to calculate the amount of Ang1 produced in a unit of time as PRA (unit, ng/ml/h). PRA and DRC assays provide different but complementary information about renin, so clinicians should fully understand the meaning of both assays to make correct interpretations and clinical decisions. On the other hand, some evidence indicates that there is a highly significant correlation between PRA and DRC results.14,15

Renin is the upstream component of the renin-angiotensin-aldosterone system (RAAS), which contains 340 amino acid residues (37 kDa) and is released from renal juxtaglomerular cells.16 Renin acts as an aspartate proteolytic enzyme to specifically hydrolyze AGT, which is synthesized by the liver.17,18 The product, decapeptide Ang1, has very minimal biological activity and is easily degraded by nonspecific proteases into angiotensin peptide products (Figure 1). Under the action of angiotensin-converting enzyme (ACE), Ang1 is cleaved into active angiotensin 2 (Ang2).19,20 Ang2 can directly affect blood pressure by stimulating the adrenal cortex to secrete aldosterone,21 thus promoting the retention of water and sodium. Ang2 can also bind G protein-associated receptors in vascular smooth muscle cells (type 1 receptors; predominantly AT1R),22 leading to vasoconstriction and eventually increasing the circulating blood volume and evaluating blood pressure. Circulating Ang2 levels can be as low as 90–110 pg/ml in healthy individuals23,24 and can be readily cleaved into many other small peptides in plasma.25 Accurate measurement of Ang2 is challenging for present measurement assays. Interestingly, studies have reported that there is a linear correlation between PRA and Ang2,26,27 and it is reasonable to measure Ang1 and PRA to reflect the condition of Ang2 and RAAS in a more practical manner;28 thus, accurate measurement of PRA plays a significant role in clinical diagnosis.

Prorenin is the precursor of renin. The normal concentration of prorenin is 10 times higher than that of renin.18,20 Inactive prorenin has an extra 43 amino acid pro-segment at the N-terminal when compared to renin28; this creates a closed conformation and cannot

![Figure 1](https://example.com/figure1.png)  
**Figure 1** Overview of the main components and interactions of the RAAS system.
bind to AGT. Under physiological conditions, prorenin can only be hydrolyzed to active renin in the kidney. However, in vitro environments such as cooling (−5°C to 4°C) or low pH (<4) can stimulate spontaneous unfolding of the pro-segment followed by cleavage by nonspecific plasma proteases, resulting in an open conformation and renin activation, known as cryoactivation, which may affect accurate measurements of PRA.

Aldosterone is a downstream effector of Ang2 in the RAAS, which belongs to the mineralocorticoid steroid hormone secreted by the adrenal cortex. It rapidly binds to MRs on target tissues with a high affinity and mediates sodium reabsorption, potassium excretion, and blood pressure elevation. MRs are found in many tissues, such as the kidney, brain, heart, vasculature, and adipose tissue, and binds mineralocorticoids and glucocorticoids with equal affinity. Despite being within the reference range, elevated aldosterone levels are associated with hypertension, renal, and metabolic disease. Aldosterone and PRA are usually simultaneously determined and are equally important for clinicians. Accurate measurement procedures (Joint Committee for Traceability of Laboratory Measurements, JCTLM, available at: https://www.bipm.org/jctlm/) and certified reference material (available at: https://www.cerilliant.com/) have been established and validated for aldosterone, which laid the foundation for building reference system and achieving metrologically traceable to the Système International units (SI units). As the standardization status of aldosterone gradually improved, we should also pay more attention to enhancing the reliability and clinical utility of PRA determination.

3 | CLINICAL UTILITY OF PRA AS A SPECIFIC BIOMARKER

PRA is a classical hypertensive biomarker and has undergone careful clinical validation. In this section, we present potential associations between PRA and multiple diseases and provide important insights into its clinical application.

3.1 | PA screening and diagnosis

PA is a common form of secondary hypertension, and previous studies reported that the risk of PA increased with more severe hypertension. PA patients suffer more cardiovascular incidences than essential hypertension patients with the same level of blood pressure. Therefore, early diagnosis and etiological treatment are vitally important. As recommended by clinical practice guidelines, the most sensitive assay for initial screening of PA is the plasma aldosterone to renin ratio (ARR). This ratio is calculated by plasma aldosterone and PRA, with the recommended cut-off value of 30 (ng/dl)/(ng/ml/h). Typical PA patients show increased aldosterone and suppressed PRA. ARR determination exhibits high patient acceptability and low cost that prompt considerable clinical practice and application, which has significantly raised the detection rate of PA in hypertension patients. Once confirmed, adrenal computed tomography and adrenal venous sampling (AVS) are used as subtype testing to distinguish between unilateral adrenal hyperplasia (UAH) and bilateral adrenal hyperplasia (BAH).

To normalize aldosterone secretion, unilateral laparoscopic adrenalectomy and MR antagonists are recommended to cure unilateral PA (i.e., aldosterone-producing adenoma or UAH) and bilateral adrenal disease (i.e., BAH), respectively. Surgical treatment might bring favorable clinical outcomes to PA patients; however, the suitable general condition and transient postoperative adrenal insufficiency could hamper the utility of surgical treatment; thus, medicinal therapy has shown to be a conservative and reliable alternative. MR antagonists such as spironolactone or eplerenone have been recommended as the first-line medicine in PA and resistant hypertension because of their notable survival benefit. PA patients treated with MR antagonists whose PRA remained suppressed had a nearly three times (95% CI: 2.11–3.80) higher risk for incident composite cardiovascular events and renal vascular risk, while unsuppressed PRA patients showed no significant difference compared with essential hypertensive patients. Recent study suggested that a more aggressive and efficient prescription of MR antagonists might be helpful in blood pressure control and cardiovascular protection. Therefore, PRA has proven to be an indispensable predictive and prognostic biomarker in early diagnosis and medicine guidance for PA.

3.2 | Evaluation of therapy of MR antagonists

MR antagonists can bind to MRs and block the interaction with aldosterone, and they have been one of the most widely used antihypertensive agents to mitigate the incidence of cardiovascular damage and target-organ deterioration. Patients treated with MR antagonists and whose PRA rose to unsuppressed tend to have a lower risk of cardiovascular events such as atrial fibrillation, diabetes, and stroke. Hundemer et al. conducted a large retrospective cohort study and found that MR antagonists-treated PA patients who maintained a suppressed PRA level showed a 2.5-fold higher risk for atrial fibrillation incidence than essential hypertensive patients. PRA could act as a coordinated biomarker to evaluate the sufficient suppression of MRs.

Moreover, studies indicated that spironolactone used as an add-on agent exerted beneficial effects on type 2 diabetes mellitus patients, specifically for insulin resistance. MRs and aldosterone also exert mediated functions on depression and anxiety in medically treated PA patients. With the prevalence of MR antagonists, PRA might be a useful biomarker for the efficacy monitoring of MR antagonists.

Notably, scholars mentioned that the definition of PRA suppression was binarily categorized with 1 ng/ml/h as the cut-off value, rather than a continuous numerical, which is because the sensitivity of the assay limits the detection for low PRA samples (<0.60 ng/ml/h). Thus, a sensitive and accurate quantitative measurement procedure for PRA is urgently needed in clinical decision-making.
### 3.3 Association with other endocrine-related diseases

Cushing’s syndrome, caused by excess adrenocorticotropic hormone (ACTH) or cortisol secretion, also accounts for endocrine hypertension.\(^{11,48}\) Cushing’s syndrome patients are usually treated with surgical intervention or targeted medical therapy. Medicine treatment mainly uses glucocorticoid receptor (GR) antagonists, such as mifepristone, to act as competitive inhibitors of cortisol. Under pathological conditions, normal levels of glucocorticoids can activate MRs and exert detrimental impacts on cardiometabolic and kidney damage. Interestingly, researchers\(^{38}\) observed increased mineralocorticoid release in Cushing’s syndrome patients, which implied that moderate MR antagonists as an add-on agent could be an aggressive strategy.

Obesity is also significantly related to excessive aldosterone secretion. Researchers\(^{47-49}\) found that adipocytes could secrete aldosterone-releasing factors and increase aldosterone, implying that obese patients with elevated aldosterone should consider the therapy of MR antagonists. Moreover, PRA levels are increased in primary adrenal hypofunction (Addison’s disease), secondary hyperaldosteronism such as Bartter’s syndrome,\(^{50}\) renin-secreting tumors, acute renal failure, and acute myocardial infarction\(^{51}\) but are reduced in the 11β-hydroxylase deficiency type of congenital adrenal hyperplasia,\(^{52,53}\) Liddle syndrome (pseudoaldosteronism),\(^{54}\) and 21-hydroxylase deficiency.\(^{55}\)

In summary, many studies have shown that PRA is an essential biomarker for multiple endocrine and metabolic disease screening, hypertension classification, prescription guidance,\(^{56}\) and efficacy assessment.\(^{57}\) However, PRA assays can be susceptible to preanalytical factors, and the results of different assays are difficult to compare; therefore, an accurate and comparable measurement procedure for PRA is urgently needed.

### 4 ADVANTAGES AND LIMITATIONS OF VARIOUS PRA DETECTION TECHNIQUES

#### 4.1 Immunoassay

Currently, in China, the mainstream immunoassays for PRA are radioimmunoassay (RIA) and chemiluminescence immunoassay (CLIA) according to information obtained from the national External Quality Assessment (EQA) programs organized by the National Center for Clinical Laboratory (NCCL, China) (data not shown).

RIA assays use a competitive principle to capture Ang1 in vitro to reflect the enzymatic activity of renin. Plasma was adjusted to pH 6.0 by adding a generation buffer containing angiotensinase inhibitors and was then incubated at 37°C and 4°C (blanks) for at least 90min. After incubation, the plasma was added to a 96-well plate coated with insufficient anti-Ang1 antibody; this endogenously produced Ang1 competes with fixed-dose \(^{125}\)I-labelled Ang1 overnight (16–24h), followed by a wash step, and the immobilized labeled antigen–antibody complex was sent to a gamma counter to detect the radioactivity signal. The PRA result was calculated by equation 1: 

\[
PRA = \frac{\text{Ang I}_{\text{radioactivity}} - \text{Ang I}_{\text{blank}}}{\text{Ang I}_{\text{radioactivity}}} \times \frac{1}{100}
\]

The CLIA assay is based on a chemiluminescent immunoassay. The assay adopts a sandwich structure where a 96-well plate was coated with Ang1-specific polyclonal antibodies. Ang1 in the samples competes with the added Ang1-HRP (horseradish peroxidase) reagent to bind the polyclonal antibodies, and nonspecific binding was prevented by washing. Luminescent substrate luminol was added and catalyzed by HRP, and the mixture was transferred to an immunoassay analyzer to detect the relative light unit value. This assay reduces the plasma incubation time to one hour and lowers the risk of cryoactivation; it is more automatic and has less radioactive hazard. Due to the advantages of economic and labor costs, CLIA diagnostic kits occupied an important position in PRA testing in the Chinese market.

The wide-recognized drawback of immunoassays was cross-reactivity. Ang1 is a decapeptide molecule, and there are many other angiotensin-like peptides in plasma, such as angiotensinogen, Ang2, angiotensin (1–7), etc. In the preparation of polyclonal antibodies, the interested standard material is used as the antigen to immunize animals to obtain antiserum, which is then purified by affinity chromatography to get specific antibodies. During this process, nonspecific antigen–antibody reactions may occur, such as some factors\(^{59}\) that inhibit antigen–antibody binding in the plasma, or other vascular peptides provided with similar target epitope compete for binding, thus affecting the accurate quantification of the Ang1 result. Although cross-reactivity has been assessed during the development of the immunoassay kit, however, due to the inherited reaction mechanism of immunoassays, it is difficult to completely avoid cross-reaction, especially for the low-activity samples, the immunoassays have been challenged and more sensitive measurement methods were needed.

#### 4.2 The ID-LC/MS/MS method

Despite the convenience and popularity of routine immunoassays, they suffer from several major drawbacks, including antibody storage stability, cross-reactivity, and low sensitivity (0.2 ng/ml/h)\(^{60,61}\); therefore, the highly sensitive and specific ID-LC/MS/MS method using stable isotope-labeled internal standards for PRA measurement has been developed and recognized as an appropriate alternative. There are similarities in sample preparation procedures between present ID-LC/MS/MS methods, generally including plasma incubation, sample extraction, and injection for LC-MS/MS analysis, and the main discrepancies existing...
| Sample preparation          | Mass spectrometry equipment | Chromatography conditions | Coefficient of variation (%) | Limit of Detection (ng/ml/h) | Recovery (%) | Method comparison (Compared with RIA) | Year (reference) |
|----------------------------|----------------------------|---------------------------|------------------------------|-----------------------------|--------------|--------------------------------------|-----------------|
| Maleic acid, neomycin, PMSF, SBTI, Acetate, 3 h/18h | PE-SCIEX API III          | Waters Nova-Pak C18(2×150 mm, 4 μm), 5 min | Within-day: 4.2–9.4          | Total: 7–15                  | 92(68–113)  | 0.92, minus bias:1.07 ng/ml/h           | 1999[17]          |
| Neomycin, EDTA, PMSF, SBTI, Acetate, 6.5 h/24h | Waters Xevo™ TQ           | Waters Xbridge C18(3.5 μm), 5.5 min | Intra-assay: 1.6–7.0        | Interassay: 6.6–13.9          | 87-106       | 0.98, minus bias:1.56 ng/ml/h           | 2012[62]          |
| AntiAng1 rabbit polyclonal antibody, 3 h | AB SCIEX API 5000         | Phenomenex Jupiter 4 μ Proteo 90Å (50×2 mm), 6 min | Intra-assay: 1.6–8.3        | Interassay: 7.2–9.4          | 0.005        | 93-105                                | 2012[60]          |
| EDTA, PMSF, Tris, 3 h     | Phenomenex Kinetex C18(100×2.1 mm, 2.6 μm), 5 min | Total: 5.0–5.8             | LOQ: 0.02                   | –                           | –            | 0.9296                               | 2013[16]          |
| EDTA, PMSF, Tris, 3 h     | Strata-X 33 μ polyester reversed-phase 96-well plate | Phenomenex Kinetex 4u Proteo 90Å(50×2.0 mm), 6 min | Total: 4.8–8.6              | Interassay: 4.0–9.0          | 0.103-107    | Spearman n’s correlation coefficient: R = 0.996 | 2021[64]          |

Abbreviations: ESI, Electrospray ionization; IS, Internal standard; LOQ, Limit of quantification; MAX, Strong anion exchange extraction; PP, Protein precipitation; SPE, Solid-phase extraction.

a Plasma incubation was performed at 37°C and pH = 5.5–6.0.
b Sample incubation was performed at 37°C and pH = 5.5–6.0.
c Absolute recovery.
d Spiking recovery.
e Competitive enzymatic EIA.
f Compared with laboratory developed test by themselves.
in each step involve the components and concentration of protease inhibitors, sample extraction modes, parameter optimization, and equalization between different types of apparatus.

In previous studies, Fredline et al. added phenylmethylsulfonyl fluoride (PMSF) and neomycin as protease inhibitors in plasma incubation and used a C18 solid-phase extraction (SPE) column for sample extraction. The absolute recovery of Ang1 was 67.9%, and the limit of detection (LOD) was 0.14 ng/mL/h with a total coefficient of variation (CV) of 7%–15%. In 2012, Carter et al. used four protease inhibitors for plasma incubation: neomycin, ethylene diamine tetra-acetic acid (EDTA), PMSF, and soybean trypsin inhibitor (SBTI); these were combined with high-performance liquid chromatography-online SPE, with relative recoveries of 87%–106%. The lower limit of quantification (LLOQ) was 0.39 ng/mL/h (following a 6.5 h incubation) with intra- and inter-assay CVs of 1.6%–7% and 6.6%–13.9%, respectively. In 2014, Owen et al. proposed a new off-line SPE alternative improved on the basis of Carter S et al. and validated that the measurement procedure was not restricted by a designed high-performance liquid chromatography-online SPE system and showed equal or better performances (total CV of 6.0%–7.5%); the LLOQ was as low as 0.065 ng/mL/h. In another study, Van Der Gugten et al. used anti-Ang1 antibodies in the incubation to capture Ang1 and prevent further degradation or processing by ACE or other angiotensinases, with an absolute recovery of 60%, spiking recoveries of 93%–105%, the LOQ was 0.005 ng/mL/h, and the intra and inter-assay imprecision ranged from 1.6%–8.3% and 7.2%–9.4%, respectively. The chromatographic separation of Ang1, Ang2, Ang 3, and Ang IV indicated that related metabolites will not interfere with the detection and quantitation of Ang1 in this assay. These findings showed that the ID-LC/MS/MS method greatly improves the sensitivity and specificity of PRA measurement. Details of the sample preparation, LC–MS/MS conditions, and performance characteristics of these methods are summarized in Table 1.

The previous studies have shown a good correlation between the ID-LC/MS/MS and RIA methods, although the RIA methods exhibited a certain mean bias compared with the ID-LC/MS/MS; this discrepancy could be attributed to the cross-reactivity of the antibody in the RIA assays. However, few studies have investigated the consistency among different ID-LC/MS/MS methods for PRA measurement. Chen F et al. compared the simultaneous quantification of aldosterone, PRA, and Ang2 with another ID-LC/MS/MS assay, which detects the three analytes separately. The 95% CI of biases for PRA ranged from −38.0% to 8.4%. According to 2019 national EQA programs organized in China, statistics showed that the CVs among different ID-LC/MS/MS assays (n = 8) ranged from 30%–72% for five EQA materials with a PRA concentration range of 1.1–21.1 ng/mL/h. This inconsistency may be due to (1) the lack of reference measurement procedures (RMPs) and certified reference materials that could be traceable to the SI units; (2) the lack of uniform commutable calibrators for in vitro diagnostic reagent manufacturers; and (3) the differences in sample preparation procedures, instrument parameters and reagent variations for the end-users. Therefore, it is evident that the standardization and harmonization of PRA measurement are urgently needed for accurate measurement and proper diagnosis.

## 5 | FACTORS AFFECTING THE PRA MEASUREMENT PROCESS

Similar to clinical enzymology measurements that are prone to be influenced by alterations in experimental conditions, such as operation personnel, facilities, or reagents, PRA assays could be affected by various conditions. To date, research has not been specifically designed to evaluate or summarize possible influencing factors related to PRA measurement. Table 2 presents an overview of the main contributing factors mentioned in the published literature, following by potential outcomes and necessary precautions during the entire analysis process. These are divided into preanalytical, analytical, and postanalytical considerations that specialists and laboratory technicians should take into account to achieve accurate and reliable determination.

### 5.1 | Preanalytical considerations

First, the PA guidelines recommended that before sample collection, patients should ensure a normal intake of sodium and potassium, have been out of bed for at least 2 h, and have been seated and rested for at least 5–15 min. Whole blood should be collected in EDTA potassium anticoagulant tubes to avoid stasis and hemolysis. Samples should be delivered and centrifuged at room temperature for no longer than 30 mins, and plasma should be removed and rapidly frozen at −80°C until analysis. For whole blood samples that cannot be transported to the laboratory within 2 h, it is mandatory to be kept at 0–5°C to avoid Ang1 production at room temperature over time.

The application of anti-hypertensive drugs can cause significant and long-lasting effects on the RAAS and interfere with the measurement of renin and aldosterone. ACE inhibitors, Ang2 receptor blockers, and dihydropyridine calcium channel blockers can lower aldosterone but give a rise in renin by the compensatory mechanism. Diuretics can rarely raise renin in PA patients but cause higher aldosterone in hypertensive patients. β-adrenergic receptor blockers would inhibit the secretion of renin and lower PRA and aldosterone levels. MR antagonists can raise renin and aldosterones in PA patients. Renin inhibitors (aliskiren) could block the enzymatic activity of renin and hamper the conversion of AGT to Ang1, and they were applied for hypertension control in 2007. A meta-analysis of 13,627 patients, with a follow-up time ranging from 4 weeks to 36 months, was implemented by Wang et al., who noted that compared with first-line antihypertensives (e.g., ACE inhibitors), renin inhibitors did not seem to have greater long-term risk reduction in blood pressure reduction or adverse events. When renin inhibitors were used before the PRA assays, they were replaced by protease inhibitors that were added to the incubation buffer and
| Year (Reference) | Analytical process | Effect factors | Condition | Findings and explanations |
|------------------|--------------------|----------------|-----------|--------------------------|
| 1. 2012 (62)     | Pre analysis       | Anticoagulant type (whole blood) | EDTA potassium vs lithium heparin | 1. No significant difference in PRA after 24 h incubation at room temperature ($n = 14$) |
| 1. 2004 (15)     |                    |                | EDTA, heparin, and citrate | 1. No significant difference in DRC was observed in variance anticoagulated plasma ($n = 15$) |
| 1. 2011 (41)     |                    | Diet           | Low salt intake | 1. Decrease aldosterone and increase PRA |
| 1. 2016 (9)      |                    | Collection time of day | Standing or lying position | 1. PRA peaks in the morning and drops in the afternoon |
| 1. 2012 (89)     |                    | Posture        | Standing or lying position | 1. The level of renin in the standing position is higher than that in the lying position, and the ARR in the standing position is more sensitive to the diagnosis of PA, thus reducing the false-negative rate |
| 1. 2010 (90)     |                    | Gender         | –          | 1. Women are susceptible to the influence of the menstrual cycle. During the luteal phase, the increase of estrogen inhibits the secretion of renin but only shows an increase in DRC |
| 1. 2009 (21)     |                    | Pregnancy      | –          | 1. Estrogen stimulates the liver to increase the synthesis of AGT; progesterones could compete with aldosterone at the MRs leading to an increase in PRA as a compensatory mechanism |
| 1. 1979 (91)     |                    |                | –          | 2. Elevated prorenin may remarkably increase PRA through cryoactivation at 4°C |
| 1. 2012 (62)     |                    |                | –          | 1. PRA decreases with age (>65 years) |
| 1. 2016 (62)     |                    |                | –          | 1. Decreased renin level/increased PRA levels |
| 1. 2005 (28)     |                    | Heart failure and liver disease | –          | 1. Diminished AGT levels lead to lower PRA |
| 1. 2004 (15)     | Storage temperature | Room temperature | –          | 1. At room temperature, whole blood samples for PRA analysis are more stable up to 72 h than in plasma after centrifugation |
| 2. 2005 (52)     |                    |                | –          | 2. Renin remains stable in plasma and whole blood for up to 24 h at room temperature; thus, centrifugation should be performed under room temperature; rapid freezing and thawing will not cause cryoactivation of prorenin |
| 3. 2009 (81)     |                    |                | –          | 3. PRA and DRC significantly decreased when whole blood was kept at room temperature for 2 h compared with samples kept at 0-5°C for 2 h before processing |
| 1. 2012 (62)     | 4°C                 |                | –          | 1. Most samples for PRA assays showed little effect after short-term 4°C storage (within 30 mins). Low PRA samples with higher concentrations of circulating prorenin or higher prorenin-to-renin ratio (such as pregnancy) are more easily affected and strikingly increased in PRA by cryoactivation for 6 h |
| 2. 2015 (92)     |                    |                | –          | 1. PRA decreased 15.2% after 5 weeks ($p < 0.02$) and 31.9% after 7 weeks ($p < 0.01$) when stored at −20°C |
| 1. 2009 (81)     | −20°C               |                | –          | (Continues)
| Year (Reference) | Analytical process | Effect factors | Condition | Findings and explanations |
|-----------------|-------------------|----------------|-----------|---------------------------|
| 1. 2016 (9)     |                   | Medication     | Hormone replacement therapy | 1. Estrogen medications (ethinylestradiol and drospirenone) lower DRC but increase PRA for 3 weeks  
2. Subdermal etonogestrel treatment (lack of estrogen) has an inference with both DRC and PRA for 6 weeks |
| 1. 2011 (73)    |                   | MR antagonists | (spironolactone, eplerenone) | 1. Lowers PRA levels; stop using for at least 4 weeks before PRA assays |
| 1. 2016 (9)     |                   | β-adrenergic receptor blockers | | 1. Inhibits the secretion of renin and lowers PRA and aldosterone levels; stop using for at least 2 weeks. |
| 1. 2009 (93)    |                   | Nonsteroidal anti-inflammatory drugs (aspirin) | | 1. A 24-hour reduction of PRA; stop using for at least 2 weeks |
| 1. 2016 (9)     |                   | Renin inhibitors (aliskiren) | | 1. Lowers PRA levels; stop using for at least 2 weeks |
| 1. 2016 (9)     |                   | Angiotensin2 type 1 receptor blockers; ACE inhibitors; Dihydropyridine calcium channel blockers | | 1. Increases PRA and lowers aldosterone levels; stop using for at least 2 weeks |
| 1. 2017 (30)    |                   | Anesthetics (pentobarbital) | | 1. Induces renin release |
| 1. 2020 (76)    |                   | Rifampin       | | 1. Rifampin elevated PRA and blood pressure in healthy volunteers without other medicines, one possible mechanism could be pregnane X receptor activation through the liver X receptor agonism by elevated 4β-hydroxycholesterol regulation pathways |
| 1. 1999 (17)    |                   | Sample dilution | | 1. Reduces the concentration of enzyme and substrate (Angiotensinogen) and causes the nonlinear production of Ang1, and different amounts of the substrate may cause different impacts on Ang1 generation |
| 1. 1999 (21) 2. 2012 (62) | In the analysis | Protease inhibitors | EDTA PMSF neomycin | 1. A mixture of EDTA, PMSF, and neomycin could stabilize Ang1 in plasma for up to 24 h  
2. A mixture of EDTA and PMSF (pH = 5.45–5.50) inhibits proteases for 3 h |
| 1. 2012 (62)    |                   | Incubation details | Time | 1. For low PRA samples (Ang1 < 2.46 ng/ml) this showed an excellent correlation between the results obtained after 6.5 h- and 24 h-incubation, suggesting that Ang1 production remains linear for 24 h and that protease inhibitors were still functional. For high-activity samples, long incubation times could underestimate PRA due to substrate exhaustion |
| 1. 2015 (83) 1991 (20) 2. 1975 (86) |                   | pH | | 1. pH 5.5–6 is suitable for renin activity; this is approximately twofold more enzymatically active than at physiological pH, and the pH is also most stable for protease inhibitor PMSF  
2. pH 5.7 is the optimum value for renin activity |
| 1. 1997 (94) 2. 2013 (18) |                   | Temperature | | 1. 45°C can achieve the maximum renin catalytic activity and is not affected by changes in pH and protease degradation. A higher incubation temperature and shorter incubation period could reduce the chance of prorenin activation and Ang1 degradation  
2. Renin will be degraded above 37°C |
TABLE 2 (Continued)

| Year (Reference) | Analytical process | Effect factors | Condition | Findings and explanations |
|------------------|--------------------|----------------|-----------|---------------------------|
| 2016 (50)        | Postanalysis       | Blank subtraction | Necessary or not | 1. For high PRA samples, a sufficient incubation time can reduce the fraction of blank, making blanks quantitatively irrelevant. |
| 2012 (62)        |                    |                |           | 2. The majority of samples had unmeasurable blank levels below the LLOQ (2.5 ng/ml). |
| 2010 (14)        |                    |                |           | 3. Blank subtraction led to an increase in interlaboratory CV and affected the accuracy of PRA measurement, especially for low renin plasma, thus leading to unsatisfactory reproducibility. |
| 2012 (62)        | Evaluation criterion | Reference range or cut-off value |           | 1. A reference range of 0.15–2.27 ng/ml/h (n = 100) was calculated on 100 healthy individuals. |
| 2005 (52)        |                    |                |           | 2. PRA <0.65 ng/ml/h was regarded as a low PRA status. |

Note: The 1, 2, 3 numbering of references of the left-most column entries correspond to the same numbering in the right-most column.
Abbreviations: ACE, angiotensin-converting enzyme; AGT, angiotensinogen; Ang1, angiotensin 1; ARR, aldosterone to renin ratio; CV, coefficient of variation; DRC, direct renin concentration; EDTA, ethylenediaminetetraacetic acid; LLOQ, lower limit of quantification; MRs, mineralocorticoid receptors; PA, primary aldosteronism; PMSF, phenylmethylsulfonyl fluoride; PRA, plasma renin activity; SBTI, soybean trypsin inhibitor.
and laboratory technicians need to get sufficient information on the previous medication regime. If necessary, sample collection and testing should be repeated after taking at least 2 weeks of substitute medicines with little interference with the RAAS.

The sample storage temperature exerts a significant influence on the PRA results, but there is still a lack of recognized stability protocols. Locsei et al.\textsuperscript{81} found that PRA and DRC significantly decreased when whole blood was kept at room temperature for 2 h compared with samples kept at 0–5°C for 2 h before processing. Other studies\textsuperscript{15,52} proposed that plasma or whole blood samples are stable up to 24–72 h at room temperature. The PRA sample results remained unaffected after short-term storage at 4°C (within 30 min). Cryoactivation may occur only when 4°C storage is up to 6–24 h.\textsuperscript{82} Low PRA samples with higher concentrations of circulating prorenin or a higher prorenin-to-renin ratio (such as pregnancy) are more easily affected by cooling stimuli. PRA decreased 15.2% after 5 weeks (p < 0.02) and 31.9% after 7 weeks (p < 0.01) when stored at −20°C.\textsuperscript{81} It seems advisable to keep samples below −70°C for long-term stability.

Substrate AGT levels also significantly affect renin enzymatic activity and PRA. For pregnant women or patients treated with hormone replacement therapy, physiological AGT levels are elevated and further increase PRA. In addition, when plasma is kept at room temperature for a long time, renin may hydrolyze AGT and lead to overconsumption of the substrate in advance. A comprehensive assessment of PRA plasma stability would be of great help in the reproducible and accurate determination. Notably, sample dilution should be avoided,\textsuperscript{83} which could alter the reaction rate irregularly due to a decreased concentration of enzyme and substrate.

5.2 | Analytical considerations

Ang1 can easily be degraded by nonspecific proteases; there are many complex degradation pathways and similar products that might bring unexpected bias.\textsuperscript{84} During the plasma incubation, Ang1 should be protected by serine protease inhibitors (PMSF or SBTI) and angiotensin-converting enzyme inhibitors (EDTA), or captured by anti-Ang1 antibodies in immunoassays. The selection and combination of inhibitors were based on previous research. In the early RIA established for PRA measurement,\textsuperscript{85,86} protease inhibitors including EDTA, neomycin, 8-hydroxyquinoline, and PMSF have been widely recognized and used in routine assays, which could achieve an 18h-inhibition during plasma incubation. Until the newly LC–MS/MS assays were continuously developed for PRA determination, they followed the main inhibitor regimen of RIA, in which EDTA, PMSF, SBTI, and neomycin were retained for the protection of Ang1.\textsuperscript{17} Recently, the incubation time was cut to 3 h, and neomycin (for inhibition of bacterial peptidase during longer incubation\textsuperscript{85}) was less used in routine laboratories.\textsuperscript{84} Therefore, the combination of EDTA, PMSF, and SBTI was chosen as the recommended inhibitors for LC–MS/MS analysis. However, despite the use of these inhibitors, approximately 2% of patient samples exhibit complete degradation of Ang1.\textsuperscript{58} Laboratory technicians need to recognize the degradation and reasonably interpret how these rare or extremely low PRA results occurred.

However, the reliability of PA diagnosis when PRA is under the decisive range (<1 ng/ml/h) has been questioned.\textsuperscript{9} For low PRA samples (<0.5 or 0.65 ng/ml/h), some laboratories used a prolonged incubation time (e.g., 18 h) to improve the sensitivity of RIA assays.\textsuperscript{52,62} However, a longer incubation time not only prolongs the run time but also may cause nonlinear production of Ang1 or significant negative bias due to substrate exhaustion or Ang1 degradation\textsuperscript{60,83}; similar to high PRA samples (>4 ng/ml/h), the incubation period should be limited to 3 h to avoid substrate exhaustion.\textsuperscript{21,58} For incubation temperature, 37°C is recommended as the most suitable temperature for renin activity because it is the physiological temperature of humans and could reproduce ‘real-life’ conditions. Different types and concentrations of generation buffers (i.e., acetate, tris, and protease inhibitors) were adopted as protease inhibitors in different laboratories\textsuperscript{16,17,50,62,64} (Table 1), which have different characteristics and capabilities of protection and exhibit variable renin activity in these assays. Therefore, careful evaluation and superior choice of sample incubation procedure need to be clarified further, hoping to achieve accurate and comparable quantification among different laboratories.

5.3 | Postanalytical considerations

Blank Ang1 levels are the variable amounts of Ang1 that exist before incubation. Theoretically, the blank should be subtracted from the equation. However, this proposal has been challenged by some researchers who claim that it is unnecessary to measure blanks. Several reasons may support this viewpoint. First, for high PRA samples, sufficient incubation could reduce the proportion of blanks and make the blanks negligible. Second, some studies demonstrated that the blanks are usually below the LLOQ, and blank measurement causes sample consumption and labor costs because larger sample volumes are required for two analyses.\textsuperscript{50,52,62,64} Samples with endogenous Ang1 above the LLOQ accounted for only 5.6–8.5% of the generated Ang1.\textsuperscript{50,64} Moreover, Morganti A et al.\textsuperscript{14} found that blank subtraction led to poor reproducibility in RIA assays, especially for low renin samples.\textsuperscript{14} However, we should avoid concluding whether need to perform blank subtraction only through think about the aspects of practical convenience or smaller proportion to the final results because there lack thorough assessment or persuasive evidence to manifest that abolishing blank subtraction would not interfere with the disease diagnosis or clinical decisions. More pertinent and comprehensive studies on blank subtraction are required to validate the influence of blank proportion. The bias caused from the blank measurement may contribute to poor consistency among assays. Therefore, a recognized protocol about the blank subtraction should be reached to facilitate the standardization of PRA determination, including whether blank subtraction needs to be performed and how blanks are measured properly.

Additionally, according to the survey on the LC–MS/MS assays for PRA carried by NCCL (data not shown), different calibrator sources,
material purity characteristics, and the self-prepared or commercial quality control samples highly contributed to the inconsistency among different laboratories. Conclusively, it should be highlighted that a preliminary consensus about incubation guidelines and commutable calibrators should be achieved through the joint endeavor between the EQA program provider and clinical laboratories. During the improvement of consistency, some detailed information, such as incubation inhibitor types, the kind of buffer, blank subtraction, and calibrator sources, should be offered by laboratories to facilitate the comparison or evaluation of results obtained from different assays.

Until now, there has been insufficient research to provide intra- and interindividual biological variation data or unified analytical performance specification for PRA, and a significant discrepancy in evaluation criteria was observed in the reporting and interpretation of results, such as different renin suppression thresholds (0.5 or 1 ng/ml/h) adopted in different studies. Therefore, it is necessary to establish PRA-specific biological variation, cut-off values, or reference intervals based on the LC–MS/MS method for healthy and hypertensive groups.64

To summarize, multiple factors in the entire analysis workflow could affect the consistency and comparability of PRA measurement. We can learn from the experience of enzymatic assays (e.g., ALT or AST), such as fixed reaction conditions and requirements in manual operation, which would reduce the influence of external environmental factors on the results. Therefore, there is an urgent need to ensure the optimal conditions for plasma preparation and then harmonize other factors such as sample collection, storage, and incubation procedure, hoping to reduce variation and achieve reproducible PRA determination results. Furthermore, considerations for quality assurance, such as performance specification and clinical validation, are needed in further research.

6 | STANDARDIZATION STATUS AND IMMEDIATE NEEDS FOR PRA ANALYSIS

Currently, the consistency of PRA assays remains unsatisfactory. In 2010, Morganti et al.14 conducted a multicenter comparative study involving 12 European medical laboratories regarding PRA measurement, and six plasma samples with a PRA range of 0.14 to 18.9 ng/ml/h were analyzed with the same RIA commercial kit, reagent, and unified operation instructions. The inter- and intra-laboratory CVs ranged from 15.5%–59.4% and 1.4%–16.5%, respectively. The interlaboratory variability markedly increased as the PRA level decreased. According to the statistics from the 2021 national EQA program in China (available at: https://www.nccil.org.cn/mainCn), five EQA materials with a PRA concentration range of 1.88 to 13.63 ng/ml/h were provided for participating laboratories. Intra-assay CVs ranged from 23% to 66% (n = 12) for LC–MS/MS assays and 34% to 111% (n = 47) for CLIA assays. The mean biases between CLIA assays and LC–MS/MS methods ranged from −12.1% to 76.5%. Remarkable inconsistency existed among different assays and laboratories for PRA determination.

As previously mentioned, the unified protocol for incubation can reduce the environment-induced variations in PRA assays. On the other hand, the issue of harmonization could be addressed by the reference measurement procedures (RMPs) and reference materials, that is, the standardization activities. The most significant steps for standardization are to establish a reference measurement system and achieve metrological traceability to the higher international standards.87 A reference measurement system consists of RMPs and higher-order reference materials with well-characterized traceability. ISO 17511 document defines the metrological traceability and hierarchy of an RMP, as well as detailed guidelines for measurands with different traceability.88 ID-LC/MS/MS technology with desirable sensitivity and specificity has been widely applied for establishing RMPs, and many ID-LC/MS/MS assays were also developed for PRA measurement. To date, there is still a lack of RMPs or reference materials developed for Ang1 or PRA in the JCTLM database. In 2016, the National Institute of Standards and Technology (NIST) provided a standard reference material (SRM 998) of Ang1, with a certified percentage of 99.9% and uncertainty of ±0.1% verified by high-performance liquid chromatography and nuclear magnetic resonance technologies. This reference material laid the foundation for metrological traceability to SI units. Reliable RMP and commutable calibrators with values assigned with the RMP are the prerequisites for the quantity values transferred from the reference system to routine analysis. The uniform commutable calibrators may effectively improve the comparability across different routine measuring systems and methods,65 which were considered more valuable and ranked higher in the traceability chain than commercial calibrators provided by manufacturers.88

A uniform consensus about the sample preparation procedure should be established. Plasma samples that could not be analyzed immediately should be kept at −70°C until analysis. Rapid freeze–thaw is necessary to prevent substrate consumption and cryo-activation. Plasma samples supplemented with incubation buffer containing protease inhibitors (PMSF, SBTI, EDTA) were recommended to be incubated under 37°C for 3 h. It is still controversial whether the blanks could be ignored, more thorough evaluation and unified consultation were indispensable in the future. Notably, for immunoassays, commercial diagnostic kits provide operation protocol and uniform calibrators that are traceable to manufacturer-claimed working calibrators, it is inconvenient for end-users to arbitrarily change the specific incubation procedure. However, most LC–MS/MS assays established in Laboratory Developed Test (LDT) laboratories have a more flexible manner to adjust the incubation procedure. It would be more feasible to prioritize the unified incubation protocol for LDT laboratories. After achieving the unification of incubation among these LC–MS/MS methods, it is time to appeal for immunoassays to coordinate with this harmonized standard (e.g., modifying their kits) and eventually improves the consistency among various laboratories and assays.

Assuming that when more clinical laboratories participate in national EQA programs to monitor analytical performance, professional institutions prepare reference materials and uniform commutable
calibrators, laboratories recognize and adopt uniform incubation guidelines, and appropriate bias or imprecision criteria are established for PRA, we believe that the standardization and consistency for PRA measurement are accessible in the foreseeable future.

7 | CONCLUSION

PRA is an essential biomarker for PA and other endocrine disorders which provides important information in hypertension classification, medical guidance, and prognosis assessment. However, poor consistency was observed between laboratories and assays. To improve the harmonization and standardization for PRA measurement, first, considering that PRA is easily susceptible to external conditions in the analysis workflow, a consensus about a standardized protocol for PRA sample incubation and preparation should be reached. Second, we appeal for establishing RMPs based on ID-LC/MS/MS for quantifying Ang1. Third, the adoption of commutable calibrators with values assigned by the RMP and participation in national EQA programs should be paid attention to, which help improve comparability and achieve traceability for routine measurement. Moreover, as retrospective studies and clinical validation accumulate, we believe that PRA may be widely used as a robust indicator and provide important utility for clinical application.

AUTHOR CONTRIBUTIONS
All authors have accepted responsibility for the entire content of this study and approved its submission.

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CONFLICT OF INTEREST
The authors declare that they have no competing interests.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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