Aldosterone and renin concentrations were abnormally elevated in a cohort of normotensive pregnant women

Valentina Pastén1 · Alejandra Tapia-Castillo1,2,3 · Carlos E. Fardella1,2,3 · Andrea Leiva4,5 · Cristian A. Carvajal1,2,3

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Abstract
Background During pregnancy, the renin–angiotensin–aldosterone system (RAAS) undergoes major changes to preserve normal blood pressure (BP) and placental blood flow and to ensure a good pregnancy outcome. Abnormal aldosterone–renin metabolism is a risk factor for arterial hypertension and cardiovascular risk, but its association with pathological conditions in pregnancy remains unknown. Moreover, potential biomarkers associated with these pathological conditions should be identified.

Aim To study a cohort of normotensive pregnant women according to their serum aldosterone and plasma renin levels and assay their small extracellular vesicles (sEVs) and a specific protein cargo (LCN2, AT1R).

Methods A cohort of 54 normotensive pregnant women at term gestation was included. We determined the BP, serum aldosterone, and plasma renin concentrations. In a subgroup, we isolated their plasma sEVs and semiquantitated two EV proteins (AT1R and LCN2).

Results We set a normal range of aldosterone and renin based on the interquartile range. We identified 5/54 (9%) pregnant women with elevated aldosterone and low renin levels and 5/54 (9%) other pregnant women with low aldosterone and elevated renin levels. No differences were found in sEV-LCN2 or sEV-AT1R.

Conclusion We found that 18% of normotensive pregnant women had either high aldosterone or high renin levels, suggesting a subclinical status similar to primary aldosteronism or hyperreninemia, respectively. Both could evolve to pathological conditions by affecting the maternal vascular and renal physiology and further the BP. sEVs and their specific cargo should be further studied to clarify their role as potential biomarkers of RAAS alterations in pregnant women.

Keywords Normotension · Pregnancy · Renin–angiotensin–aldosterone system · Extracellular vesicles

Introduction

During pregnancy, the renin–angiotensin–aldosterone system (RAAS) undergoes major changes. Aldosterone and renin are upregulated during pregnancy to ensure an increase in maternal volemia and good placental perfusion [1, 2]. Circulating prorenin and renin increase fourfold by 10 weeks of gestation and plateau at 22 weeks of gestation [3, 4]. Aldosterone and its regulator angiotensin II (Ang-II) also increase during pregnancy [5]. The actions of Ang-II, including aldosterone synthesis, are mediated through the Ang-II type-1 receptor (AT1R) [6–8]. AT1R is expressed in the placenta and is increased in pathologies such as preeclampsia [9, 10]. However, to date, the regulation of AT1R abundance in the plasma of pregnant women is unknown.

Circulating aldosterone binds to mineralocorticoid receptor (MR) [11–13] and plays a crucial role in regulating
Body fluid volume and blood pressure (BP). Aldosterone increases approximately tenfold at term pregnancy [4]; however, the pathophysiological effects of increased aldosterone on MR are mitigated in pregnancy by progesterone, which acts as an antagonist of MR [14, 15] and by inhibiting CYP11B2 activity in adrenal tissue [16, 17]. MR activation by aldosterone increases the circulating and urinary levels of lipocalin-2 protein (LCN2, also called NGAL), which has been proposed as a surrogate biomarker of MR activation by aldosterone [13, 18, 19].

Currently, experimental and clinical studies have demonstrated that changes in the concentration, size, and/or cargo of sEVs or exosomes are potential biomarkers of disease in nonpregnant and pregnant populations [20–24]. The presence of LCN2 in small extracellular vesicles (sEVs) has also been suggested as an early marker of renal injury and MR activation [13, 19, 25], but there is no information suggesting whether changes in this novel biomarker are associated with high BP, aldosterone, or renin levels in pregnancy. Like LCN2 [19], the presence of AT1R [26, 27] in circulating sEV associated with changes in RAAS in pregnant women remains unknown.

The aim of this study was to measure aldosterone levels and plasma renin in a cohort of normotensive pregnant women to identify women with abnormal levels of these hormones. In addition, we explored the presence of plasma sEVs and their protein cargo LCN2 and AT1R in these pregnant women.

**Material and methods**

**Subjects**

This study was performed in 54 Chilean normotensive women in the third trimester of pregnancy (>37 weeks of gestation) from the Hospital Clínico UC-CHRISTUS, Chile. Normotension was determined according to the 2017 ACC/AHA Guidelines for High BP [28]. All the women included in this study had single pregnancies; no intrauterine infections or obstetric complications; no chronic pathologies such as kidney failure, heart failure, chronic liver damage, or endocrinopathies; and the absence of exogenous treatment with mineralocorticoids or glucocorticoids. Subjects with pregestational and gestational diabetes, intrauterine growth restriction, fetal malformations, chronic hypertension, hypertensive disorders of pregnancy, or other maternal pathologies were excluded from this study. Women previously diagnosed with secondary causes of hypertension, such as primary aldosteronism, familial hyperaldosteronism (OMIM 103900), apparent mineralocorticoid excess (OMIM 218030), hypercortisolism, and renovascular disease, were also excluded from this study. General maternal (i.e., age, height, weight, and BP) and neonatal (i.e., sex, gestational age, weight, and height) variables were obtained from the clinical records. All subjects included in this study signed a written informed consent in accordance with the Helsinki Declaration and were approved by the ethical Committee of the Faculty of Medicine (CEC-18081004), Pontificia Universidad Católica de Chile.

**Classification of pregnant women based on aldosterone and renin levels**

Pregnant women were classified into three groups: controls with normal aldosterone and renin levels and women with high aldosterone or renin levels [29, 30]. There were 44 control subjects with normal aldosterone and renin levels (Fig. 1). Women with serum aldosterone greater than the 75th percentile (115.3 ng/dL) with a concomitant low renin concentration (lower than the 25th percentile: 34.9 µUI/mL) were categorized as elevated aldosterone and low renin (EALR) (Fig. 1A). Otherwise, women with...
a low aldosterone (lower than the 25th percentile: 37 ng/dL) and a concomitant renin concentration greater than the 75th percentile (62.5 µIU/mL) were categorized as low aldosterone and elevated renin (LAER) (Fig. 1B).

**Biochemical assay**

Serum aldosterone (LIAISON® Aldosterone (310450)) and direct plasma renin concentration (DRC) (LIAISON® Direct Renin (code 310470)) were measured by chemiluminescent immunoassay technology on an automated chemiluminescent analyzer (Liaison XL/DiaSorin, Saluggia, Vercelli, Italy), with reported coefficients of variation of 9.5% for 6.7 ng/dL and 5.6% for 28.8 ng/dL for plasma aldosterone and 13% for 5.8 mIU/mL and 7.3% for 107.5 mIU/mL for DRC in the clinical laboratory of the Hospital Clínico UC-CHRISTUS. Plasma electrolytes (sodium and potassium) were evaluated with methods previously described [31]. Serum cortisol and cortisone were quantified using mass spectrometry (LC–MS/MS) and validated according to the parameters suggested by the Food and Drug Administration and Clinical and Laboratory Standards Institute using deuterated internal standards of cortisol and cortisone (cortisol-d4 and cortisone-d2) in Agilent 1200 series HPLC equipment coupled to an AB Sciex 4500 QTrap mass spectrometer [32].

**Isolation of small EVs from maternal plasma samples**

Small EVs (exosomes) were isolated by ultracentrifugation (UCF) from plasma samples (500 µL) from 18 pregnant women (including 4 EALR, 4 LAER, and 10 control women) as described [33]. Briefly, samples were diluted in phosphate-buffered saline (PBS) supplemented with calcium and magnesium (DPBS) and centrifuged (4 °C, 2000 × g, 30 min) to eliminate cell debris. The supernatant was centrifuged (4 °C, 12,000 × g, 45 min), transferred to UCF tubes, filtered with a 0.22-µm filter, and then centrifuged (4 °C, 200,000 × g, 1.5 h) (UCF Thermo-Sorvall WX80+). The obtained supernatant was discarded, and the pellet was resuspended in 4 mL of DPBS and centrifuged again (4 °C, 200,000 × g, 1.5 h). Finally, sEV pellets were resuspended in 100 µL of PBS or RIPA buffer and stored at −20 °C.

**Quantification of sEVs by nanoparticle tracking analysis (NTA)**

sEV samples were diluted with PBS to obtain a concentration range between 20 and 100 particles per frame (optimal greater than 20 particles per frame). The samples were analyzed using an NS300 instrument (Malvern, UK) with NanoSight NTA 3.0 Nanoparticle Tracking and Analysis software (Version Build 0064). The videos (two videos per sample) were processed and analyzed as previously described and informed the mean, mode, and median particle size together with an estimated number of particles per mL of plasma [34].

**Determination of sEV morphology by transmission electron microscopy**

sEV shape and size were determined by transmission electron microscopy as described [34]. For this, a 15-µL sEV pellet was added onto a carbon-coated copper grid (300 mesh) for 1 min and stained with 2% uranyl acetate for 1 min. The grids were visualized at 80 kV in a Tecnai transmission electron microscope (Phillips, Finland).

**Identification of characteristic EV proteins, LCN2, and AT1R by western blot**

sEVs were resuspended in RIPA buffer (Thermo Fisher Scientific, USA), and the protein content was determined with the bicinchoninic acid (micro-BCA) Protein Assay kit (Thermo Fisher Scientific, USA). Proteins (30 µg) from the EV lysate were separated by polyacrylamide gel electrophoresis under denaturing and reducing conditions, transferred to polyvinylidene difluoride membranes, and later probed with primary rabbit polyclonal anti-TSG101 (1:10,000, 18 h, 4 °C) (Abcam, UK) and anti-AT1R (1:200, 18 h, 4 °C) (Sigma–Aldrich, USA) antibodies and goat polyclonal anti-LCN2 (1:200, 18 h, 4 °C) (R&D Systems, USA) antibodies. After washing, the membranes were incubated (1 h, room temperature) with secondary horse-radish peroxidase-conjugated goat anti-rabbit (Thermo Fisher Scientific, USA) or rabbit anti-goat (Abcam, UK) antibodies as previously described [35]. Proteins were detected by enhanced chemiluminescence, and a semi-quantitative densitometry analysis was performed using ImageJ software (NIH, USA).

**Statistical analysis**

Values are presented as the median [interquartile range] or mean ± standard deviation (range). Comparisons between two groups were performed by Student’s t test for parametric data or the Mann–Whitney U-test for nonparametric data. A value of p < 0.05 was considered statistically significant. Data analysis and plotting were performed with GraphPad Prism 7.0 software (GraphPad Software Inc., USA).
Glucocorticoids

Plasma electrolytes

Mineralocorticoids
cortisol-to-cortisone ratio

CCR

women with elevated aldosterone and low renin, EALR performed by Student’s t test for parametric data or Mann–Whitney U-test for nonparametric data. *p < 0.05, **p < 0.001, ***p < 0.0001 vs. corresponding values in control group. Clinical and biochemical data are presented as median and interquartile range.

EALR women with elevated aldosterone and low renin, LAER women with low aldosterone and elevated renin, ARR aldosterone to renin ratio, CCR cortisol-to-cortisone ratio

| Table 1 Clinical and biochemical characteristics of pregnant women and their newborns |
|----------------|----------------|----------------|----------------|
| Variables       | Total (n = 54) | Control (n = 44) | EALR (n = 5) |
| Maternal variables |               |                 |              |
| Age [years]     | 31 [25.8–34.3] | 30 [26–32.8]    | 31 [28–34.5] |
| Height [cm]     | 161 [158–165]  | 161 [158–165]   | 165 [163–168]|
| Weight 3rd trimester [kg] | 79 [70.5–86.5] | 79 [71–87] | 79 [70.5–91] |
| Weight gain [kg] | 12 [8.5–14]   | 12 [9.5–14]    | 12 [9.5–19.5]|
| BMI at 3rd trimester [kg/m²] | 30.5 [27.1–33.4] | 30.5 [27.3–33.2] | 29 [26.5–32.4] |
| SBP at 3rd trimester [mmHg] | 107.5 [102.8–112.3] | 108 [102–112] | 107 [102.5–110.5] |
| DBP at 3rd trimester [mmHg] | 67 [62–70] | 66.5 [62–70] | 68 [65.5–69] |
| Mineralocorticoids |               |                 |              |
| Plasma aldosterone [ng/dL] | 65.2 [36.8–116.6] | 65.8 [40.6–96] | 130 [122.5–205]*** |
| Renin concentration [µUI/mL] | 44.3 [35–60.9] | 44.7 [34.9–58.3] | 34.1 [6–35]* |
| ARR | 1.5 [0.7–2.7] | 1.5 [0.8–2.3] | 4.9 [3.6–29.8]*** |
| Plasma electrolytes |               |                 |              |
| Na+ (mEq/L) | 132.8 [129.9–135.3] | 133.2 [131.1–135.6] | 134.5 [132.4–136.9] |
| K+ (mEq/L) | 4.1 [4.0–4.4] | 4.2 [4.0–4.4] | 3.9 [3.8–4.2] |
| Na+/K+ ratio | 32.4 [30.8–35.2] | 31.7 [30.6–34.6] | 34.5 [32.5–36.8] |
| Glucocorticoids |               |                 |              |
| Cortisol [µg/dL] | 28.9 [21.6–41.6] | 27.9 [21.5–41.1] | 35 [27.9–52.7] |
| Cortisone [µg/dL] | 6.4 [5.6–7.3] | 6.2 [5.6–7.1] | 7.3 [6.2–7.9] |
| CCR | 4.6 [3.7–5.7] | 4.3 [3.36–5.6] | 5 [4.3–7] |
| Newborn variables |               |                 |              |
| Sex [female/male] | 32/22 | 7/6 | 4/1 |
| Gestational age [weeks] | 39.3 [38.6–40] | 39.3 [38.7–40] | 38.9 [38.3–39.4] |
| Birth weight [g] | 3315 [3090–3635] | 3300 [3090–3635] | 3630 [3395–3905] |
| Height [cm] | 50 [49–51] | 50 [49–51] | 51 [50.5–52.5] |
| Ponderal index [g/cm³ × 100] | 2.6 [2.6–2.8] | 2.6 [2.5–2.8] | 2.7 [2.6–2.8] |

Results

Identification of groups of normotensive pregnant women with either high plasma aldosterone or high renin levels

All women participating in this study had normal BP (SBP < 120 mmHg and DBP < 80 mmHg), with an average mean BP of 102.5 mmHg (Table 1). We identified 44 pregnant women with normal aldosterone or normal renin levels, which were considered controls (Table 1) (Fig. 1). In our cohort, 9% (5/54) were identified as having EALR (Table 1) (Fig. 1A). In the EALR group, the aldosterone levels were twofold higher in pregnant women than in controls (130 ng/dL vs. 65.6 ng/dL, p < 0.0001), and the renin levels were 64% significantly lower than those in controls (34.1 µUI/mL vs. 44.7 µUI/mL, p < 0.05). The ARR was threefold higher in women with EALR than in controls (4.9 vs. 1.5, p < 0.0001) (Table 1).

Similarly, 9% (5/54) of pregnant women were identified as having LAER (Table 1) (Fig. 1B). These five women were different than those identified in the EALR group. The data corresponding to control women are shown in Table 1 and Fig. 1B. In pregnant women with LAER, aldosterone levels were 70% lower (30.8 ng/dL vs. 65.8 ng/dL, p < 0.05), renin levels were higher (74.2 µUI/mL vs. 44.7 µUI/mL, p < 0.05) and ARR was fourfold lower (0.36 vs. 1.5, p < 0.001) compared to control pregnant women (Table 1).

Serum cortisol, cortisone, and the cortisol-to-cortisone ratio were similar in different groups (Table 1). Regarding the neonatal variables, we did not observe any difference in the EALR and LAER groups compared to control neonates (Table 1).
Plasma electrolytes in normotensive pregnant women

Plasma electrolytes \(Na^+\) and \(K^+\) and the respective \(Na^+/K^+\) ratio were determined. Although EALR group had a trend to higher sodium and lower potassium levels, both were similar to control or LAER group (Kruskal–Wallis test, pNS). Unfortunately, no urine samples were obtained from these women to measure urinary \(Na^+\) or \(K^+\) electrolytes.

Identification and characterization of plasma extracellular vesicles in the EALR and LAER groups

Plasma sEVs were isolated from 18 pregnant women in our cohort, including 10 controls, 4 EALRs, and 4 LAERs. The particle size ranged between 50 and 150 nm for all samples (Fig. 2A). The morphology of the isolated sEVs was determined by transmission electron microscopy. sEVs showed the characteristic morphology of a round donut shape between 30 and 150 nm (Fig. 2B). The presence of the sEV marker TSG101 was confirmed by western blot in the isolated sEVs (Fig. 2C). No significant differences were observed in sEV concentration and mode in the different study groups (Table 2), with the exception of the higher mode of particles (102.4 ± 0.8 vs. 79.8 ± 19.8, \(p < 0.05\)) in the LAER group compared to the controls.

Table 2

| Group         | Particle concentration (particles/mL plasma) | Mode of particles (nm) |
|---------------|---------------------------------------------|------------------------|
| Controls      | \(7.9 \times 10^8 \pm 3 \times 10^8\) (4.7 \(\times 10^8\)–1 \(\times 10^9\)) | 79.8 ± 19.8 (48.2–102.9) |
| EALR          | \(7.3 \times 10^8 \pm 1 \times 10^8\) (6.6 \(\times 10^8\)–8.0 \(\times 10^8\)) | 78.2 ± 14.9 (67.6–88.7) |
| LAER          | \(7.1 \times 10^8 \pm 3 \times 10^8\) (5.3 \(\times 10^8\)–8.9 \(\times 10^8\)) | 102.4 ± 0.8 (101.8–102.9)* |

Mann–Whitney U-test was used to identify differences between groups. Data are presented as mean ± S.D (range). EALR women with elevated aldosterone and low renin, LAER women with low aldosterone and elevated renin. *\(p < 0.05\)

Determination of AT1R and LCN2 proteins in plasma sEVs from pregnant women

A qualitative and semiquantitative analysis by western blot for AT1R and LCN2 protein was performed in plasma EV
lysates from EALR and LAER pregnant women compared to controls. AT1R and LCN2 proteins from EVs of EALR women showed no significant differences compared to controls (Fig. 2D), although LCN2 protein abundance tended to be higher in the EALR group compared to controls ($p = 0.057$) (Fig. 2D). In addition, the protein abundances of AT1R and LCN2 in sEVs were similar between LAER and the control group (Fig. 2E).

**Discussion**

In the present study, we evaluated the levels of aldosterone and renin in a cohort of 54 normotensive pregnant women. We also described for the first time the presence of AT1R and LCN2 proteins in sEVs isolated from maternal circulation, which have been considered potential biomarkers of RAAS and MR activity, respectively.

Currently, there is no normal range that identifies lower and upper thresholds for normal circulating concentrations of aldosterone and renin (DRC) during pregnancy, which is helpful to determine when these physiological changes can become pathophysiological changes. Plasma renin activity (PRA) has traditionally been used to calculate the ARR; however, measurement of DRC has become increasingly popular. DRC assays are still in evolution, and generally a conversion factor of PRA (ng/mL/h) to DRC (mU/L) is $8.2 - 12$ is accepted [36]. We propose the use of the interquartile range (25th percentile–75th percentile) to identify a normal range for renin and aldosterone in a normotensive group of pregnant women. Our results confirmed that aldosterone and renin levels are higher in pregnancy than in nonpregnant women [3, 4]. Although certain groups of the women showed either elevated levels of aldosterone or renin (EALR and LAER groups), none of these pregnant women showed clinical symptomatology of high BP during pregnancy.

In the EALR group (Fig. 1A), we suggest that these women have increased renin-independent aldosteronism, which is a condition similar to that seen in primary aldosteronism [28, 37–39], where aldosterone secretion is independent of renin and could be attributed generally to the presence of an autonomous secretion of aldosterone, which may be due to adrenal hyperplasia, aldosterone-producing cell clusters, or aldosterone-producing adenoma [40–42]. In this regard, previous studies in normotensive populations have shown the existence of a continuum of renin-independent aldosteronism [30] and a higher risk of developing arterial hypertension in normotensive subjects with elevated ARR [29]. Considering these findings, we suggest that normotensive pregnant women with EALR have an increased likelihood of future developing hypertension or CV diseases, especially in presence of a second factor or challenge (e.g., ischemia/reperfusion, excessive high salt intake, electrolytic imbalance, exogenous/endogenous metabolites, placental sEVs, miRNAs, etc.). Hence, complementary, and longitudinal studies in these pregnant women are strongly encouraged.

With respect to the women from the LAER group (Fig. 1B), we suggest that the hyperreninemic phenotype caused by an increase in circulating renin mainly derived from the placenta and maternal decidua could favor the synthesis and activity of Ang-II [43, 44]. In this regard, Lumbers et al. postulated that the oversecretion of placental renin and placental exosomes enriched with renin and other functional components of the renin–angiotensin system (e.g., renin, Ang-II, AT1R, AT1R-AA, miRNAs) could directly affect maternal vascular physiology and BP [44].

In respect to plasma electrolytes Na$^+$ and K$^+$, we did not find differences between groups. These results are similar to previous studies in normotensive pregnant women [45, 46], and suggest the aldosterone and renin changes in these women are not a compensatory response to sodium loading or electrolytic imbalance, and should respond to alterations of the normal aldosterone or renin physiology.

Recent evidence concerning the role of sEVs (exosomes) in the pathophysiology of pregnancy highlights the impact of sEVs and their cargo on metabolism and function over receptor cells and tissues. Studies about the cargo of sEVs and MR activation have been carried out in human and animal models [47, 48]. However, studies on the sEV concentration and cargo in normotensive pregnancies with alterations in aldosterone–renin metabolism have not been performed to date. In this study, we detected for the first time the presence of AT1R and LCN2 proteins in plasma sEVs from normotensive pregnant women. sEVs from EALR and LAER pregnancy did not show any changes in the protein abundance of EV-AT1R or EV-LCN2; however, there was a trend toward increased levels of EV-LCN2 in the EALR group. Although this finding was not significant, it could become so if a larger cohort of pregnant women were studied because increased EV-LCN2 expression has been previously associated with MR activation by aldosterone [18, 19]. Moreover, a significant increase in the sEV mode was found in the LAER group compared to the controls, suggesting that in LAER, the nanovesicles found in plasma are larger and may have a different biogenesis [49] (Table 2).

In summary, we found normotensive pregnant women having either a high circulating aldosterone (9%) or a high plasma renin (9%) level. Despite both normal BP and normal plasma electrolytes, these abnormal ARRs suggest subclinical status similar to primary aldosteronism or to hyperreninemia, respectively. In these women, both are subclinical conditions that could evolve to pathological conditions by altering the maternal vascular and renal physiology and, subsequently the BP [44, 50]. In this
respect, the presence of AT1R and LCN2 proteins in plasma sEVs isolated from pregnant women should be further explored as potential biomarkers of subclinical conditions associated with an unregulated RAAS.

Author contributions V.P., A.L., C.A.C., C.E.F. and A.T.-C. designed the study, collected, analyzed, interpreted the patients data, and wrote the first draft of the manuscript. All authors contributed to the discussion, reviewed the manuscript, and approved the final version.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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