Retrospective Observational Studies for Biomarker Evaluation:

Experience with Routine service for Primary Aldosteronism by LC-MS/MS

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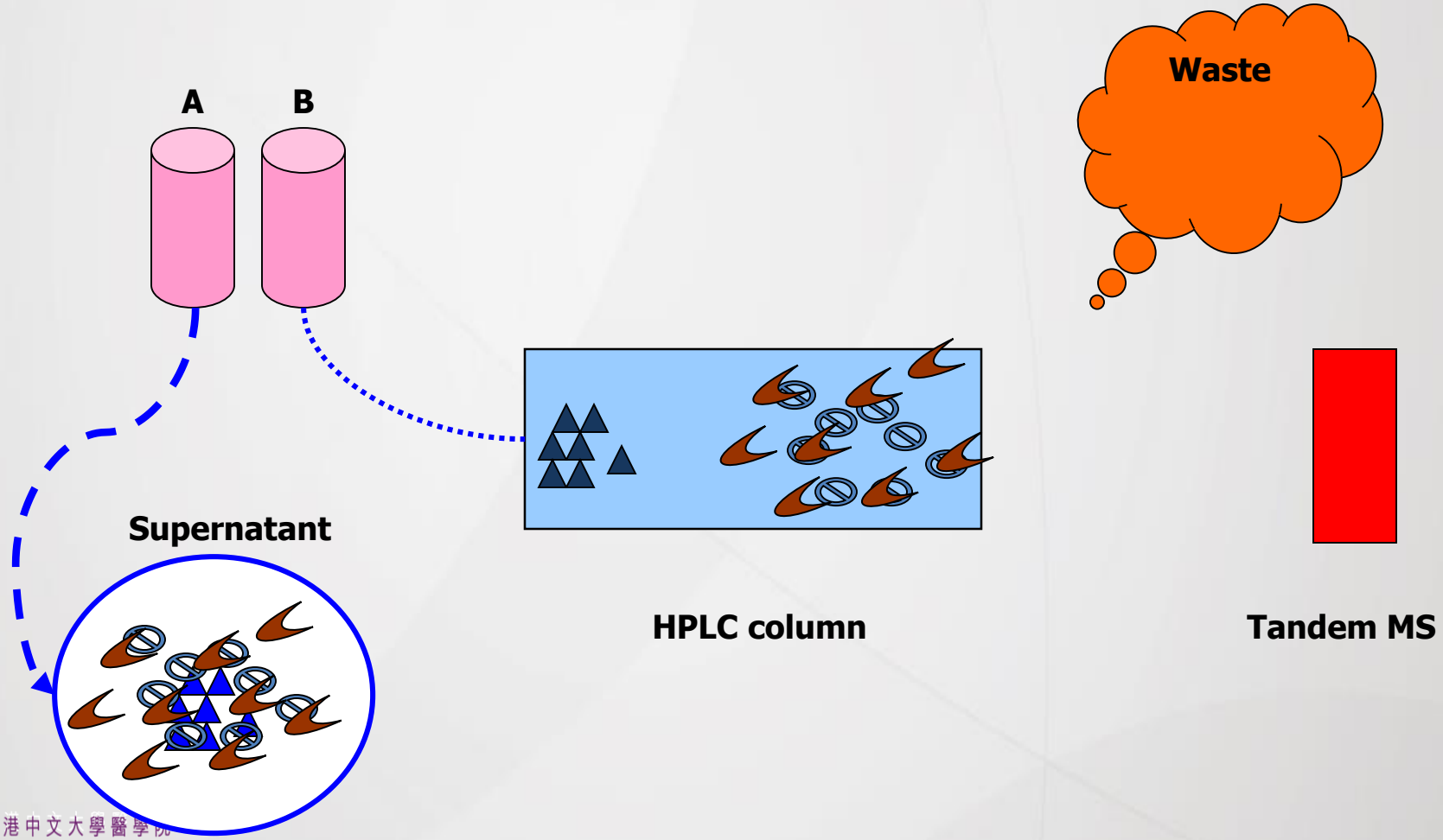
Biomedical Mass Spectrometry Unit (1995 -)

Prof. NM Hjelm

Micromass Quattro II
1995 - 2007

8 LC-MS/MS
3 LC-TOF

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

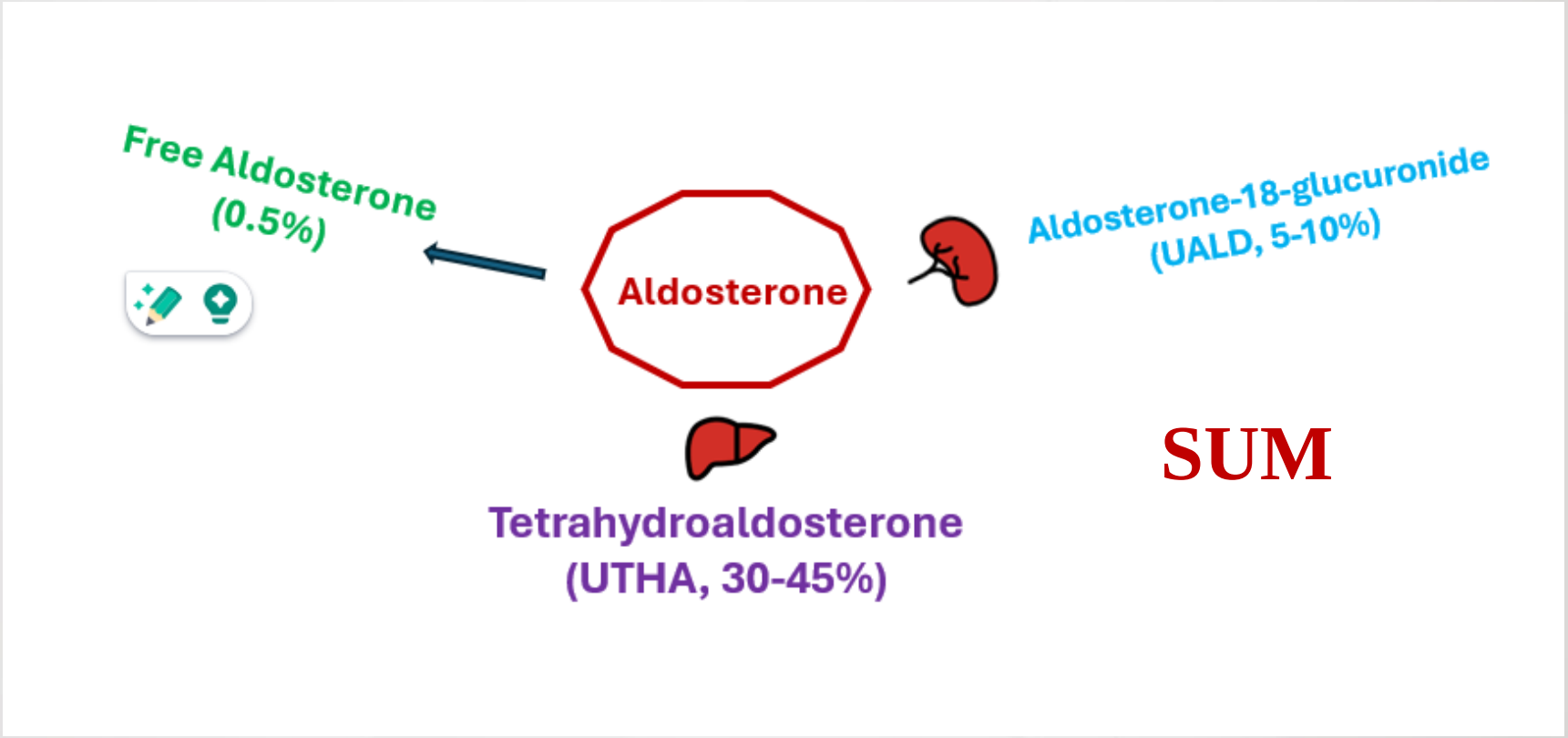


Biomedical Mass Spectrometry Unit (Historical events)

- First routine service 2003:
  - Blood Tacrolimus
- Second routine service 2004:
  - Serum 17OH-progesterone
- …

- Closing down the RIA laboratory 2013:
  - Plasma renin activity (PRA) + aldosterone (PALD)
  - PALD/PRA ratio (ARR)
  - Urine aldosterone (UALD) + Tetrahydroaldosterone (UTHA)

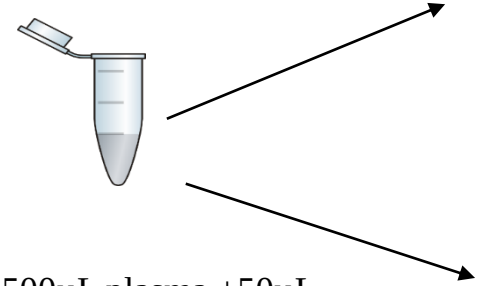
Excretion of Aldosterone Metabolites



PRA Sample preparation

Background angiotensin I

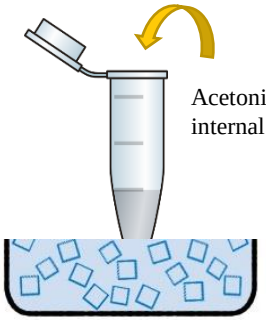
4°C



500µL plasma +50µL generation buffer

Angiotensin I generated

37 °C



Acetonitrile+ 10%FA+ internal standard

Ice bath

Stop reaction and sample extraction

3 hours

Pro-renin

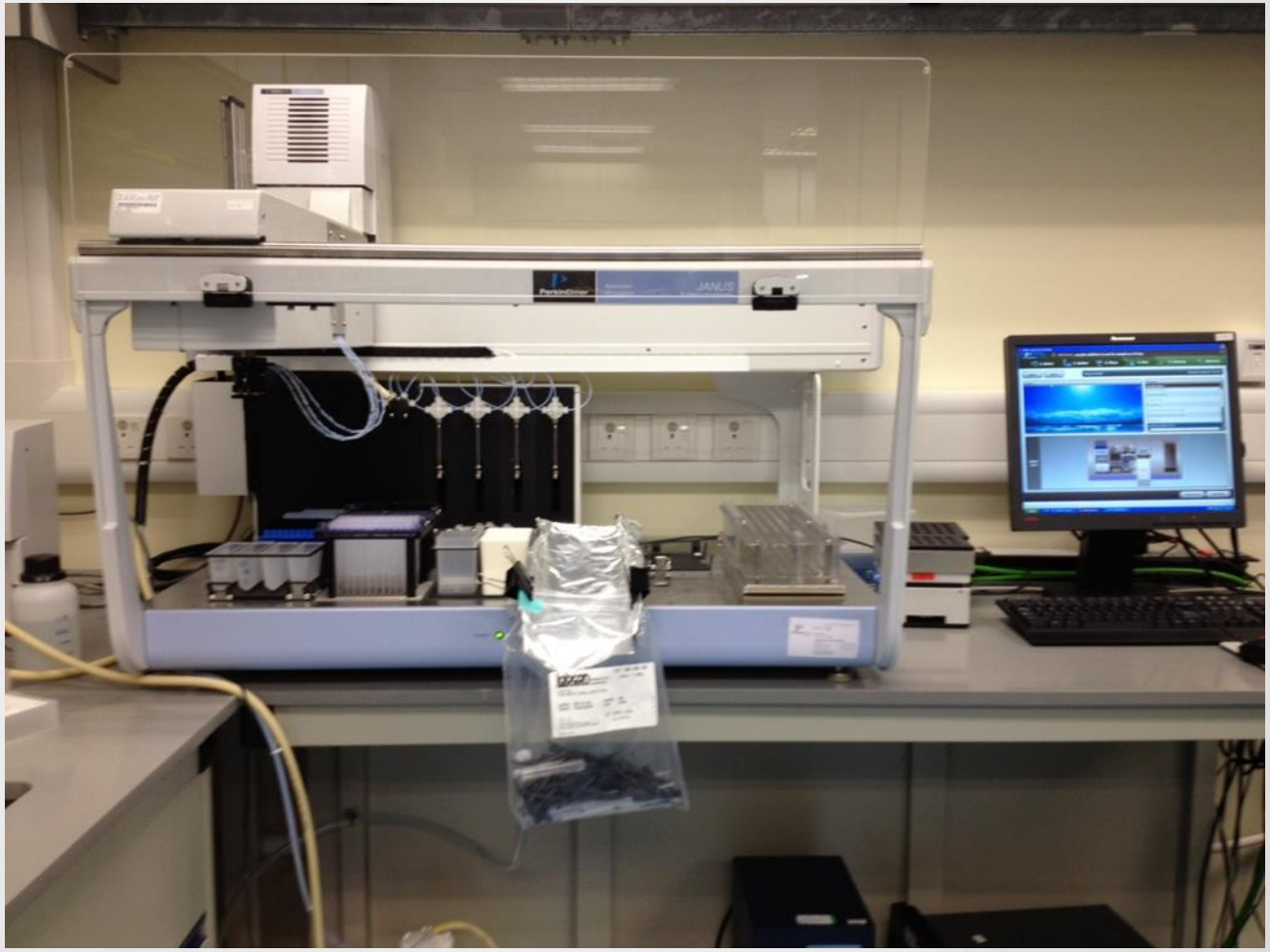
Angiotensinogen → Renin at pH6.1, 37 °C → Angiotensin I

Falsely low PRA

Angiotensin I → Angiotensin 1-3

Endogenous peptidases

Semi-automated sample preparation



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40 samples per batch

PALD, UALD & UTHA sample preparation

Procedures	PALD	UALD/ UTHA
Glucuronidase digestion		Y
MBTE extraction & freeze at -80 C for an hour	Y	Y
Remove MBTE & reconstitute with 90% methanol	Y	
Remove MBTE & reconstitute with 10% ACN		Y
Hexane wash	Y	
Remove methanol & reconstitute with 10% ACN	Y	
Ready for LC-MS/MS analysis	Y	Y

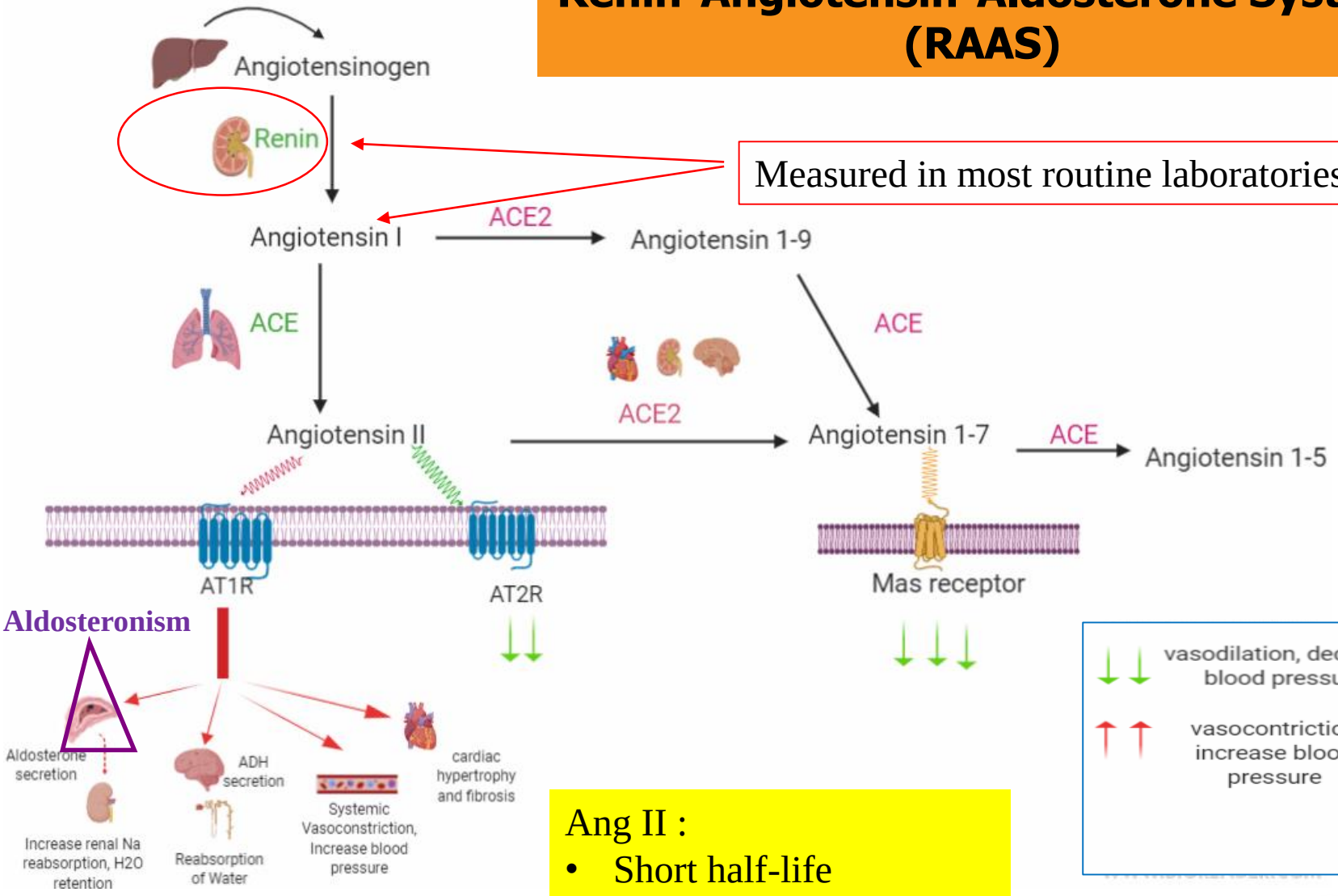

Acquity
Acquity
Xevo

Performance of different biomarkers

Indicators	PRA	PALD	UALD / UTHA
Electrospray ionization	Positive	Negative	Negative
Injection-to injection time (min)	12	9	9
Lower Limit of Quantitation	0.07 ng/mL-h (0.2 ng/mL Ang I)	50 pmol/L	0.5 nmol/L
Linearity	500 ng/mL Ang I	5160 pmol/L	2777 nmol/L
Between-batch precision (CV%)	<9.2	<5.0	<5.2

Carryover, matrix interference:
Not significant

Renin-Angiotensin-Aldosterone System (RAAS)



Ang II :

- Short half-life
- Low concentration
- difficult to be measured

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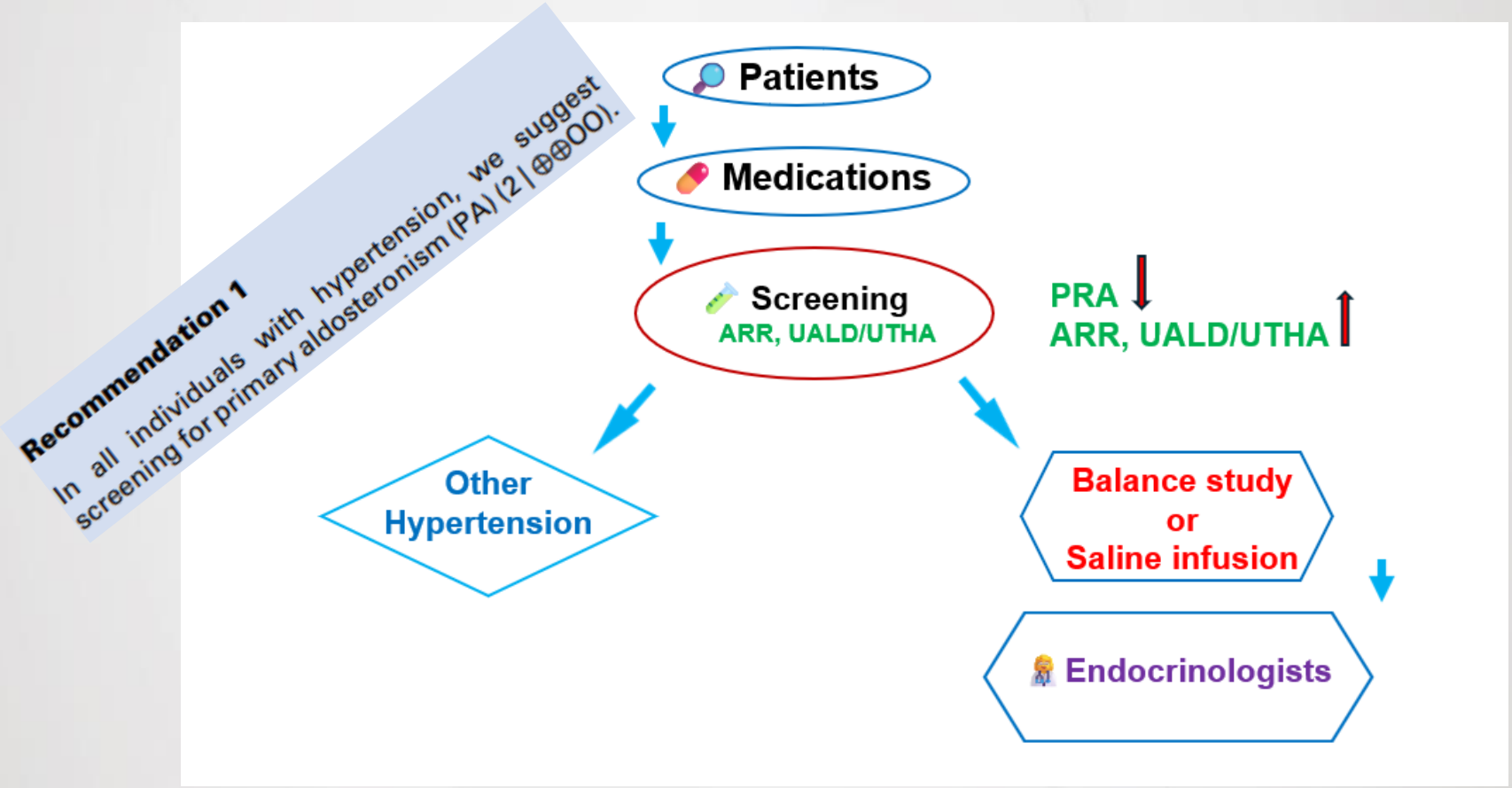
Aldosteronism

- Primary aldosteronism (PA)
  - High prevalence among hypertensive patients
  - Increased cardiovascular morbidity and mortality compared to essential hypertension
  - Treatable (adenoma by surgery, bilateral hyperplasia by medication)

- Secondary aldosteronism
  - Renal artery stenosis/renin-secreting tumor
  - Increase PRA and PALD

Routine workup for PA



Primary Aldosteronism: An Endocrine Society Clinical Practice Guideline (JCEM 2025)

Evaluation of biomarkers by LC-MS/MS for Primary Aldosteronism workup

RETROSPECTIVE OBSERVATIONAL STUDIES

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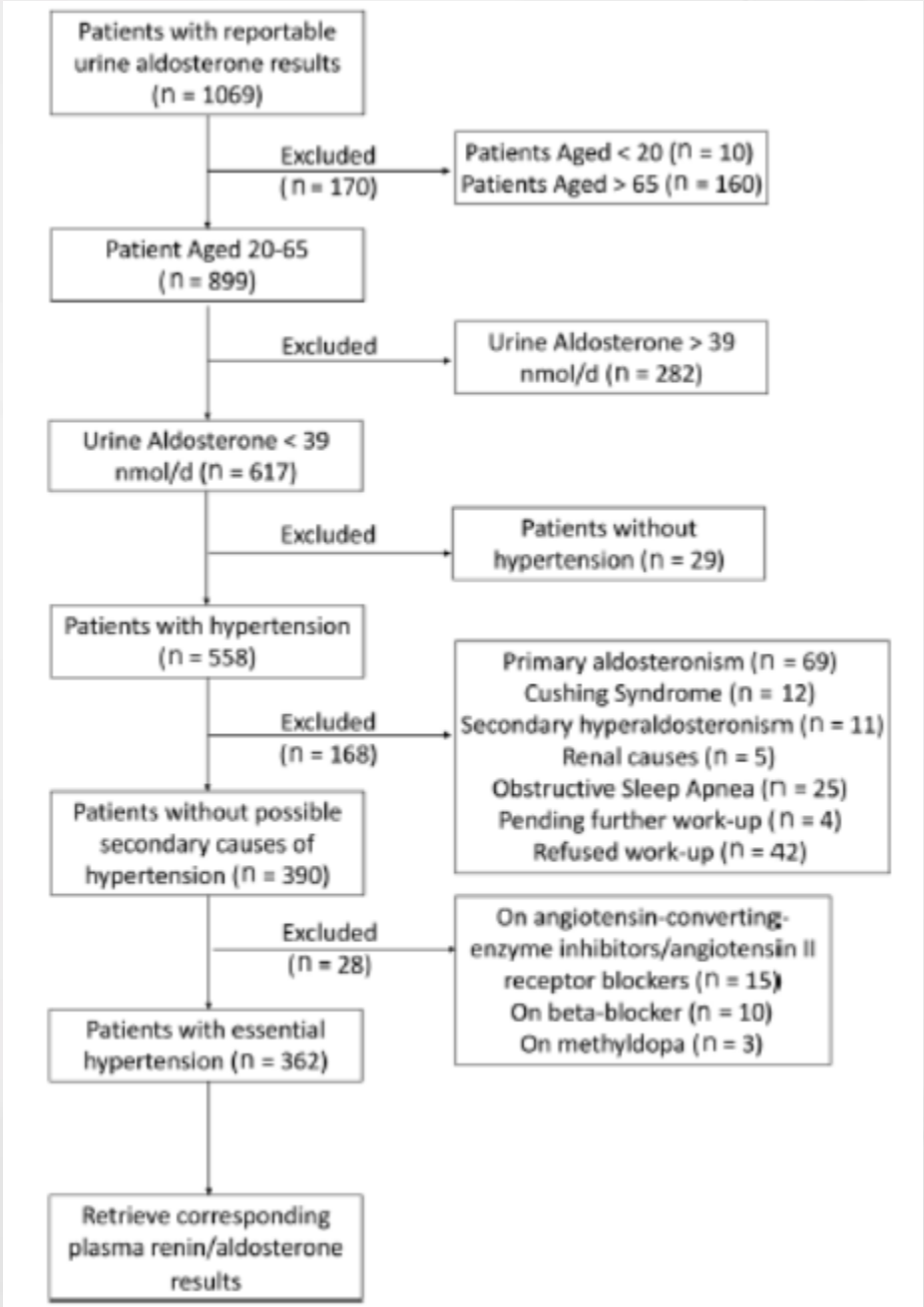
Reference intervals for biomarkers

- Recruiting reference subjects [239]
  - Apparently healthy adults (<60 years)
  - Not on hypertensive medications
  - Written consent signed
  - Questionnaire, blood pressure measurement, blood, 24 hr urine

- Reference subjects included [170]
  - Excluded 46 hypertensives (19.2%)
  - Excluded 4 with hypokalaemia
  - Excluded 19 due to past medical history

- A prospective study design

Hypertensive reference intervals



October 2013 - December 2020

From 1069 to 362

A retrospective observational study design

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Pros and Cons

Aspects	Retrospective Observational	Prospective
Timeline	Fast (months; historical data)	Slow (years, forward data collection)
Cost	Low (historical data analysis)	High (recruitment, sample analyses, data analysis, etc)
Real-world reflection	High (routine practice, e.g. UTHA)	Moderate (controlled settings)
Bias risk	Higher (selection bias)	Lower (randomisation possible)
Evidence strength	Good for hypothesis (association)	Stronger for casualty

Clin Chem Lab Med 2022; 60(10): 1640–1647 DE GRUYTER

Jenny Yeuk-Ki Cheng*, Felix Chi-Kin Wong, Edith Wing-Kar Chow, Wendy Wan-Hang Lau, Kitty Kit-Ting Cheung, Timothy Hua-Tse Cheng, Teresa Kam-Chi Tsui, Alan Shek-Lun Chan, Clara Wai-Shan Lo and Chung-Shun Ho

Chinese normotensive and essential hypertensive reference intervals for plasma aldosterone and renin activity by liquid chromatography-tandem mass spectrometry

Integrating urine biomarkers with plasma ARR for PA screening

A RETROSPECTIVE OBSERVATIONAL STUDY

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Objective

- Hypothesis: PA patients with autonomous secretion of aldosterone from the adrenal gland(s), and UALD & UTHA should also be increased.
- Endocrine Society clinical practice guidelines for PA: screening by plasma ARR.

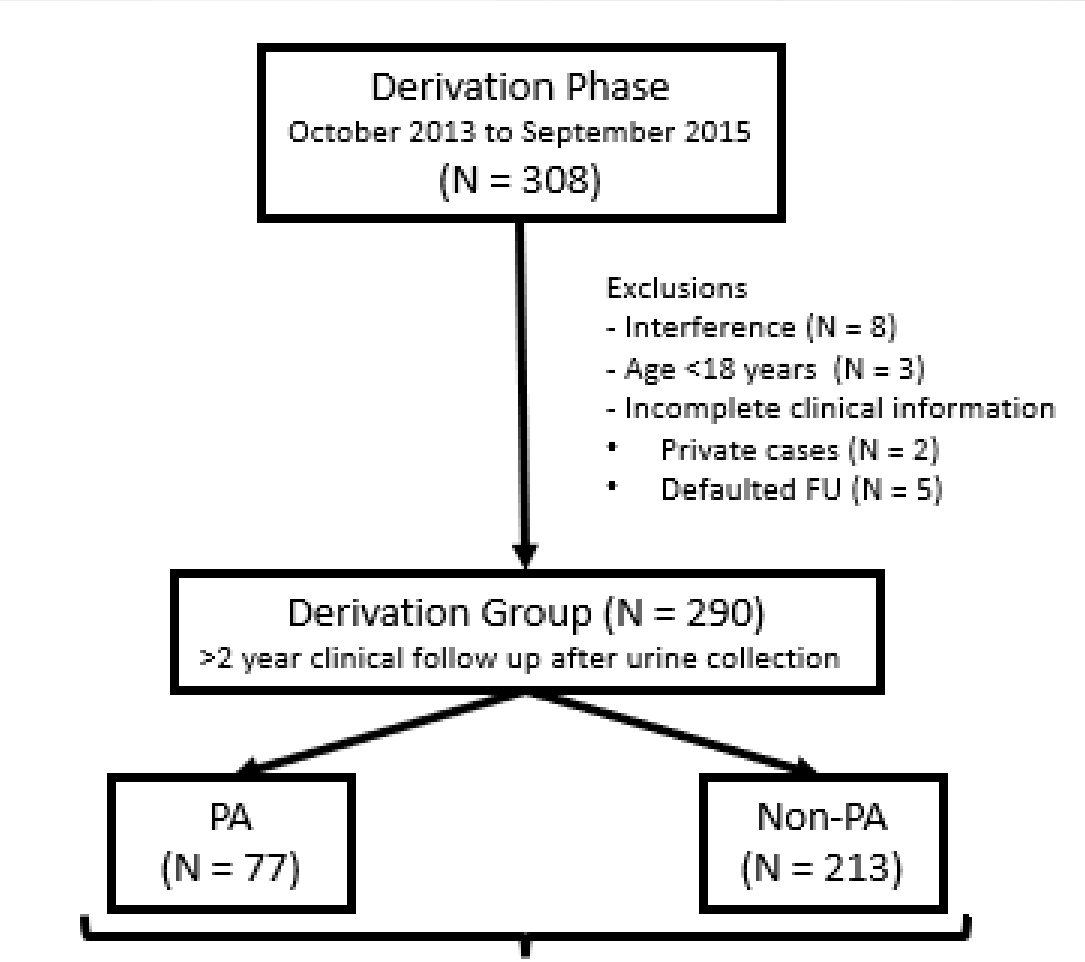
- Retrospective observational study: evaluate the performance of urine biomarkers for PA screening and compare with plasma ARR

Study design

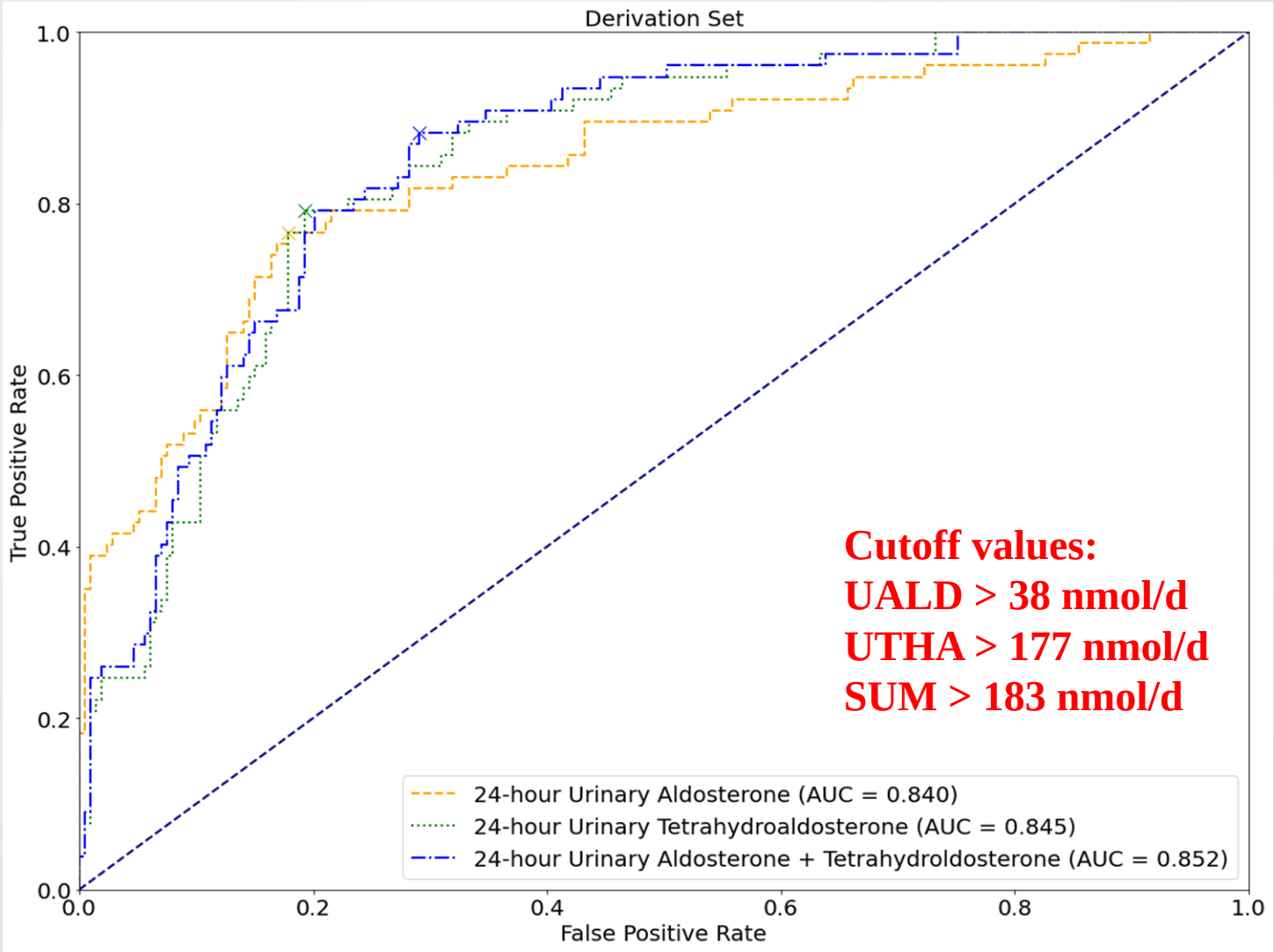
- Retrieved all UALD requests from October 2013 to September 2020
- Clinical records were reviewed
- A follow-up period of 2 to 7 years after UALD collection before establishing or excluding the diagnosis of PA

- The data were divided into two phases
  - Derivation phase (to establish optimal cutoff values)
  - Validation phase (to validate the established cutoff values and compare the performance with plasma ARR)

Results


Derivation Phase
October 2013 to September 2015
(N = 308)
Exclusions
- Interference (N = 8)
- Age <18 years (N = 3)
- Incomplete clinical information
• Private cases (N = 2)
• Defaulted FU (N = 5)
Derivation Group (N = 290)
>2 year clinical follow up after urine collection
PA
(N = 77)
Non-PA
(N = 213)

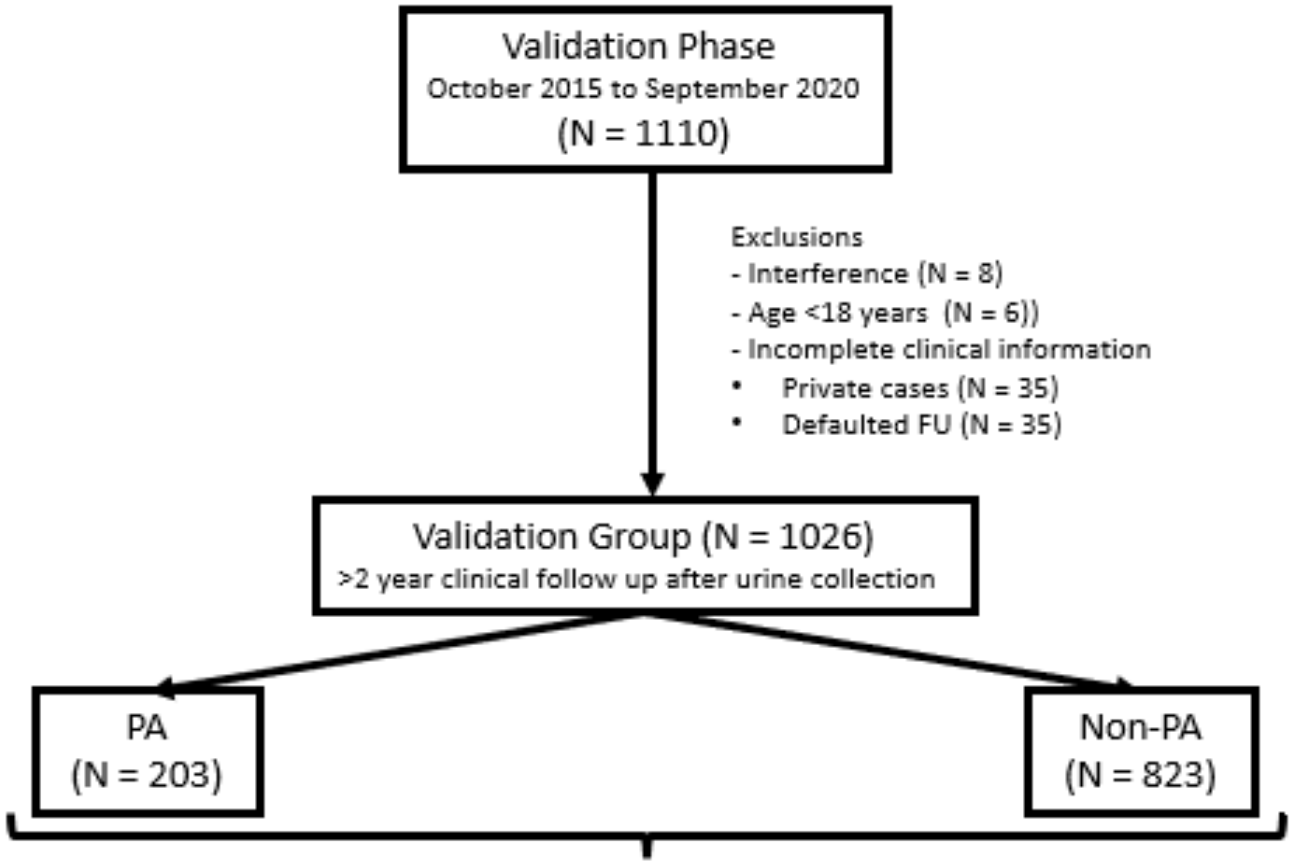
Results



Cutoff values:
UALD > 38 nmol/d
UTHA > 177 nmol/d
SUM > 183 nmol/d

Results

811 patients with plasma ARR within 90 days of urine collection:
171 with PA and 640 with non-PA



Results

	Derivation Phase (N = 290)	Validation Phase (N = 1026)	P-value
Age (Years)	51 (20)	51 (23)	0.5132
Sex (Female; Male)	149; 141	479; 547	0.1579
Hypertension (N (%))	266 (92%)	966 (94%)	0.1355
Hypokalemia (N (%))	158 (54%)	498 (49%)	0.0739
Presence of adrenal nodule (N (%))	89 (31%)	297 (29%)	0.5652
Urine volume (L)	2.0 (1.1)	2.0 (1.1)	0.9805
Urinary sodium (mmol/L)	160 (110)	165 (106)	0.3598
Primary Aldosteronism (N (%))	77 (27%)	203 (20%)	0.0130

Results

Biomarkers	Cutoff values (nmol/d)	Sensitivity (%)	Specificity (%)
UALD	>38	74	83
UTHA	>177	72	78
SUM	>183	84	71
ARR	>550	68	81
ARR or suppressed PRA	>550 or <1	80	60

Biomarkers with false positives

Biomarkers	No. of false positive cases	% False positive
UALD	123	19
UTHA	148	23
SUM	199	31
ARR	119	19
ARR or PRA<1	254	40

ARR + Urine Biomarkers:
Picked up 19% PA cases
False Positive by 24%

How to integrate?

- SUM 199 false positive
  - ARR 52 true positives
  - SUM 147 false positive
    - PRA >3 67 cases (NOT PA)
    - SUM 57 false positive

Biomarkers with false negatives

- One case with incomplete sample collection + ARR false negative
- One case with borderline negative results + ARR false negative

Summary

- Integration of urine biomarkers with plasma ARR improves the screening of PA

- One 24 hr urine collection can be used for screening of 3 different secondary hypertensions: PA, Cushing syndrome, and phaeochromocytoma

Integrating 24-hour Urine Aldosterone and Tetrahydroaldosterone with Plasma Screening to Improve Detection of Primary Aldosteronism: A Seven-Year Retrospective Study

Timothy Hua-Tse Cheng[1], Jenny Yeuk Ki Cheng[1,2], Edith Wing Kar Chow[3,6], Wendy Wan Hang Lau[3], Kitty Kit Ting Cheung[3], Clara Wai Shan Lo[1,2], Teresa Kam Chi Tsui[1,2], Risa Ozaki[3], Ronald Ching Wan Ma[3,4,5,6], Chung Shun Ho[1]

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The Balance Study to confirm and subtype Primary Aldosteronism

A RETROSPECTIVE OBSERVATIONAL STUDY

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Balance Study

- To confirm and subtype PA
  - Oral salt loading to confirm
  - Postural stimulation to subtype
- Objectives
  - Evaluate the performance of different biomarkers
  - Establish cutoff values for routine service
- Retrospective observation
  - Patients with Balance study April 2013 – March 2024

Balance Study Protocol

Pre-admission

- Sodium chloride tablets (5 days)
- Potassium supplement if needed

Admission

- Spot urine & blood check
- Collect 24 hr urine UALD

Posture Stimulation

- Supine position since midnight
- 9 a.m. Collect supine PRA & PALD
- 4 hours of ambulation
- Collect erect PRA & PALD

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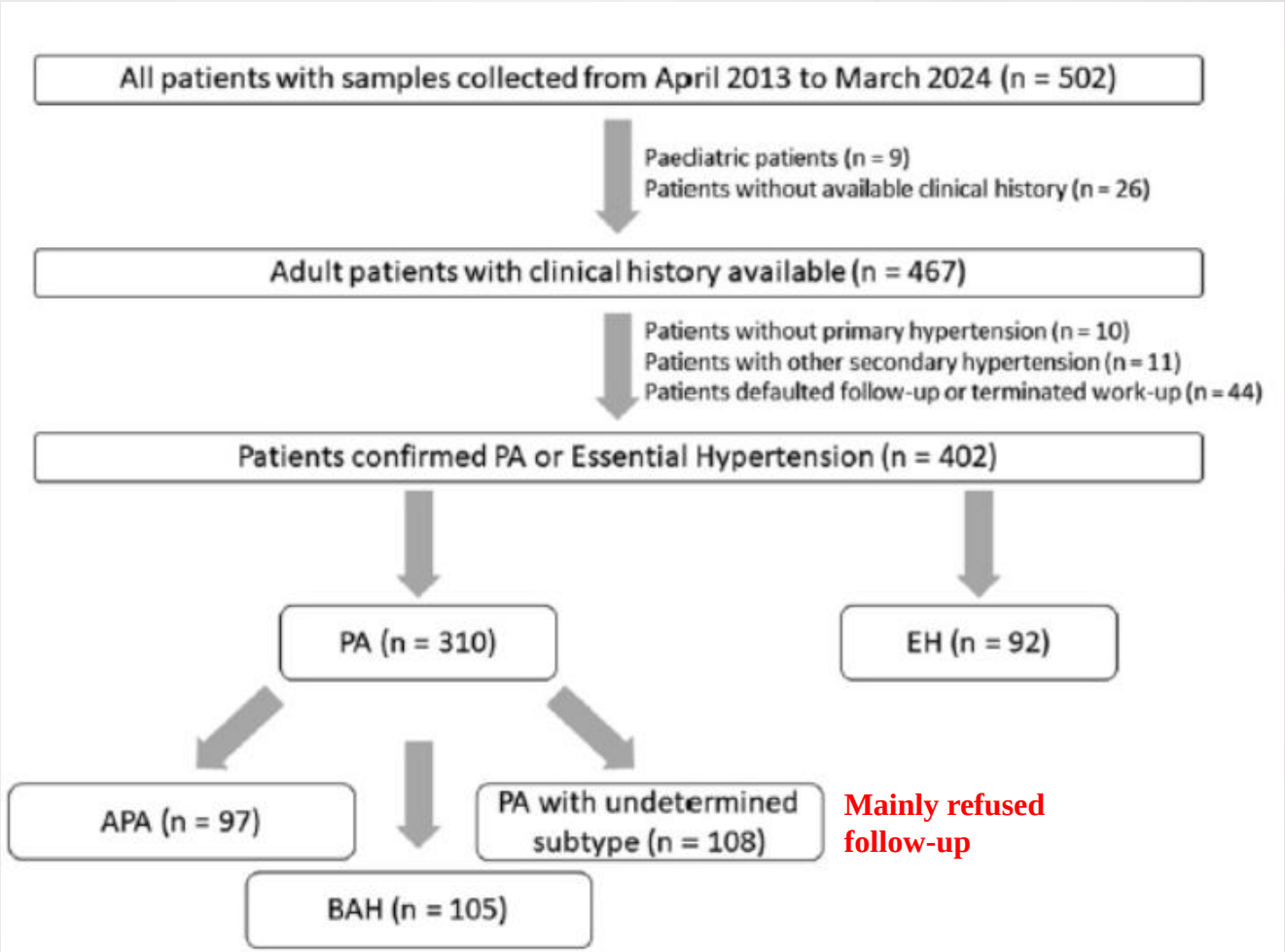


Figure 1: Flow diagram of data extraction. APA: aldosterone-producing adenoma; BAH: bilateral adrenal hyperplasia; EH: essential hypertension; PA: primary aldosteronism.

Table 1: Comparison of parameters measured in the balance study between patients with primary aldosteronism (PA) and essential hypertension (EH).

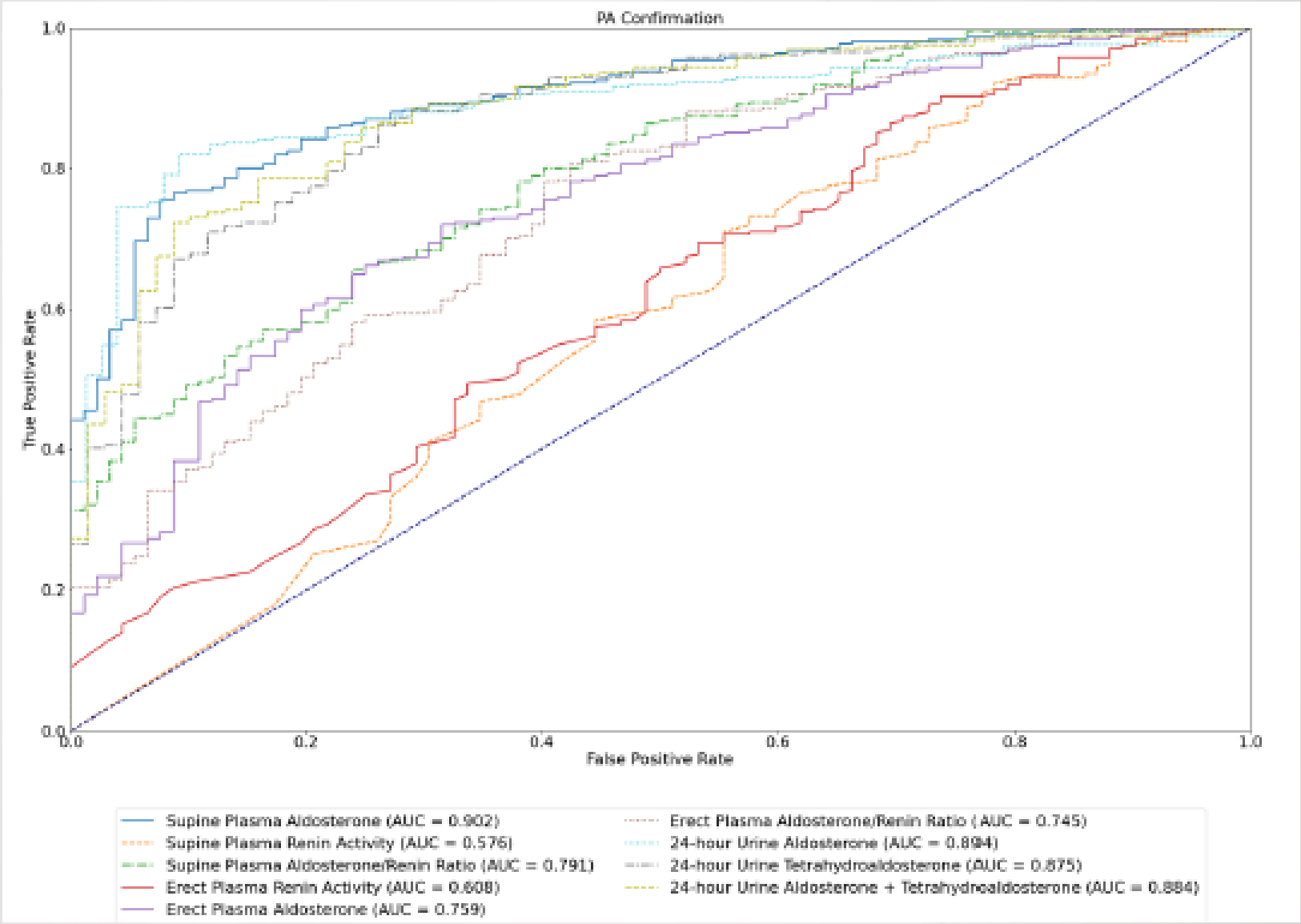
	PA (n=310)	EH (n=92)	p-Value
24-h salt-loaded urine			
Aldosterone, nmol/day	62 (57–66)	24 (21–26)	**<0.001**
Tetrahydroaldosterone, nmol/day	293 (272–320)	115 (101–132)	**<0.001**
Plasma: Supine			
Renin activity, ng/mL-h	0.17 (0.15–0.20)	0.23 (0.17–0.40)	**0.026**
Aldosterone, pmol/L	423 (400–456)	160 (143–175)	**<0.001**
Aldosterone-renin ratio, pmol/L/ng/mL-h	2,073 (1,828–2,553)	624 (438–843)	**<0.001**
Plasma: Erect			
Renin activity, ng/mL-h	0.40 (0.35–0.44)	0.61 (0.42–0.82)	**0.002**
Aldosterone, pmol/L	540 (497–574)	289 (247–338)	**<0.001**
Aldosterone-renin ratio, pmol/L/ng/mL-h	1,226 (1,049–1,538)	439 (305–608)	**<0.001**

p-Value <0.05 are bolded. Numerical results are expressed in median (95 % confidence interval).

Table 2: Comparison of parameters measured in the balance study between patients with unilateral aldosterone-producing adenoma (APA) and bilateral adrenal hyperplasia (BAH).

	APA (n=97)	BAH (n=105)	p-Value
24-h salt-loaded urine			
Aldosterone, nmol/day	75 (61–81)	57 (50–64)	**0.016**
Tetrahydroaldosterone, nmol/day	329 (281–360)	267 (237–296)	**0.009**
Plasma: Supine			
Renin activity, ng/mL-h	0.13 (0.10–0.17)	0.26 (0.17–0.39)	**<0.001**
Aldosterone, pmol/L	605 (546–689)	343 (276–392)	**<0.001**
Aldosterone-renin ratio, pmol/L/ng/mL-h	4,448 (2,769–6,713)	1,107 (871–1,872)	**<0.001**
Plasma: Erect			
Renin activity, ng/mL-h	0.28 (0.22–0.38)	0.75 (0.50–0.99)	**<0.001**
Aldosterone, pmol/L	373 (306–467)	595 (542–665)	**<0.001**
Aldosterone-renin ratio, pmol/L/ng/mL-h	1,359 (902–1,888)	829 (613–1,201)	**0.015**

p-Value <0.05 are bolded. Numerical results are expressed in median (95 % confidence interval).


PA Confirmation
True Positive Rate
False Positive Rate
0.0
0.2
0.4
0.6
0.8
1.0
Supine Plasma Aldosterone (AUC = 0.902)
Supine Plasma Renin Activity (AUC = 0.576)
Supine Plasma Aldosterone/Renin Ratio (AUC = 0.791)
Erect Plasma Renin Activity (AUC = 0.608)
Erect Plasma Aldosterone (AUC = 0.759)
Erect Plasma Aldosterone/Renin Ratio (AUC = 0.745)
24-hour Urine Aldosterone (AUC = 0.894)
24-hour Urine Tetrahydroaldosterone (AUC = 0.875)
24-hour Urine Aldosterone + Tetrahydroaldosterone (AUC = 0.884)

Performance of Biomarkers to confirm PA by ROC analyses

Biomarkers	AUC	Sensitivity	Specificity
Supine PALD (>273 pmol/L)	0.902	76	92
UALD (>38 nmol/d)	0.894	82	91
UTHA (>160 nmol/d)	0.875	86	74
SUM (>259 nmol/d)	0.884	72	91

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Overall: 94% SN, 76% SP

Performance of Biomarkers to subtype PA by ROC analyses

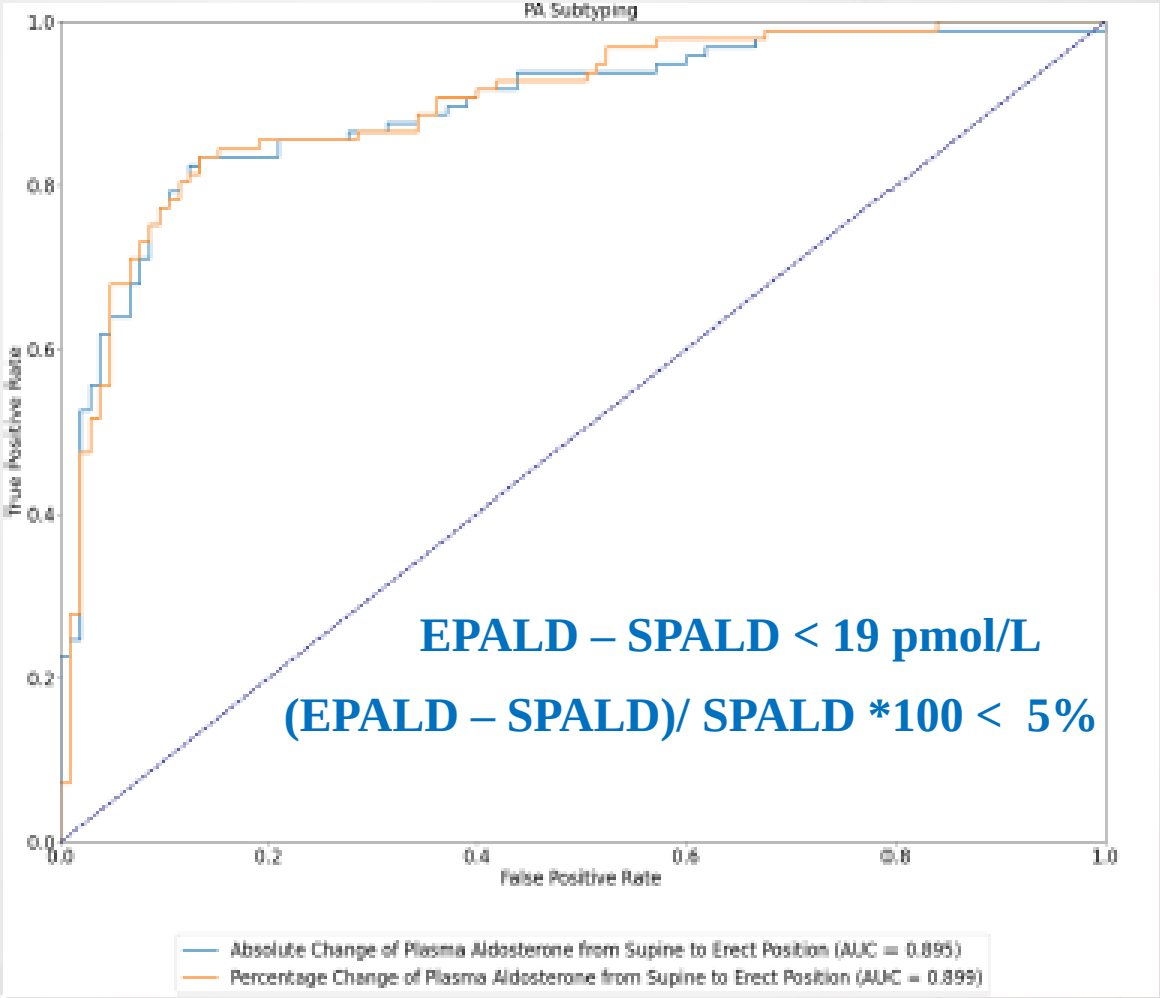

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Table 3: Performance of postural stimulation test in different studies.

Study	Number of samples	Methodology for PALD	Time for supine and erect PALD	AUC	Cut-off of erect/supine PALD	Sensitivity	Specificity
Fontes et al. [25]	89 APA and 57 BAH	RIA	Supine: morning Erect: 2–4 h after ambulation	/	<30 % increase	85.4 %	80.7 %
Fuss et al. [20]	55 APA and 29 BAH	RIA; CLIA	Supine: 8–9 am Erect: 4 h after ambulation	0.724	>28 % decrease	36.4 %	100 %
Wu et al. [19]	314 APA and 217 BAH	CLIA	Supine: 5 am Erect: 2 h after ambulation	0.604	<30 % increase	73.8 %	46.2 %
Current study	108 APA and 105 BAH	LC-MS/MS	Supine: 9 am Erect: 4 h after ambulation	0.90	<5 % increase	84 %	87 %

AUC, area under the curve; CLIA, chemiluminescence immunoassay; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PALD, plasma aldosterone; RIA, radioimmunoassay.

Oral salt loading before Postural Stimulation

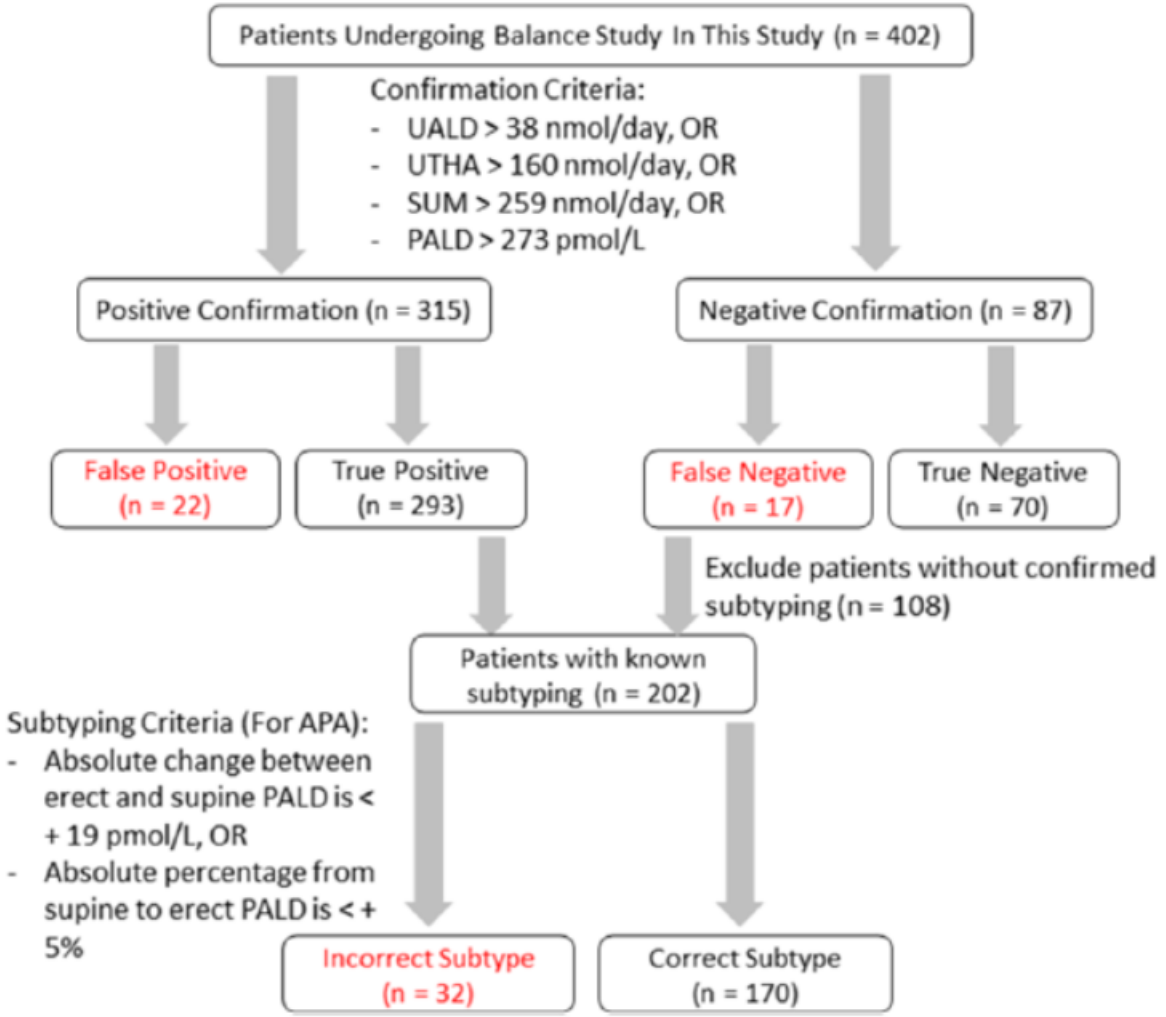

Figure 3: Flow diagram of patients' diagnosis based on proposed cut-offs.

Summary

- The Balance Study can help PA confirmation and provide direction on PA subtyping in a non-invasive manner
- UTHA and supine PALD could complement UALD for PA confirmation
- Erect/supine PALD ratio could assist in PA subtyping in cases with equivocal AVS results or in regions where AVS is not readily available

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Clin Chem Lab Med 2025; aop

Jenny Yeuk Ki Cheng*, Wai Shan Clara Lo, Teresa Kam Chi Tsui, Wing Kar Edith Chow, Kitty Kit Ting Cheung, Ronald Ching Wan Ma, Risa Ozaki and Chung Shun Ho

Oral salt loading combined with postural stimulation tests for confirming and subtyping primary aldosteronism

Aldosterone/ Angiotensin II Ratio (AAIIR)

A RETROSPECTIVE COHORT STUDY

Dr. Clara Lo PhD project

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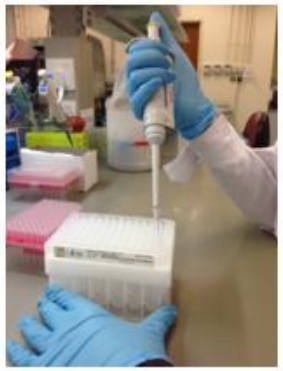
LCMS method for plasma Ang II measurements

300uL plasma+30µL IS+100 µL 4% FA



Sample added to MAX µElution plate

Sample was extracted by off-line SPE with Waters positive pressure processor

Extracted samples were measured by LCMS

Sample preparation time for 40 samples : 1 hour
Injection time: 6.5min

Between batch CV <7%

LOQ : 3.3 pmol/L
Linearity range :3.3-700 pmol/L

Not interfered by other angiotensin peptides

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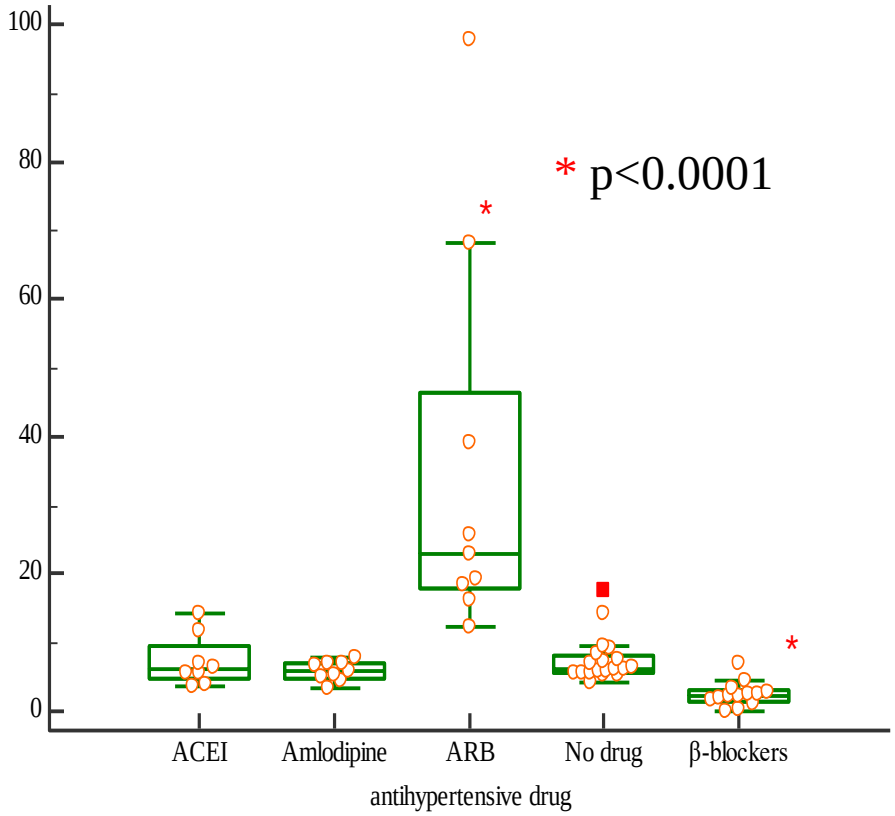
Ang II levels in patients taking different anti-hypertensive drugs



Compared with untreated hypertensive patients:
ARB-significantly higher
Beta-blocker- significantly lower

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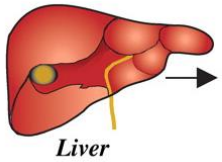
Biomedical Chromatography WILEY

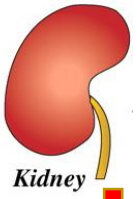
RESEARCH ARTICLE

Quantitation of plasma angiotensin II in healthy Chinese subjects by a validated liquid chromatography tandem mass spectrometry method

Clara Wai-Shan Lo[1] | Teresa Kam-Chi Tsui[1] | Ronald Ching-Wan Ma[2,3] | Michael Ho-Ming Chan[1] | Chung-Shun Ho[1]

Primary Aldosteronism (PA)

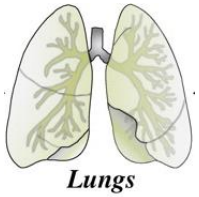




Decrease in renal perfusion

Renin

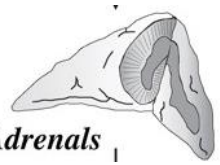
Angiotensinogen → Angiotensin I



ACE1

Angiotensin II

Aldosterone



Laboratory test results

- Plasma aldosterone (PALD) ↑
- Plasma renin activity(PRA) ↓↓

$$ARR = \frac{PALD}{PRA} \uparrow$$

Laboratory test results

- Plasma aldosterone (PALD) ↑
- Plasma angiotensin II (Ang II) ↓↓

$$AAIIR = \frac{PALD}{AngII} \uparrow$$

Ang II- active mediator
?? Better screening marker than ARR for PA

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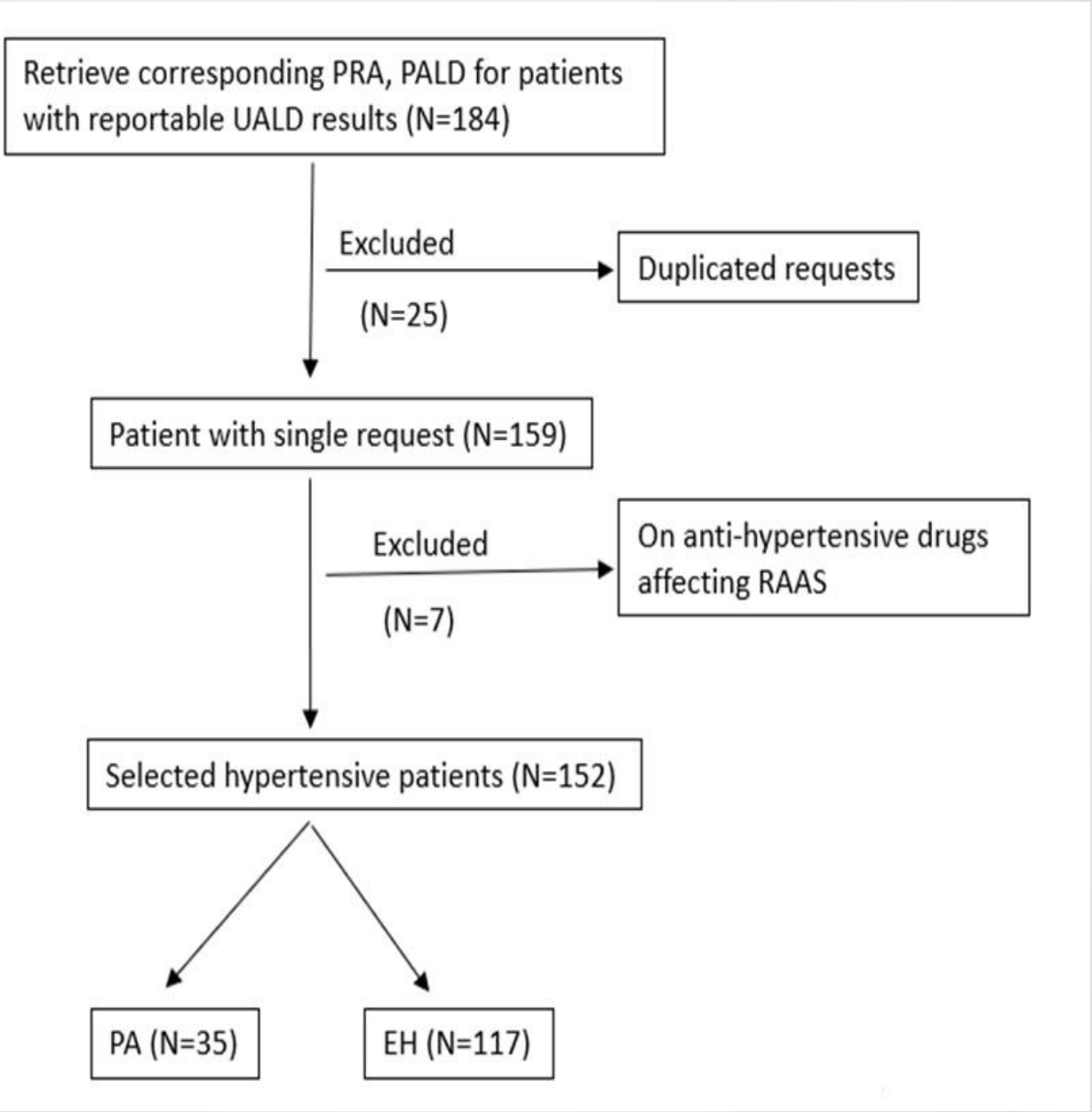
Retrieve corresponding PRA, PALD for patients with reportable UALD results (N=184)
Excluded
(N=25)
Duplicated requests
Patient with single request (N=159)
Excluded
(N=7)
On anti-hypertensive drugs affecting RAAS
Selected hypertensive patients (N=152)
PA (N=35)
EH (N=117)

Characteristics of the selected patients

	Patient's group		
	Non-PA (n = 120)	PA (n = 35)	P
Female	62	21	
Male	58	14	
Age	45.3±11.7	53.9 ± 9.6	<0.0001
24hr urine aldosterone (nmol/day)	29.2 ± 4.3	63.0 ± 7.6	<0.0001
24hr urine sodium (mmol/day)	162 ± 14.7	173 ± 21.5	0.3774
PRA (ng/mL-hour)	1.13 ± 0.22	0.39 ± 0.19	<0.0001
PALD (pmol/L)	198± 24.6	468 ± 83.4	<0.0001
Ang II (pmol/L)	7.6 ± 0.2	5.0 ± 0.74	<0.0001
ARR	180 ± 45	1613 ± 541	<0.0001
AAIIR	27.2 ± 3.39	112.8 ± 20.75	<0.0001

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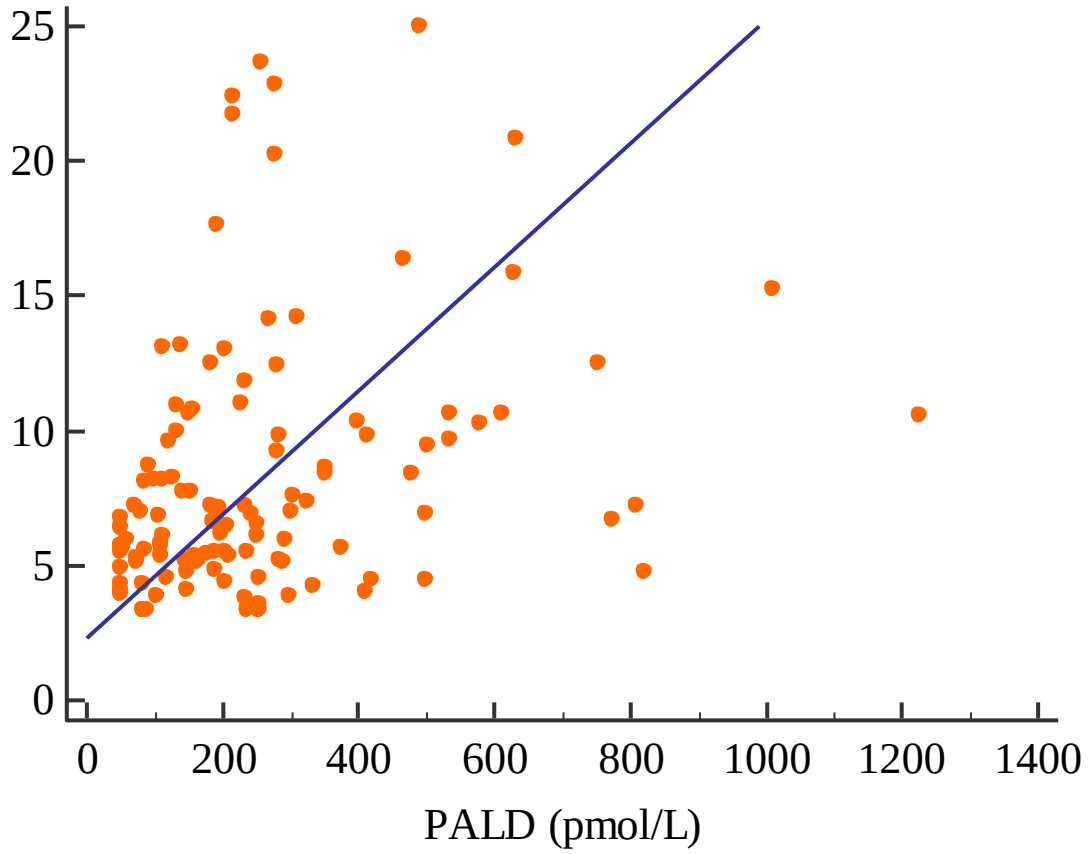
Correlation of AngII and PALD in Non-PA patients

(n=120, p=0.0004, r=0.32)



Significantly correlated

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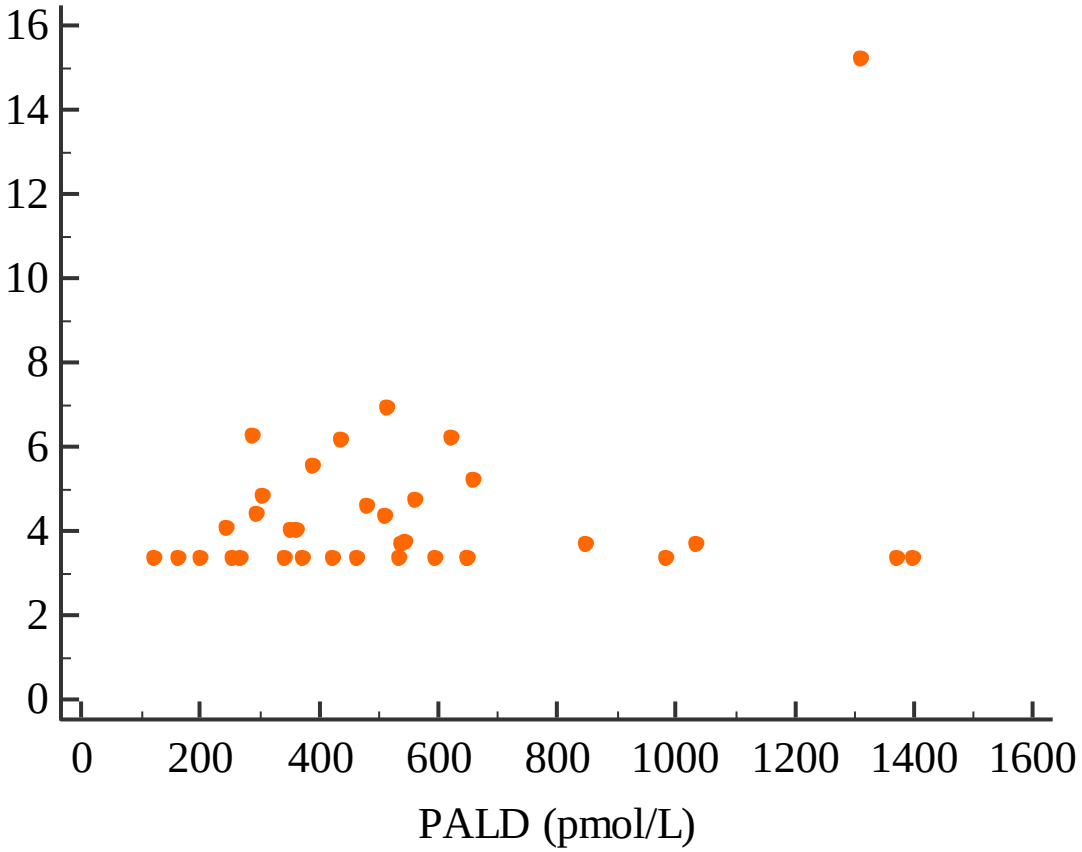
Correlation of Ang II and PALD in PA patients

(n=35, p=0.8)



NOT correlated

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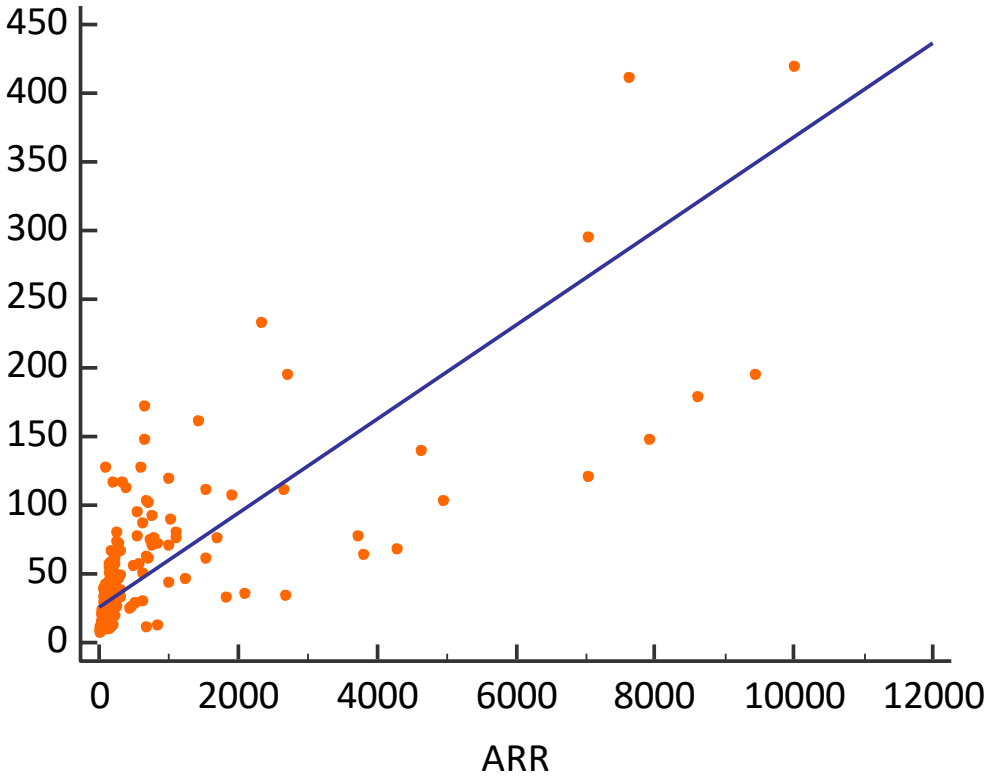
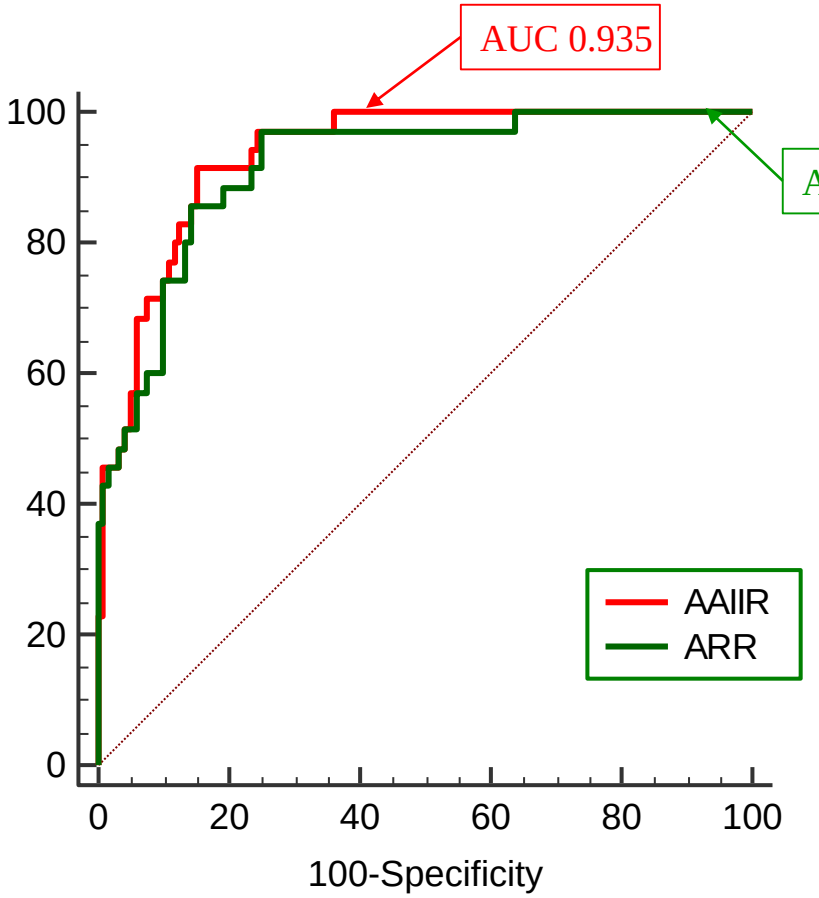
Correlation of AAIIR and ARR in patients

(n=155, p<0.0001, r=0.78)



Significantly correlated

Comparison of the ROC curves of AAIIR and ARR for patients(n=155)



No significant difference in AUC

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ROC curve analysis (2)

	AAIIR	ARR
Sensitivity (%)	91	86
Specificity (%)	85	87
Area under ROC curve AUC	0.935	0.916
Cut Off	>60	>577
Likelihood ratio(positive)	6	6
Likelihood ratio(negative)	0.1	0.2

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AAIIR is an acceptable test for screening PA

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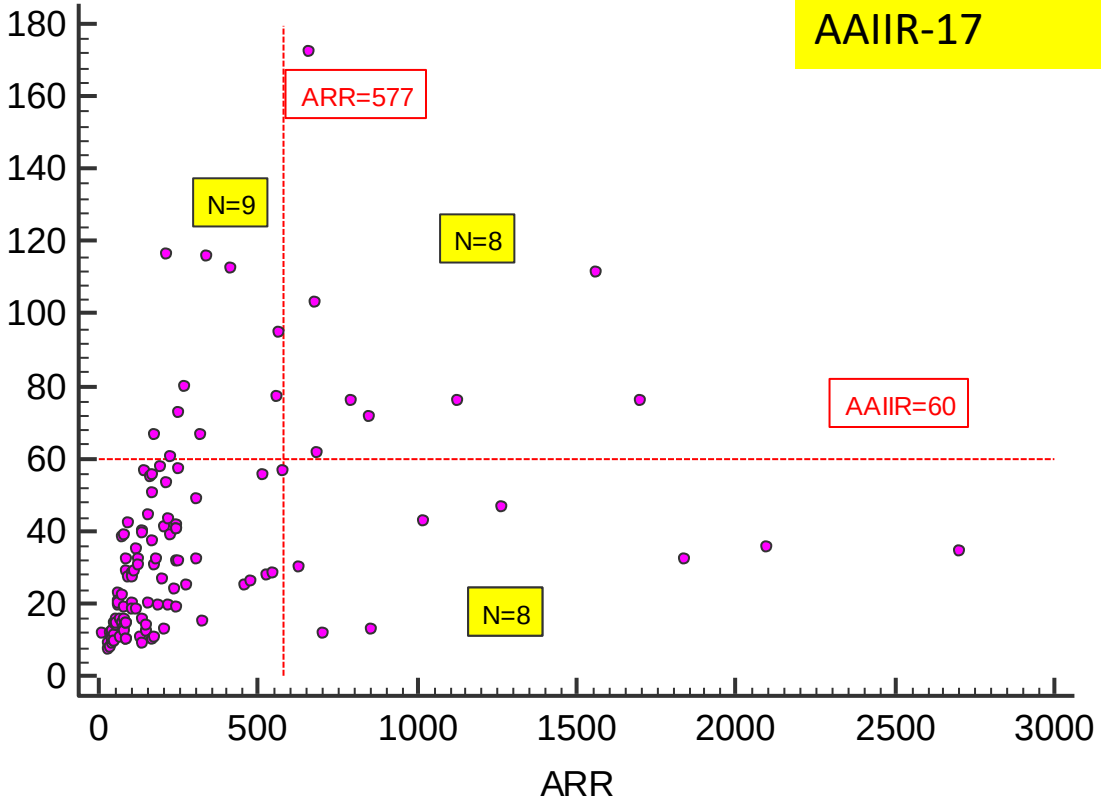
Concordance of AAIIR and ARR for non-PA patients

N=120

False positive cases
ARR-16
AAIIR-17



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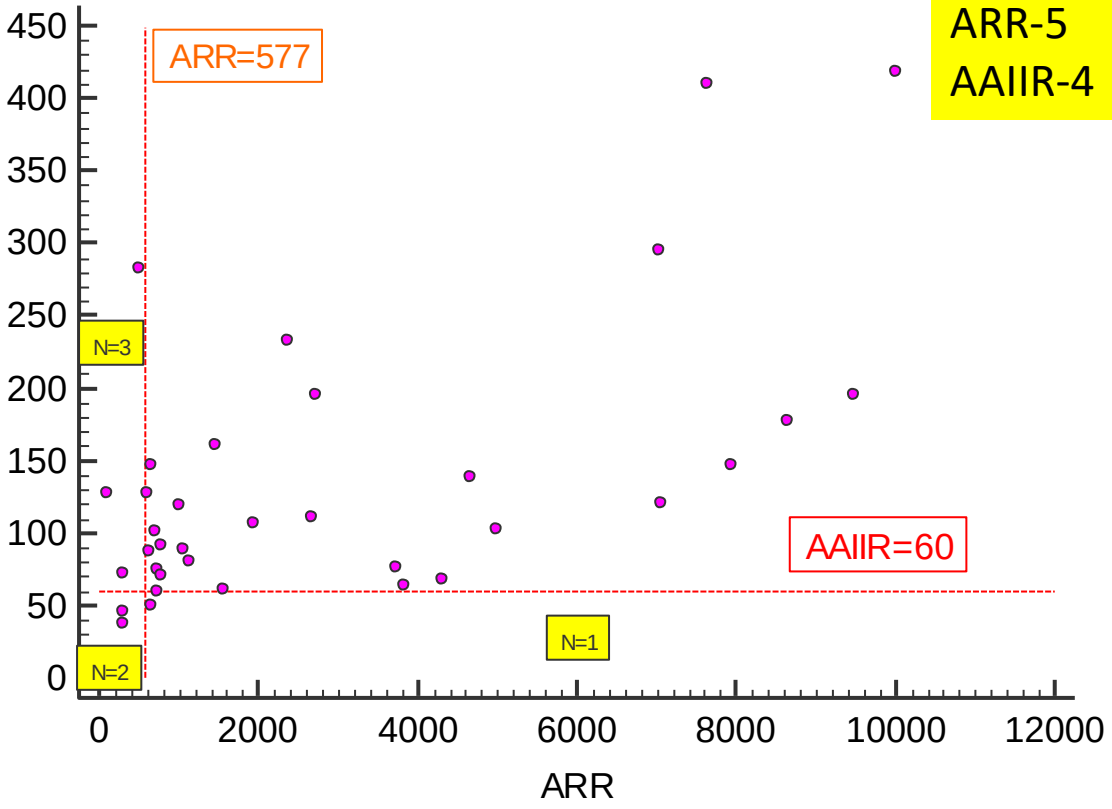
Concordance of AAIIR and ARR for PA patients

N=35

False negative cases
ARR-5
AAIIR-4



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Advantages of using AAIIR

	ARR	AAIIR
Analysis time (40 samples)	22 hours	10 hours
Affected by endogenous proteases	Yes	No
Specificity	Indirect measurement on RAS	Active mediator of RAS

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DE GRUYTER

Clin Chem Lab Med 2025; aop

Clara Wai-Shan Lo*, Jenny Yeuk-Ki Cheng, Teresa Kam-Chi Tsui, Ronald Ching-Wan Ma, Michael Ho-Ming Chan, Risa Ozaki and Chung-Shun Ho

Screening primary aldosteronism by plasma aldosterone-to-angiotensin II ratio

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Keywords: primary aldosteronism screening; aldosterone-to-angiotensin II ratio; diagnostic performance

Future direction for the PA routine service

RETROSPECTIVE OBSERVATIONAL STUDY

- Reporting of the urine biomarkers for the screening and the Balance study
- An improved LC-MS/MS method for the simultaneous quantification of PRA and plasma Ang II
- Obstacles to overcome: EQA programs for these new biomarkers

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