RESEARCH

Adrenomedullin: new inhibitory regulator for cortisol synthesis and secretion

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Abstract

Preterm birth is associated with immaturity of several crucial physiological functions notably those prevailing in the lung and kidney. Recently, a steroid secretion deficiency was identified in very preterm neonates, associated with a partial yet transient deficiency in 11β-hydroxylase activity, sustaining cortisol synthesis. However, the P450c11β enzyme is expressed in preterm adrenal glands, we hypothesized an inhibition of cortisol production by adrenomedullin (ADM), a peptide highly produced in neonates and whose effect on steroidogenesis remains poorly known. We studied the effects of ADM on three models: 104 cord-blood samples of the PREMALDO neonate cohort, genetically targeted mice overexpressing ADM, and two human adrenocortical cell lines (H295R and HAC15 cells). Mid-regional-proADM (MR-proADM) quantification in cord-blood samples showed strong negative correlation with gestational age ($P = 0.0004$), cortisol production ($P < 0.0001$), and 11β-hydroxylase activity index ($P < 0.0001$). Mean MR-proADM was higher in very preterm than in term neonates (1.12 vs 0.60 nmol/L, $P < 0.0001$). ADM-overexpression mice revealed a lower 11β-hydroxylase activity index ($P < 0.05$). Otherwise, aldosterone levels measured by LC-MS/MS were higher in ADM-overexpression mice (0.83 vs 0.46 ng/mL, $P < 0.05$). More importantly, the negative relationship between adrenal ADM expression and aldosterone production found in control was lacking in the ADM-overexpression mice. Finally, LC-MS/MS and gene expression studies on H295R and HAC15 cells revealed an ADM-induced inhibition of both cortisol secretion in cell supernatants and *CYP11B1* expression. Collectively, our results converge toward an inhibitory effect of ADM on glucocorticoid synthesis in humans and should be considered to explain the steroid secretion deficiency observed at birth in premature newborns.

Key Words

- adrenomedullin
- adrenal
- cortisol
- *hCYP11B1*
- prematurity

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Introduction

Corticosteroid hormones are synthesized in the adrenal cortex, an endocrine gland that produces mineralocorticoids in the *zona glomerulosa*, glucocorticoids in the *zona fasciculata,* and adrenal androgens in the *zona reticularis*. Each zone is thereby dedicated to a specific steroidogenic pathway under specific regulatory mechanisms (Miller & Auchus 2011). Glucocorticoid production and secretion are mainly under the control of ACTH whereas mineralocorticoid synthesis responds to angiotensin II (Ang II) and potassium (K^+) (Miller & Auchus 2011).

Prematurity is known to be associated with renal tubular immaturity that leads to poor electrolyte and water homeostasis, with major urinary sodium waste (Sulyok *et al.* 1979, Holtbäck & Aperia 2003). This transient physiological condition is linked to an inappropriate response to corticosteroid hormones at birth, as recently shown by the PREMALDO cohort (Martinerie *et al.* 2015).

An exhaustive steroidomic analysis by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) revealed in this cohort a defect of both cortisol and aldosterone secretion in very preterm neonates. This defect was supported by a partial yet transient deficiency in 11β-hydroxylase activity (P450c11β, encoded by *CYP11B1*), at birth and at day 3 of life (Travers *et al.* 2017*a*), despite the fact that this enzyme is clearly expressed in adrenal glands at this developmental stage (Naccache *et al.* 2016). This latter enzymatic activity (P450c11β), encoded by the *CYP11B1* gene, is required to transform 11-deoxycortisol (S) into cortisol (F) (Schiffer *et al.* 2015). However, the expression of *CYP11B1* has never been quantified according to the degree of prematurity. The reason for this partial deficiency remains unclear, but several hypotheses were made.

In order to elucidate the mechanisms of such a defect in steroidogenesis, we took a particular interest in the 52-amino acid peptide adrenomedullin (ADM), highly expressed in newborns (Marinoni *et al.* 1999, De Martin *et al.* 2014), whose plasma concentration is known to be increased during birth stress (Boldt *et al.* 1998) and used as a biomarker for brain injury in preterm infants (Gazzolo *et al.* 2001, Risso *et al.* 2012).

However, no study has assessed the action of ADM on cortisol synthesis, and the impact of ADM upon aldosterone production remains controversial. On one hand, ADM was shown to stimulate the renin-angiotensin-aldosterone system (RAAS) by increasing renin secretion through its vasodilatory effect (Jensen *et al.* 1997), to enhance steroid secretion *ex vivo* (Mazzocchi *et al.* 1996*a*) and to increase aldosterone synthase (P450c11AS, encoded by *CYP11B2*) expression in H295R cells, a human adrenal cell line model (Thomson *et al.* 2001). On the other hand, ADM was reported to exert *ex vivo* and *in vivo* an inhibitory effect over aldosterone production in the rat (Yamaguchi *et al.* 1995, 1996, Mazzocchi *et al.* 1996*b*). These results are further complicated by the possibility of an autocrine and paracrine role of ADM produced in the human adrenal medulla (Thomson *et al.* 2001, 2003).

ADM production is mainly reported in the lung, kidney, placenta, adrenal medulla (hence its name), and cardiovascular system, as ADM is mainly synthesized and secreted by endothelial and vascular smooth muscle cells (Schönauer *et al.* 2017). The human ADM is encoded by a single gene located on chromosome 11 and transcripts lead to the prepro-ADM, a precursor converted into the pro-ADM that is then processed into four peptides: pro-ADM N-terminal 20 peptide, mid regional pro-ADM (MR-proADM) lacking any biological activity (Ishimitsu *et al.* 1994), immature ADM, and adrenotensin (Ishimitsu *et al.* 1994, Kitamura *et al.* 2002). Immature ADM is transformed into a bioactive peptide by amidation of its C-terminal part (Kitamura *et al.* 1998). ADM's half-life is relatively short (22 min), whereas MR-proADM's is several hours long (Valenzuela-Sánchez *et al.* 2016). As MR-proADM is secreted in equimolar quantity than ADM, MR-proADM is largely used as a biomarker of ADM production (Vigué *et al.* 2016, Gille *et al.* 2017, Krintus *et al.* 2018).

ADM effects are mediated through a multiprotein complex receptor consisting of the calcitonin-like receptor (CLR) and a co-receptor (receptor activity-modifying protein or RAMP) (Schönauer *et al.* 2017). Three types of RAMP have been described, and the association of RAMP2 or RAMP3 with CLR is known to have the highest specificity for ADM binding (McLatchie *et al.* 1998). Homozygous knockout mouse models for ADM are embryonic lethal (Caron & Smithies 2001), whereas an ADM-overexpressing mouse model presents with cardiac hyperplasia (Wetzel-Strong *et al.* 2014) improved lymphangiogenesis (Trincot *et al.* 2019), and revealed that ADM played a role in the innate immune milieu of the placenta (Li *et al.* 2013).

In this study, we hypothesized that the partial defect in steroid secretion observed during the first 3 days of human life, in very preterm neonates, was linked to the ADM signaling pathway. We measured MR-proADM in cord-blood samples from the PREMALDO cohort and performed *in vitro* studies to explore the underlying mechanisms that could lead to the partial defect in *CYP11B1* expression in this fragile population. We also used an ADM-overexpressing mouse model to investigate

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the potential impact of ADM *in vivo* and demonstrated that ADM inhibits cortisol production and secretion in two human H295R and HAC15 adrenocortical cell lines.

Materials and methods

Reagents

Forskolin (FK), adrenomedullin (ADM), and angiotensin II (Ang II) were obtained from Merck–Sigma–Aldrich. Another supplier for ADM was ThermoFisher but showed no pharmacological activity.

Newborn samples

MR-proADM quantification was performed on 104 cord-blood sample leftovers of the PREMALDO cohort already described (Martinerie *et al.* 2015). Newborns were sorted according to their gestational age: 30 very preterm (<33 gestational weeks (GW)), 43 preterms (33–36 GW), and 31 term neonates (≥37 GW). Precise gestational ages in days were available for 66 neonates. Steroidomic profiles were previously performed on these cord-blood samples and discussed elsewhere (Travers *et al.* 2017*a*).

ADM-overexpression mouse model

A mouse model overexpressing ADM (*Adm*$^{hi/hi}$) has been previously described. Briefly, the endogenous ADM mRNA was stabilized by replacing the 3'-UTR of *Adm* gene with that of the bovine growth hormone in a C57BL/6J genetic background, which allows approximately to triple the basal circulating ADM concentrations (Wetzel-Strong *et al.* 2014). The *Adm*$^{hi/hi}$ mice are maintained on an isogenic C57BL/6J homozygous background breeding scheme. Therefore, *Adm*$^{hi/hi}$ and C57BL/6J WT mice used in these studies were maintained and generated on isogenic genetic backgrounds. We studied seven WT male mice aged from 117 to 133 days, and six *Adm*$^{hi/hi}$ male mice aged from 34 to 159 days, with blood samples and two adrenal glands from all mice. Animals were housed in specific pathogen-free housing with Tecniplast Greenline ventilated micro isolator cages. The mice had a 12 h light: 12 h darkness cycle from 07:00 on to 19:00 h off, with a temperature ranging between 68 and 74°F (20–23°C) and humidity between 30 and 70%. Blood samples were collected *via* retro-orbital bleeds. Whole blood was collected in BD Microtainer tubes with BD SST additive. Whole blood was placed on ice for 30 min, then spun at 8000 ***g*** at 4°C. The supernatant was collected, then frozen at –80°C prior to shipping on dry ice. Mice were euthanized with carbon dioxide and secondary cervical dislocation before being dissected for adrenal glands.

All animal studies were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Cell culture

The human adrenocortical carcinoma H295R (RRID:CVCL_0458) and HAC15 (RRID:CVCL_S898) cells were tested and the latter were known for their higher steroidogenic capacities (Rainey *et al.* 2004). Cells were cultured in DMEM/HAM'S F12 medium (GE Healthcare) supplemented with 10% fetal calf serum (Biowest, Nuaillé, France), 20 mM HEPES (Life Technologies), penicillin (100 IU/mL), streptomycin (100 µg/mL), 2 mM glutamine, and 50 nM sodium selenite (Sigma), 5 µg/mL transferrin (Life Technologies), and 5 µg/mL insulin (Sigma), at 37°C in a humidified atmosphere of 95% air and 5% CO_2. HAC15 cells were cultured on type I collagen-coated plates (final solution concentration: 40 µg/mL, with 300 µL in each 12-well plates). Type I collagen was provided by Institut de Biotechnologies Jacques Boy (Reims, France).

For stimulation experiments, cells were seeded at a density of 150 × 10^3 cells/well. After 3 days of cell division with daily renew medium, cells were incubated in a complete medium supplemented with 10% dextran-coated charcoal serum and FK (20 µM), ADM (1 µM), Ang II (1 µM), potassium (K^+, 10 mM), or the combination (FK+ADM) at described concentrations. Three hours stimulation was conducted for gene expression study, whereas 24 h stimulation was necessary for steroid secretion determination.

Hormone assays

Steroids levels in cell culture supernatants or in plasma/serum samples were assessed by liquid chromatography coupled to tandem mass spectrometry as previously described (Travers *et al.* 2017*b*). Briefly, supernatants were collected from cell cultures and centrifuged at 9500 ***g*** for 5 min. Two hundred microliters of the supernatant were then extracted by the SPE method with deuterated internal standards and injected into the chromatographic system. Steroid hormones quantified are referred as follows: 11-deoxycorticosterone (DOC), corticosterone (B), 18-hydroxy-corticosterone (18OHB), aldosterone (Aldo), 11-deoxycortisol (S), and cortisol (F).

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MR-proADM was assessed in cord-blood samples using TRACE (time-resolved amplified cryptate emission) technology, on a Kryptor compact PLUS® (ThermoFisher Scientific) (Caruhel *et al.* 2009).

Reverse transcription quantitative PCR (RT-qPCR)

RT-qPCR was performed as previously described (Viengchareun *et al.* 2009). Briefly, total RNAs were extracted using the Nucleospin RNAII® (Macherey Nagel, Hoerdt, France). After DNAse I treatment (Biolabs), total RNAs were reverse-transcribed using the High-Capacity cDNA RT Kit (Life Technologies). Samples were analyzed by quantitative PCR using the Power SYBR Green PCR Master Mix (Life Technologies) with the indicated primers (from Eurogentec, Liège, Belgium, Table 1) in a QuantStudio 6 Instrument (Life Technologies). For *hCYP11B2* expression quantification, we used a TaqMan probe (Life Technologies) whose sequence was CGCCTTCAACACTAC.

Relative expression analysis was determined with a standard curve. For the preparation of standards, amplicons were subcloned into pGEMT-easy plasmid (Promega) and sequenced to confirm the identity of the sequence. Standard curves were generated using serial dilutions of linearized standard plasmids, spanning 6 orders of magnitude. Standard and sample values were amplified in duplicate and analyzed from two independent experiments. Results are presented as mean ± S.D. and represent the relative expression of target genes normalized to *36b4* expression for mouse samples, and generally to geometric means of three housekeeping genes (*ACTB*, *36B4*, *GAPDH*) for *in vitro* human cell samples, using the BestKeeper program (Pfaffl *et al.* 2004).

Statistical analyses

Results were analyzed on GraphPad Prism Software (v.8). Statistical tests are indicated in each figure legend, and statistical significance is as follow, according to *P*-value: $*P<0.05$; $**P<0.01$; $***P<0.001$; $****P<0.0001$. Parametric correlations were performed using Pearson analysis, whereas non-parametric correlations were assessed using Spearman method.

Results

ADM quantification in PREMALDO cohort

We used the cord-blood leftovers of the PREMALDO cohort (5), composed of 30 samples from the very preterm (VTP)

Table 1 Primers used for RT-qPCR experiments. Forward and reverse primers are indicated for both human and murine genes explored.

Gene	Access number	Forward	Forward localization	Reverse	Reverse localization	Size (bp)
hCYP11B1	NM_000497.4	GGAGACACTAACCCAAGAGGACAT	Exon 9	ACGTGATTAGTTGATGGCTCTGAA	Exon 9	100
hCYP11B2	NM_000498.3	ATCTACCAGGAACTGGCCTTCA	Exon 5	TGACAGTTCCGCCTTCAACA	Exon 5	81
hADM	NM_001124.3	CCGAGTGTTTGCCAGGCTTA	Exon 4	GGTGACACGCCGTGAGAAAT	Exon 4	119
hCLR	NM_005795.6	AAGATTATGCAAGACCCCATTCA	Exon 5	ATCAGGGCAGAGCTGCATTG	Exon 6	120
hRAMP2	NM_005854.3	ACCAGATCCACTTTGCCAAC	Exon 4	CCTGGGCCTCACTGTCTTTA	Exon 4	153
hRAMP3	NM_005856.3	GCCATGGAGACTGGA	Exon 1	CATCATGTCTGCGAAAG	Exon 2	146
hACTB	NM_001101.5	GCATGGGTCAGAAGGATTCCT	Exon 3	ACACGCAGCTCATTGTAGAAGG	Exon 3	150
h36B4	NM_001002.4	CCCATTCTATCATCAACGGGTACAA	Exon 7	CAGCAAGTGGGAAGGTGTAATCC	Exon 7	75
hGAPDH	NM_002046.7	TGCACCACCAACTGCTTAGC	Exon 7	GGCATGGACTGTGGTCATGAG	Exons 7 and 8	86
mADM	NM_009627.2	GAGCGAAGCCCCACATTCGT	Exon 4	GAAGCGGCATCCATTGCT	Exon 4	75
mCYP11B1	NM_001033229.3	ACAAGCTGTAGACTTGTGGT	Exon 9	GAAACGCACTAAACAATTTC	Exon 9	53
mCYP11B2	NM_009991.4	TGAGTATGCCAACAGATGTA	Exon 4 & 5	ACAATGCCACTGTAGGTCT	Exon 5	77
m36B4	NM_007475.5	AGCGCGTCCTGGCATTGTCTGT	Exon 6	GGGCAGCAGTGGTGGCAGCAGC	Exon 7	128

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group, 43 from the preterm (PT) group, and 31 from the term neonate group. We highlighted a decrease in cord-blood ADM as pregnancy progresses. Therefore, VPT secrete much more ADM at birth than term neonates (median and 25th–75th percentile): 1.11 nmol/L (0.62–1.61) vs 0.66 nmol/L (0.35–0.84), $P < 0.0001$, as for PT whose ADM secretion is higher than term newborns (0.87 nmol/L (0.62–1.16), $P < 0.05$) (Fig. 1A). For 66 newborns whose gestational age was available in days, a negative correlation was observed between MR-proADM levels and gestational age ($r = -0.43$, $P = 0.0004$, Fig. 1B).

Moreover, studying the relationship between ADM and cortisol concentrations reported in Travers *et al.* (Travers *et al.* 2017*a*) for the PREMALDO cohort revealed a strong negative relationship ($y = -29.55 \times + 55.52$; $r = -0.53$, $P < 0.0001$; Fig. 1C). In addition, the activity of the P450c11β, estimated by the enzyme activity index (EAI) defined as a product-to-substrate ratio (F/S), was negatively correlated to MR-proADM levels ($y = -10.15 \times + 20.33$; $r = -0.40$, $P < 0.0001$; Fig. 1D). On the contrary, no relationship was found between MR-proADM levels and aldosterone secretion (Fig. 1E).

It is very likely from these results that the more premature the birth is, the more ADM is secreted. As preterm neonates are known to exhibit a steroid secretion deficiency at birth, we aimed at exploring the potential link between steroid production or secretion and ADM by studying steroid metabolomics in a genetically engineered mouse model overexpressing ADM, the $Adm^{hi/hi}$ mouse model (Kitamura *et al.* 2002).

ADM overexpression in mouse is associated with loss of the inverse relation between aldosterone secretion and ADM levels observed in WT mice

We first quantified ADM expression by RT-qPCR in total adrenal glands (Fig. 2A) and showed, as expected, a ~2-fold higher ADM expression in $Adm^{hi/hi}$ mice compared to WT controls ($P < 0.01$), consistent with prior characterization of the ADM-overexpression model.

Steroidomic profiling by LC-MS/MS showed no difference regarding corticosterone (B) secretion (Fig. 2B) but a lower corticosterone-to-11-deoxycorticosterone (B/DOC) ratio in $Adm^{hi/hi}$ mice ($P < 0.05$, Fig. 2C). As B can be formed from DOC by P450c11β or P450cAS, this latter (B/DOC) ratio reflects activities from both enzymes (P450c11β and P450cAS). As 18OHB/B and Aldo/18OHB

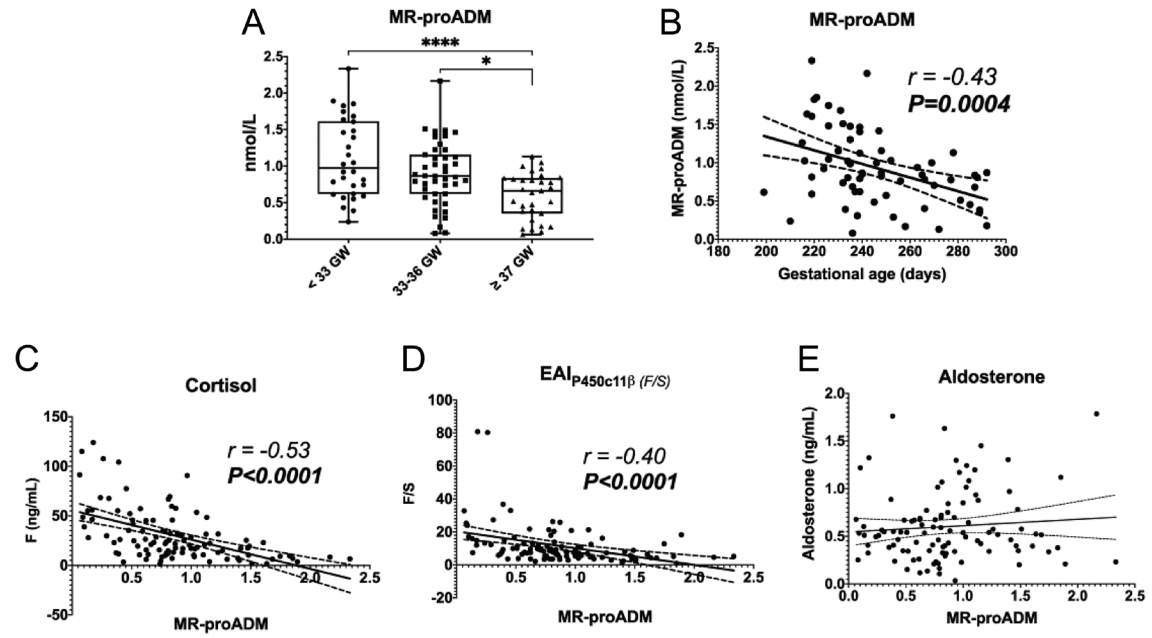

Figure 1
Adrenomedullin and corticosteroid levels in cord blood of human newborns. MR-proADM has been assessed by TRACE technology on 104 cord-blood sample left overs. Among them, gestational ages of 66 neonates were collected in days. (A) MR-proADM levels in cord blood according to gestational age (all values, median, and interquartiles). ANOVA and Tukey's post-tests: *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$ – GW, gestational weeks. (B) Relationship between MR-proADM levels and gestational age (Pearson: $r = -0.43$, $P = 0.0004$). (C) Relationship between cortisol and MR-proADM levels at birth (Pearson: $r = -0,53$, $P < 0.0001$). (D) Relationship between $EAI_{P450c11\beta}$ (F/S ratio) and MR-proADM levels at birth (Pearson: $r = -0,40$, $P < 0.0001$). (E) Relationship between MR-proADM and aldosterone levels at birth (ns).

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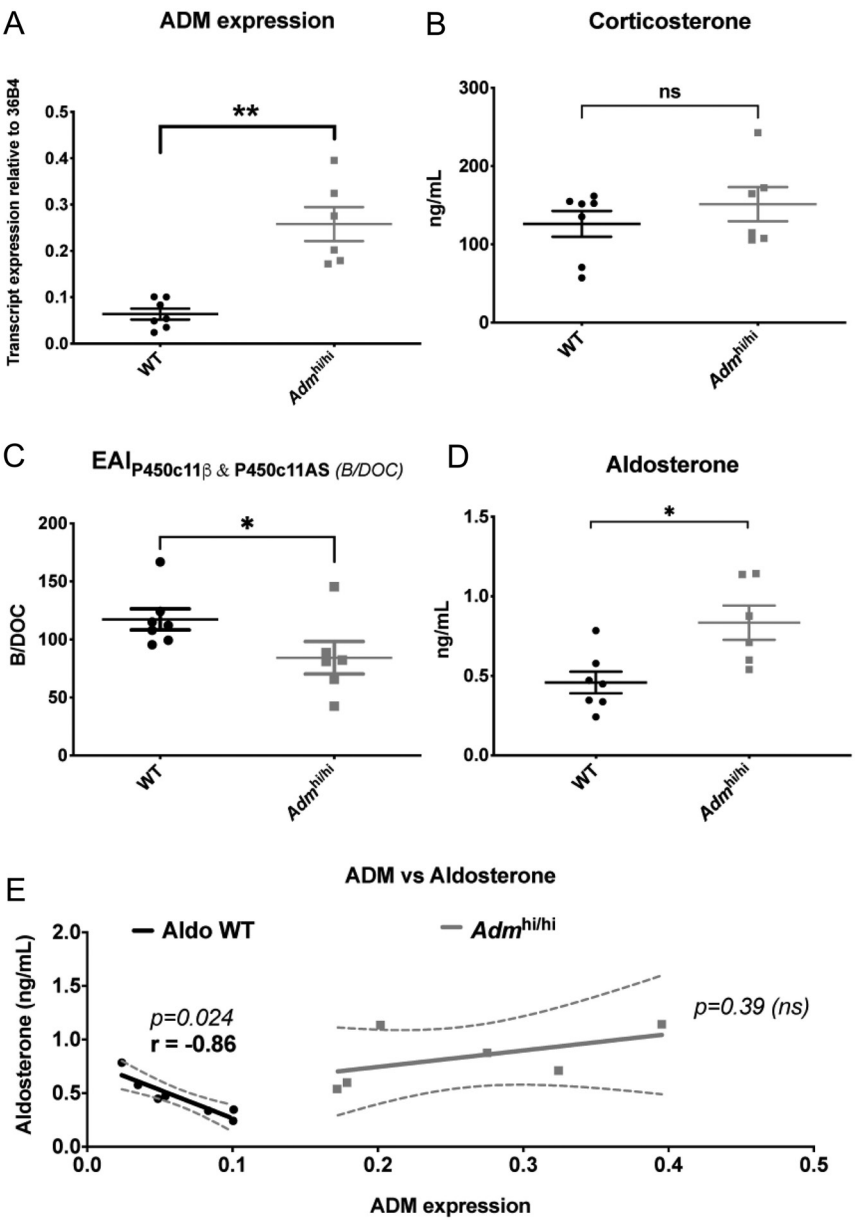

Figure 2
ADM expression and steroid secretion of *Adm*$^{hi/hi}$ mice and WT littermates. ADM expression was quantified by RT-qPCR on adrenal glands for each animal after mRNA extraction. Steroids levels were assessed on serum samples by LC-MS/MS. (A) ADM expression (RT-qPCR/*36B4*; non-parametric Mann–Whitney *U-test*: $P = 0.001$). (B) Corticosterone secretion (LC-MS/MS; non-parametric Mann–Whitney *U-test*: *ns*). (C) EAI of P450c11β and P450c11AS (B/DOC ratio) (non-parametric Mann–Whitney *U-test*: $P = 0.03$). (D) Aldosterone secretion (LC-MS/MS; non-parametric Mann–Whitney *U-test*: $P = 0.01$). (E) Relationship between ADM expression and aldosterone secretion. *Adm*$^{hi/hi}$, mice overexpressing; ADM, correlation performed by Spearman analysis.

ratios that reflect P450cAS activity were unchanged (data not shown), the decreased B/DOC ratio could mainly be explained by P450c11β inhibition.

However, no difference in *mCyp11b1* or *mCyp11b2* expression was observed in adrenal glands of both groups, as determined by RT-qPCR (data not shown).

Surprisingly, steroid metabolomic studies allowed us to show an increase in serum aldosterone (0.83 ± 0.11 vs 0.46 ± 0.07 ng/mL, $P < 0.05$; mean ± S.E.M., Fig. 2D) and 18-hydroxy-corticosterone concentrations in *Adm*$^{hi/hi}$ mice (data not shown), suggesting an increase in mineralocorticoid secretion.

Interestingly, by studying the relationship between expressed ADM and serum aldosterone concentration, we found a strong negative correlation in WT mice (Fig. 2E). Thus, linear regression found a –5.3 ± 1.0 slope (mean ± S.D.) with $r^2 = 0.84$. Correlation Spearman test found a $r_{Spearman} = -0.86$ ($P = 0.024$). On the contrary, this relationship was lacking in the *Adm*$^{hi/hi}$ mice, suggesting that the strong relationship between aldosterone secretion and ADM production observed in WT animals was abolished in *Adm*$^{hi/hi}$ mice. To better understand the regulatory impact of ADM on steroidogenesis, we conducted *in vitro* experiments on two human adrenal cell models.

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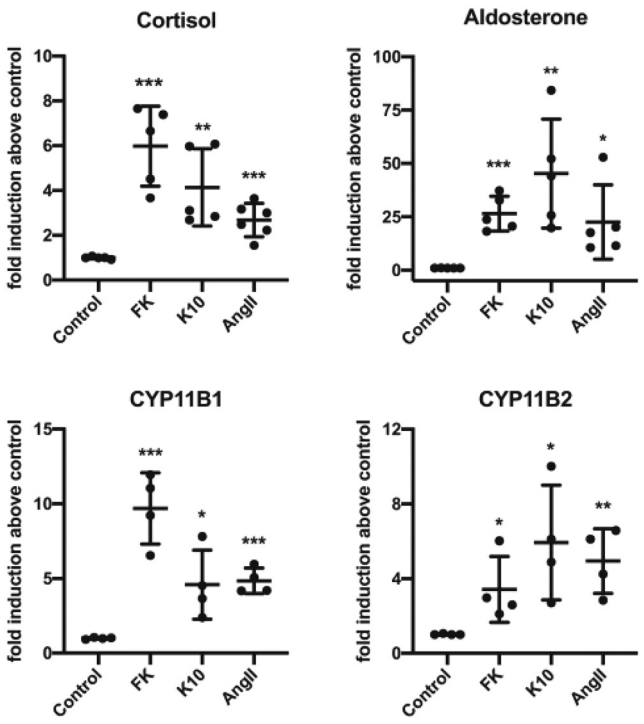

Figure 3
Effects of various agonists on steroid secretion and gene expression of H295R cells. H295R cells were stimulated with forskolin 20 µM (FK), potassium 10 mM (K10), and angiotensin II 1 µM (Ang II) for 3 h for *CYP11B2* and *CYP11B1* expression studies (RT-qPCR) for 24 h for steroid secretion quantification (LC-MS/MS). (Upper panel) Mean ± S.D. of cortisol and aldosterone increases in cell supernatants after 24 h stimulation. (Lower panel) Mean ± S.D. of the rise of *CYP11B1* and *CYP11B2* expressions after 3 h stimulation. Each point represents an independent experiment and is the mean of 4 to 6 wells. ANOVA followed by Tukey post-tests *vs control: *P* < 0.05*; **P* < 0.01*; ***P* < 0.001.

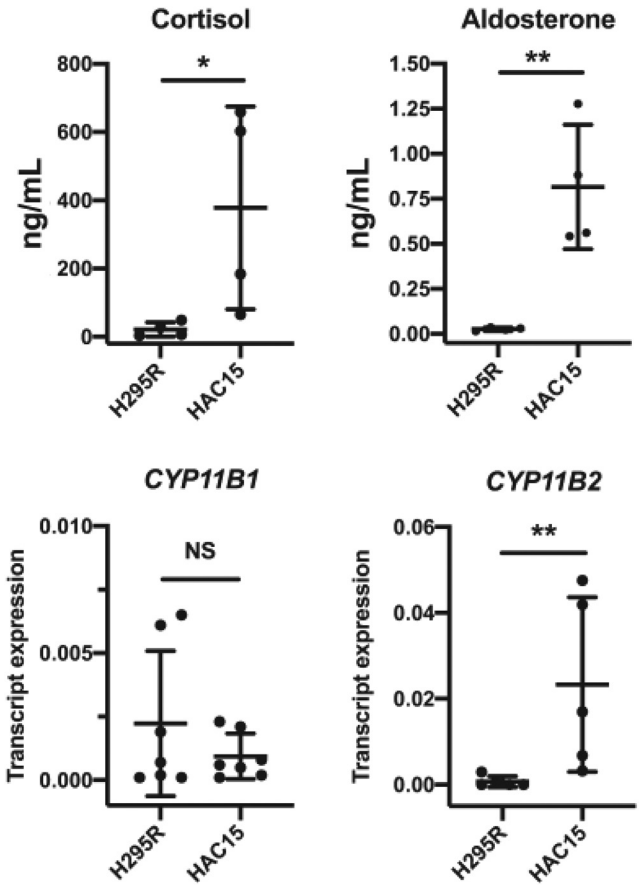

Figure 4
Comparison of basal steroid secretion and enzyme expression between adrenocortical human H295R and HAC15 cells (mean ± S.D.). Cortisol and aldosterone were measured in cell supernatants by LC-MS/MS, and *CYP11B1* and *CYP11B2* mRNA were quantified by RT-qPCR (expressed to geometric means of three housekeeping genes (*GAPDH, 36B4,* and *ACTB*)). Each point represents an independent experiment and is the mean of 4 to 6 wells. Non-parametric Mann–Whitney *U*-tests: **P* < 0.05*; **P* < 0.01.

Comparison of adrenocortical carcinoma cells responses to various stimuli

Since H295R cellular responses to different agonists have been described as highly variable according to culture conditions (Rainey *et al.* 2004), we first examined the experimental conditions in which this cell model was responding to classical stimulations. Figure 3 presents how the H295R cells are able to respond to FK (20 µM), potassium (10 mM), and Ang II (1 µM). As anticipated, steroid overproductions are observed on the secretions of aldosterone (mean-fold induction over control ± S.D.: 26.5 ± 8.2 with FK; 45.2 ± 25.5 with K; 22.6 ± 17.4 with Ang II) and of cortisol (6.0 ± 1.9 with FK; 4.1 ± 1.9 with K; 2.7 ± 0.7 with Ang II) after 24 h stimulation while *CYP11B2* (3.4 ± 1.8 with FK; 5.9 ± 3.1 with K; 4.9 ± 1.7 with Ang II) and *CYP11B1* expressions (9.7 ± 2.4 with FK; 3.5 ± 1.5 with K; 5.0 ± 1.2 with Ang II) were significantly increased in H295R cells after 3 h incubation.

Although H295R cells appeared to be a good model to investigate the regulation of corticosteroid synthesis, the mineralocorticoid pathway remains poorly expressed, as very little basal aldosterone is secreted and scarce quantity of *CYP11B2* transcript is found.

We tested another human adrenal cell model, derived from H295R cells: the HAC15 cells, known to produce more mineralocorticoids (Parmar *et al.* 2008). Indeed, we found, under basal conditions, a higher secretion of cortisol (over 14-times higher, $P=0.028$) and aldosterone (over 31-times higher, $P=0.004$), along with a higher expression of *CYP11B2* ($P=0.008$) compared to H295R cells (Fig. 4).

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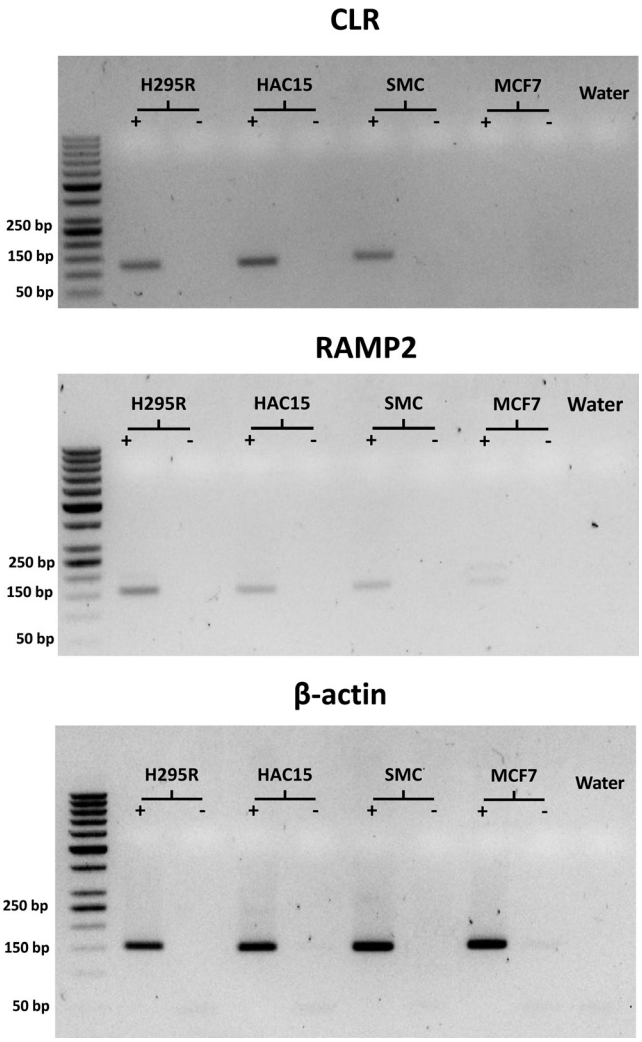

Figure 5
Expression of ADM receptor complex in H295R and HAC15 cells. The expression of CLR and RAMP2 was assessed by RT-PCR (from 1 µg RNA) and PCR fragments (10 µL) were analyzed after agarose (2%) electrophoresis. The ladder molecular weight marker (GeneRuler 50 bp DNA Ladder, ThermoScientific) was shown in the left part of each gel. Note on the right panel the expression of β-actin used as control. (Left panel) CLR expression (120 bp) 1, H295R cells; 2, HAC15 cells; 3, SMC cells; 4, MCF7 cells. (Right panel) RAMP2 expression (153 bp). 1, H295R cells; 2, HAC15 cells; 3, SMC cells; 4, MCF7 cells. (Lower panel) β-actin expression (150 bp). 1, H295R cells; 2, HAC15 cells; 3, SMC cells; 4, MCF7 cells.

However, H295R and HAC15 cells showed no significant difference in *CYP11B1* expression.

Adrenocortical carcinoma cells are sensitive to ADM

Furthermore, we tested whether H295R and HAC15 cells were able to express ADM complex receptor. Identification of ADM complex receptor (CLR+RAMP2) was performed by classical RT-PCR and agarose gel electrophoresis, and breast cancer MCF7 cells were used as a negative control. Both cell lines express the ADM-receptor complex and, therefore, should be also able to respond to ADM treatment (Fig. 5). Therefore, we decided to pursue our study on both H295R and HAC15 cells.

ADM inhibits steroidogenesis *in vitro*

H295R and HAC15 cells were cultured on collagen I-coated dishes and stimulated with ADM (1 µM) or FK (20 µM) alone or in combination. Cortisol and aldosterone secretions were quantified by LC-MS/MS in the supernatants after 24 h stimulation and *CYP11B1* and *CPY11B2* expression quantified by RT-qPCR after 3 h stimulation.

ADM alone failed to show any effect on steroid secretions nor on *CYP11B1* and *CYP11B2* expression on both cell lines (Fig. 6).

However, regarding glucocorticoid production, ADM reduced FK-induced cortisol secretion in both H295R cells (6.4-fold vs 4.2-fold, $P=0.02$; Fig. 6A) and HAC15 cells (4.9-fold vs 2.5-fold, $P=0.04$; Fig. 6D). The cortisol-to-11-deoxycortisol (F/S) ratio, assessing the P450c11β activity, significantly increased with FK in H295R cells and HAC15 cells (6.5-fold, $P<0.0001$; Fig. 6B and 5.2-fold, $P<0.0001$; Fig. 6E), respectively. Moreover, ADM significantly reduced this FK-induced stimulation of F/S ratio in H295R cells (3.4-fold, $P=0.04$; Fig. 6B) and in HAC15 cells (3.0-fold, $P=0.03$; Fig. 6E). Similarly, ADM reduced FK-induced *CYP11B1* expression in both H295R (19.8-fold vs 8.6, $P=0.04$; Fig. 6C) and HAC15 cells (6.5-fold *vs.* 2.7-fold, $P=0.03$; Fig. 6F).

However, both H295R and HAC15 cells did not allow us to elucidate the effects of ADM on the mineralocorticoid synthesis pathway as supported by the literature, whether in a positive or negative manner. Thus, no effect of ADM was observed neither on aldosterone secretion nor on *CYP11B2* expression (data not shown).

Discussion

Prematurity, accounting for 10% of births worldwide, is linked to numerous risks and complications as a result of organ immaturity (Frey & Klebanoff 2016). Steroidogenesis is one of the many biochemical processes impacted by prematurity, with a partial defect in steroid secretion at birth (Travers *et al.* 2017*a*) along with a partial yet transient defect in 11-hydroxylase activity despite the fact that P450c11β is expressed in the fetal adrenal cortex (Naccache *et al.* 2016) but never evaluated according to

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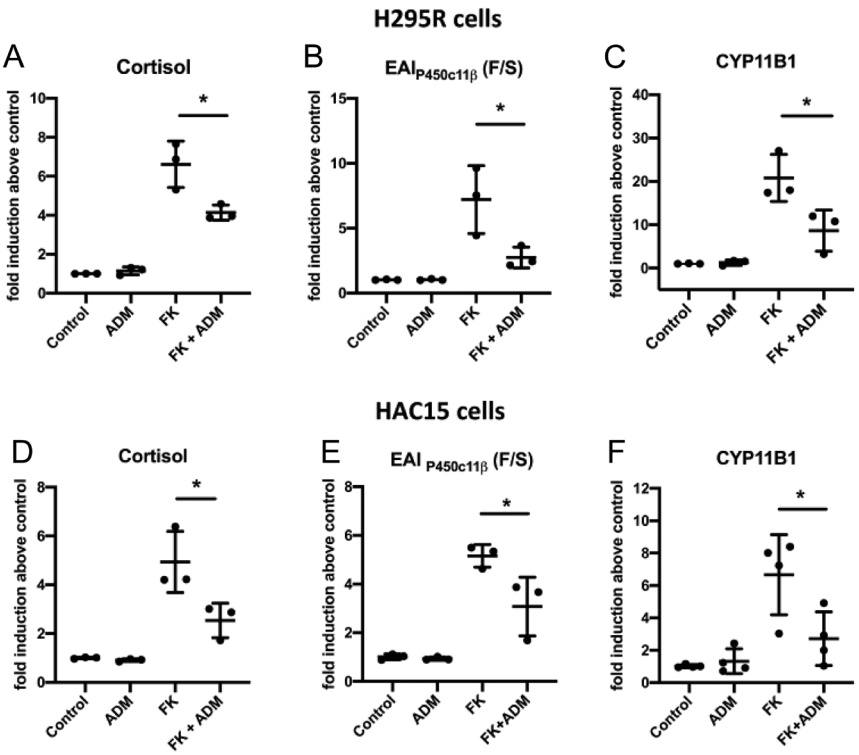

Figure 6
Effects of ADM on steroidogenesis in H295R and HAC15 cells. H295R (A, B, C) and HAC15 (D, E, F) cells were incubated or not with forskolin (FK, 20 µM) and/or ADM (1 µM) for 24 h for cortisol (F) and 11-deoxycortisol (S) analyses by LC-MS/MS and 3 h for *CYP11B1* expression measured by RT-qPCR. (A and D) cortisol secretion; (B and E) F/S ratio assessing P450c11β activity; (C and F) *CYP11B1* expression. Data are presented as mean ± S.D., and each point represents an independent experiment and is the mean of 4 to 6 wells. Non-parametric Mann–Whitney *U*-tests FK *vs* FK+ADM *$P < 0.05$.

the prematurity degree. Many regulators of corticosteroid synthesis have been thoroughly described (hypothalamic-pituitary axis (HPA), RAAS, kalemia), while some others have been recently discovered: bile acids (Liu *et al.* 2019), catecholamines (Mokuda *et al.* 1992), serotonin (Lefebvre *et al.* 2001), substance P (Wils *et al.* 2020), or adrenomedullin (ADM) (Kita *et al.* 2010).

To our knowledge, the effects of ADM on glucocorticoid production have not been studied, and those upon mineralocorticoid synthesis remain largely controversial.

The present study, performed on the cord-blood sample leftovers of the PREMALDO cohort, reveals that MR-proADM secretion is negatively correlated with gestational age. This biomarker has been chosen for its stability with higher plasma half-life than ADM itself, and for its reflection of ADM secretion (Schönauer *et al.* 2017). Previous papers already reported an increase of ADM in preterm neonates with intracerebral hemorrhage (Gazzolo *et al.* 2001), with bronchopulmonary dysplasia (Gong *et al.* 2020), or with perinatal infection (Admaty *et al.* 2012). It is also well established that ADM levels are higher in neonates than in adults (Koch *et al.* 2011) and that they normalized 2 days after birth in term neonates but remain elevated in preterm infants (Admaty *et al.* 2012). The biological function of ADM during human gestation, mostly synthesized by the placenta, remains to be fully elucidated. However, it is clear that ADM is required for placental vascular tonicity (Di Iorio *et al.* 1997), neonatal vascular adaptation (Boldt *et al.* 1998, Rudolph 1998), and local regulation of the immune system (Li *et al.* 2013).

Regarding cortisol synthesis, we highlight a strong negative correlation between ADM and cortisol productions, along with a similar negative relationship between ADM levels and F/S ratio, which constitutes an index of 11-hydroxylase activity. Investigation of a mouse model overexpressing ADM shows a decrease of the B/DOC ratio only in *Adm*$^{hi/hi}$ mice compared to WT littermates. These results are strongly in favor of a diminished 11-hydroxylase activity linked to ADM overexpression. Finally, in both human H295R and HAC15 cell models of corticosteroidogenesis, we demonstrate an inhibitory effect of ADM on cortisol secretion induced by FK associated with an inhibition of *CYP11B1* expression.

Glucocorticoid synthesis may be impacted by ADM at several levels. Even though our LC-MS/MS method did not allow us to quantify pregnenolone nor 17-hydroxypregnenolone, we could not exclude that ADM could inhibit 3-hydroxysteroid dehydrogenase activity. The synthesis of ADM is clearly ubiquitous in humans, including in the adrenal glands, making even more complex the interpretation of ADM effects on the HPA axis. *In vitro*, ADM inhibits ACTH secretion (Samson *et al.* 1995), an effect confirmed *in vivo* in sheep (Parkes & May 1995). In late gestation, the placenta secretes corticotrophin-releasing

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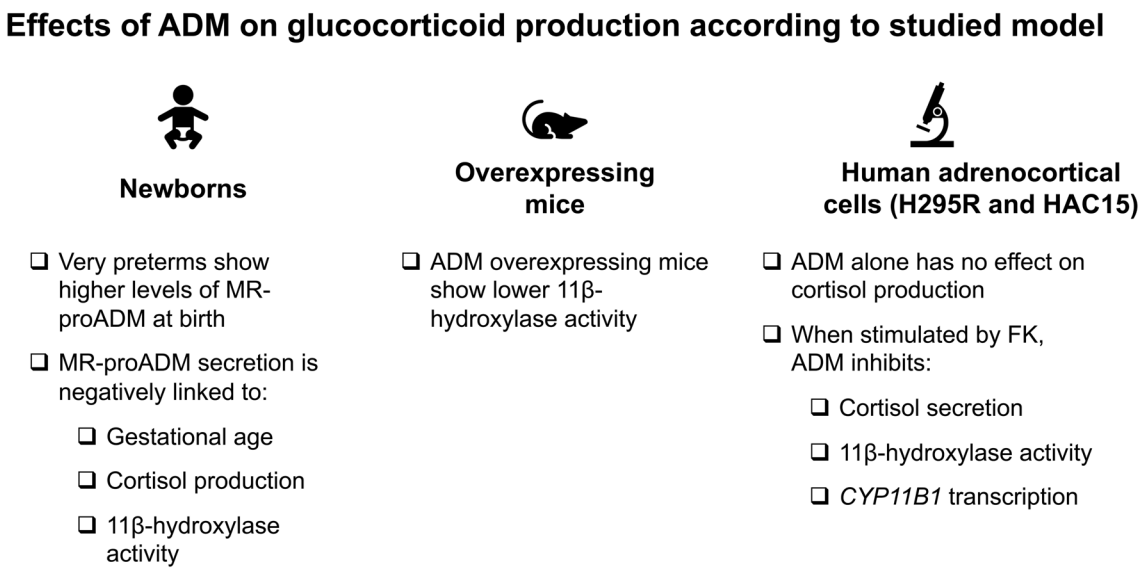

Figure 7
Effects of ADM on glucocorticoid production according to the model studied. Summary of main findings regarding the effects of ADM on glucocorticoid production in newborns at birth, in ADM-overexpressing mice, and in two human adrenocortical cell lines (H295R and HAC15).

hormone (CRH) (Smith & Nicholson 2007). It remains plausible that the placental ADM regulates the placental CRH secretion, thereby affecting steroid synthesis, both in the placenta and the fetus. Labor and delivery constitute a highly stressful period, with elevated circulating cortisol levels (Miller *et al.* 2019). It is likely that ADM plays a role in the regulation of the stress responses related to this critical period, as it has also been shown that ADM levels increase during labor (Boldt *et al.* 1998). It is worth considering that prenatal betamethasone administration is often given to pregnant mothers at risk of preterm delivery to facilitate lung maturation of the fetuses and ultimately to reduce the mortality of the very preterm neonates. However, this glucocorticoid administration could contribute to the HPA and corticosteroid synthesis suppression observed at birth in preterm neonates (Ng *et al.* 1997, Karlsson *et al.* 2000, Roberts *et al.* 2017) which cannot, therefore, be explained only by elevated ADM concentrations.

ADM alone showed no effect, whereas when used in combination with a steroidogenesis-stimulating compound, it clearly inhibits *CYP11B1* expression, reducing P450c11β activity and decreasing cortisol secretion. Figure 7 summarizes our main findings of ADM effects on glucocorticoid synthesis among the three models studied here (human newborns, ADM-overexpressing mice, and two human adrenocortical cell lines).

In 2003, Charles *et al.* found that ADM decreased the Ang II-induced aldosterone secretion. Similarly, an aldosterone decrease along with an increased plasma renin activity was found (Charles *et al.* 2003), consistent with the increase of aldosterone-to-renin ratio found in preterm neonates of PREMALDO cohort (Martinerie *et al.* 2015). However, we failed to observe an inhibitory effect of ADM regardless of the model used. Similarly, in 2000, Lainchbury *et al.* also reported an increase of renin secretion in healthy volunteers under ADM perfusion, without any modification of aldosterone levels (Lainchbury *et al.* 2000). Nevertheless, we observed a strong negative correlation between ADM and plasma aldosterone in the WT control mice. Such a negative relationship between ADM and ARR has been also highlighted in healthy volunteers of the KORA F4 study (Then *et al.* 2016*a*,*b*). ADM perfusion was shown to decrease aldosterone secretion in patients with primary aldosteronism as well as in control subjects (Kita *et al.* 2010).

Otherwise, in our mouse model, genetically engineered mice overexpressing ADM present with a paradoxical increase of aldosterone secretion. It has also been reported that ADM could increase steroid secretion (Thomson *et al.* 2001) and aldosterone synthase expression in H295R cells (Thomson *et al.* 2001). On the contrary, inhibition of mineralocorticoid production by ADM has also been reported, in the rat both *ex vivo* and *in vivo* (32–34). Collectively, our results did not provide any conclusive effect of ADM on mineralocorticoid synthesis, yet this topic remains to be further examined in other studies.

In our mouse model, the loss of the relationship between ADM levels and serum aldosterone concentrations in $Adm^{hi/hi}$ mice is quite intriguing. It appears that aldosterone synthesis regulation could be different according to an

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acute or chronic ADM production and its duration, as previously reported for ACTH stimulation (Cozza *et al.* 1989). Thus, under a physiological state, ADM could exert a fine regulation of aldosterone secretion along with what we found for cortisol production, indicating an inhibitory effect of ADM. On the contrary, in a situation where ADM is overexpressed starting from early developmental stages, it is likely that an adaptive mechanism to the chronic ADM-induced vasodilatation may exist. Therefore, the continuous stimulation of RAAS leads to an increase in aldosterone production.

In conclusion, we provide the first evidence for a direct relationship between plasma ADM and corticosteroid levels in newborns according to their gestational age. We also report the first plasma steroid metabolome of a genetically engineered mouse model overexpressing ADM. Our findings support an inhibitory effect of ADM at least on glucocorticoid synthesis. The strong negative correlation found in newborns and investigations on human adrenocortical cells further validate our hypothesis of a functional link between elevated ADM concentrations in preterm neonates and impaired 11-hydroxylase activity.

Declaration of interest

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

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Author contribution statement

M L and E P: these senior authors contributed equally to this work.

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References

Admaty D, Benzing J, Burkhardt T, Lapaire O, Hegi L, Szinnai G, Morgenthaler NG, Bucher HU, Bührer C & Wellmann S 2012 Plasma midregional proadrenomedullin in newborn infants: impact of prematurity and perinatal infection. *Pediatric Research* **72** 70–76. (https://doi.org/10.1038/pr.2012.38)

Boldt T, Luukkainen P, Fyhrquist F, Pohjavuori M & Andersson S 1998 Birth stress increases adrenomedullin in the newborn. *Acta Paediatrica* **87** 93–94. (https://doi.org/10.1080/08035259850157949)

Caron KM & Smithies O 2001 Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional adrenomedullin gene. *PNAS* **98** 615–619. (https://doi.org/10.1073/pnas.021548898)

Caruhel P, Mazier C, Kunde J, Morgenthaler NG & Darbouret B 2009 Homogeneous time-resolved fluoroimmunoassay for the measurement of midregional proadrenomedullin in plasma on the fully automated system brahms kryptor. *Clinical Biochemistry* **42** 725–728. (https://doi.org/10.1016/j.clinbiochem.2009.01.002)

Charles CJ, Lainchbury JG, Nicholls MG, Rademaker MT, Richards AM & Troughton RW 2003 Adrenomedullin and the renin-angiotensin-aldosterone system. *Regulatory Peptides* **112** 41–49. (https://doi.org/10.1016/s0167-0115(03)00021-1)

Cozza EN, Ceballos NR & Lantos CP 1989 Effects of ACTH on the last step of aldosterone biosynthesis. *Journal of Steroid Biochemistry* **33** 1253–1255. (https://doi.org/10.1016/0022-4731(89)90438-x)

De Martin S, Paliuri G, Belloni A, Orso G, Zanarella E, Stellin G, Milanesi O, Basso G, Ruga EM, Frasson C, *et al*. 2014 Expression and distribution of the adrenomedullin system in newborn human thymus. *PLoS ONE* **9** e97592. (https://doi.org/10.1371/journal.pone.0097592)

Di Iorio RD, Marinoni E, Scavo D, Letizia C & Cosmi EV 1997 Adrenomedullin in pregnancy. *Lancet* **349** 328. (https://doi.org/10.1016/S0140-6736(05)62827-9)

Frey HA & Klebanoff MA 2016 The epidemiology, etiology, and costs of preterm birth. *Seminars in Fetal and Neonatal Medicine* **21** 68–73. (https://doi.org/10.1016/j.siny.2015.12.011)

Gazzolo D, Marinoni E, Giovannini L, Letizia C, Serra G & Di Iorio R 2001 Circulating adrenomedullin is increased in preterm newborns developing intraventricular hemorrhage. *Pediatric Research* **50** 544–547. (https://doi.org/10.1203/00006450-200110000-00020)

Gille J, Ostermann H, Dragu A & Sablotzki A 2017 MR-proADM: a new biomarker for early diagnosis of sepsis in burned patients. *Journal of Burn Care and Research* **38** 290–298. (https://doi.org/10.1097/BCR.0000000000000508)

Gong X, Qiu J, Qiu G & Cai C 2020 Adrenomedullin regulated by miRNA-574-3p protects premature infants with bronchopulmonary dysplasia. *Bioscience Reports* **40** BSR20191879. (https://doi.org/10.1042/BSR20191879)

Holtbäck U & Aperia AC 2003 Molecular determinants of sodium and water balance during early human development. *Seminars in Neonatology* **8** 291–299. (https://doi.org/10.1016/S1084-2756(03)00042-3)

Ishimitsu T, Kojima M, Kangawa K, Hino J, Matsuoka H, Kitamura K, Eto T & Matsuo H 1994 Genomic structure of human adrenomedullin gene. *Biochemical and Biophysical Research Communications* **203** 631–639. (https://doi.org/10.1006/bbrc.1994.2229)

Jensen BL, Krämer BK & Kurtz A 1997 Adrenomedullin stimulates renin release and renin mRNA in mouse juxtaglomerular granular cells. *Hypertension* **29** 1148–1155. (https://doi.org/10.1161/01.hyp.29.5.1148)

Karlsson R, Kallio J, Toppari J, Scheinin M & Kero P 2000 Antenatal and early postnatal dexamethasone treatment decreases cortisol secretion in preterm infants. *Hormone Research* **53** 170–176. (https://doi.org/10.1159/000023563)

Kita T, Tokashiki M & Kitamura K 2010 Aldosterone antisecretagogue and antihypertensive actions of adrenomedullin in patients with primary aldosteronism. *Hypertension Research* **33** 374–379. (https://doi.org/10.1038/hr.2010.8)

Kitamura K, Kato J, Kawamoto M, Tanaka M, Chino N, Kangawa K & Eto T 1998 The intermediate form of glycine-extended adrenomedullin is the major circulating molecular form in human plasma. *Biochemical and Biophysical Research Communications* **244** 551–555. (https://doi.org/10.1006/bbrc.1998.8310)

Kitamura K, Kangawa K & Eto T 2002 Adrenomedullin and pamp: discovery, structures, and cardiovascular functions. *Microscopy Research and Technique* **57** 3–13. (https://doi.org/10.1002/jemt.10052)

Koch L, Dabek MT, Frommhold D & Poeschl J 2011 Stable precursor fragments of vasoactive peptides in umbilical cord blood of term and preterm infants. *Hormone Research in Paediatrics* **76** 234–239. (https://doi.org/10.1159/000329285)

Krintus M, Kozinski M, Braga F, Kubica J, Sypniewska G & Panteghini M 2018 Plasma midregional proadrenomedullin (MR-proADM) concentrations and their biological determinants in a reference population. *Clinical Chemistry and Laboratory Medicine* **56** 1161–1168. (https://doi.org/10.1515/cclm-2017-1044)

Lainchbury JG, Troughton RW, Lewis LK, Yandle TG, Richards AM & Nicholls MG 2000 Hemodynamic, hormonal, and renal effects of short-term adrenomedullin infusion in healthy volunteers. *Journal of Clinical Endocrinology and Metabolism* **85** 1016–1020. (https://doi.org/10.1210/jcem.85.3.6422)

Lefebvre H, Compagnon P, Contesse V, Delarue C, Thuillez C, Vaudry H & Kuhn JM 2001 Production and metabolism of serotonin (5-HT) by the human adrenal cortex: paracrine stimulation of aldosterone secretion by 5-HT. *Journal of Clinical Endocrinology and Metabolism* **86** 5001–5007. (https://doi.org/10.1210/jcem.86.10.7917)

Li M, Schwerbrock NMJ, Lenhart PM, Fritz-Six KL, Kadmiel M, Christine KS, Kraus DM, Espenschied ST, Willcockson HH, Mack CP, *et al*. 2013 Fetal-derived adrenomedullin mediates the innate immune milieu of the placenta. *Journal of Clinical Investigation* **123** 2408–2420. (https://doi.org/10.1172/JCI67039)

Liu L, Panzitt K, Racedo S, Wagner M, Platzer W, Zaufel A, Theiler-Schwetz V, Obermayer-Pietsch B, Müller H, Höfler G, *et al*. 2019 Bile acids increase steroidogenesis in cholemic mice and induce cortisol secretion in adrenocortical H295R cells via S1PR2, ERK and SF-1. *Liver International* **39** 2112–2123. (https://doi.org/10.1111/liv.14052)

Marinoni E, Di Iorio R, Alò P, Villaccio B, Alberini A & Cosmi EV 1999 Immunohistochemical localization of adrenomedullin in fetal and neonatal lung. *Pediatric Research* **45** 282–285. (https://doi.org/10.1203/00006450-199902000-00021)

Martinerie L, Pussard E, Yousef N, Cosson C, Lema I, Husseini K, Mur S, Lombès M & Boileau P 2015 Aldosterone-signaling defect exacerbates sodium wasting in very preterm neonates: the Premaldo study. *Journal of Clinical Endocrinology and Metabolism* **100** 4074–4081. (https://doi.org/10.1210/jc.2015-2272)

Mazzocchi G, Musajo F, Neri G, Gottardo G & Nussdorfer GG 1996*a* Adrenomedullin stimulates steroid secretion by the isolated perfused rat adrenal gland in situ: comparison with calcitonin gene-related peptide effects. *Peptides* **17** 853–857. (https://doi.org/10.1016/0196-9781(96)00109-x)

Mazzocchi G, Rebuffat P, Gottardo G & Nussdorfer GG 1996*b* Adrenomedullin and calcitonin gene-related peptide inhibit aldosterone secretion in rats, acting via a common receptor. *Life Sciences* **58** 839–844. (https://doi.org/10.1016/0024-3205(96)00017-3)

McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, Solari R, Lee MG & Foord SM 1998 RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* **393** 333–339. (https://doi.org/10.1038/30666)

Miller WL & Auchus RJ 2011 The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocrine Reviews* **32** 81–151. (https://doi.org/10.1210/er.2010-0013)

Miller N, Asali AA, Agassi-Zaitler M, Neumark E, Eisenberg MM, Hadi E, Elbaz M, Pasternak Y, Fishman A & Biron-Shental T 2019 Physiological and psychological stress responses to labor and delivery as expressed by salivary cortisol: a prospective study. *American Journal of Obstetrics and Gynecology* **221** 351.e1–351.e7. (https://doi.org/10.1016/j.ajog.2019.06.045)

Mokuda O, Sakamoto Y, Kawagoe R, Ubukata E & Shimizu N 1992 Epinephrine augments cortisol secretion from isolated perfused adrenal glands of guinea pigs. *American Journal of Physiology* **262** E806–E809. (https://doi.org/10.1152/ajpendo.1992.262.6.E806)

Naccache A, Louiset E, Duparc C, Laquerrière A, Patrier S, Renouf S, Gomez-Sanchez CE, Mukai K, Lefebvre H & Castanet M 2016 Temporal and spatial distribution of mast cells and steroidogenic enzymes in the human fetal adrenal. *Molecular and Cellular Endocrinology* **434** 69–80. (https://doi.org/10.1016/j.mce.2016.06.015)

Ng PC, Wong GW, Lam CW, Lee CH, Fok TF, Wong MY, Wong W & Chan DC 1997 Pituitary-adrenal suppression and recovery in preterm very low birth weight infants after dexamethasone treatment for bronchopulmonary dysplasia. *Journal of Clinical Endocrinology and Metabolism* **82** 2429–2432. (https://doi.org/10.1210/jcem.82.8.4152)

Parkes DG & May CN 1995 ACTH-suppressive and vasodilator actions of adrenomedullin in conscious sheep. *Journal of Neuroendocrinology* **7** 923–929. (https://doi.org/10.1111/j.1365-2826.1995.tb00737.x)

Parmar J, Key RE & Rainey WE 2008 Development of an adrenocorticotropin-responsive human adrenocortical carcinoma cell line. *Journal of Clinical Endocrinology and Metabolism* **93** 4542–4546. (https://doi.org/10.1210/jc.2008-0903)

Pfaffl MW, Tichopad A, Prgomet C & Neuvians TP 2004 Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: best keeper excel-based tool using pair-wise correlations. *Biotechnology Letters* **26** 509–515. (https://doi.org/10.1023/b:bile.0000019559.84305.47)

Rainey WE, Saner K & Schimmer BP 2004 Adrenocortical cell lines. *Molecular and Cellular Endocrinology* **228** 23–38. (https://doi.org/10.1016/j.mce.2003.12.020)

Risso FM, Sannia A, Gavilanes DAW, Vles HJ, Colivicchi M, Ricotti A, Li Volti G & Gazzolo D 2012 Biomarkers of brain damage in preterm infants. *Journal of Maternal-Fetal and Neonatal Medicine* **25** (Supplement 4) 101–104. (https://doi.org/10.3109/14767058.2012.715024)

Roberts D, Brown J, Medley N & Dalziel SR 2017 Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. *Cochrane Database of Systematic Reviews* **3** CD004454. (https://doi.org/10.1002/14651858.CD004454.pub3)

Rudolph AM 1998 Adrenomedullin: its role in perinatal adaptation. *Acta Paediatrica* **87** 235–236. (https://doi.org/10.1080/08035259850157246)

Samson WK, Murphy T & Schell DA 1995 A novel vasoactive peptide, adrenomedullin, inhibits pituitary adrenocorticotropin release. *Endocrinology* **136** 2349–2352. (https://doi.org/10.1210/endo.136.5.7720684)

Schiffer L, Anderko S, Hannemann F, Eiden-Plach A & Bernhardt R 2015 The CYP11B subfamily. *Journal of Steroid Biochemistry and Molecular Biology* **151** 38–51. (https://doi.org/10.1016/j.jsbmb.2014.10.011)

Schönauer R, Els-Heindl S & Beck-Sickinger AG 2017 Adrenomedullin: new perspectives of a potent peptide hormone. *Journal of Peptide Science* **23** 472–485. (https://doi.org/10.1002/psc.2953)

Smith R & Nicholson RC 2007 Corticotrophin releasing hormone and the timing of birth. *Frontiers in Bioscience: Journal and Virtual Library* **12** 912–918. (https://doi.org/10.2741/2113)

Sulyok E, Varga F, Györy E, Jobst K & Csaba IF 1979 Postnatal development of renal sodium handling in premature infants. *Journal of Pediatrics* **95** 787–792. (https://doi.org/10.1016/s0022-3476(79)80737-4)

Then C, Rottenkolber M, Lechner A, Meisinger C, Heier M, Koenig W, Peters A, Rathmann W, Bidlingmaier M, Reincke M, *et al*. 2016*a* Altered relation of the renin-aldosterone system and vasoactive peptides in type 2 diabetes: the KORA F4 study. *Atherosclerosis* **252** 88–96. (https://doi.org/10.1016/j.atherosclerosis.2016.07.905)

Then C, Rottenkolber M, Lechner A, Meisinger C, Heier M, Koenig W, Peters A, Rathmann W, Bidlingmaier M, Reincke M, *et al*. 2016*b* Dataset of the associations of aldosterone to renin ratio with MR-proANP and MR-proADM. *Data in Brief* **8** 1395 – 1399 . (https://doi.org/10.1016/j.dib.2016.08.008)

Thomson LM, Kapas S, Carroll M & Hinson JP 2001 Autocrine role of adrenomedullin in the human adrenal cortex. *Journal of Endocrinology* **170** 259–265. (https://doi.org/10.1677/joe.0.1700259)

Thomson LM, Kapas S & Hinson JP 2003 Paracrine effects of PAMP and adrenomedullin on the human adrenal H295R cell line: pamp but not adrenomedullin stimulates DHEA secretion. *Regulatory Peptides* **112** 3–7. (https://doi.org/10.1016/S0167-0115(03)00016-8)

Travers S, Martinerie L, Boileau P, Lombès M & Pussard E 2017*a* Alterations of adrenal steroidomic profiles in preterm infants at birth. *Archives of Disease in Childhood: Fetal and Neonatal Edition* **103** F143–F151. (https://doi.org/10.1136/archdischild-2016-312457)

Travers S, Martinerie L, Bouvattier C, Boileau P, Lombès M & Pussard E 2017*b* Multiplexed steroid profiling of gluco- and mineralocorticoids pathways using a liquid chromatography tandem mass spectrometry method. *Journal of Steroid Biochemistry and Molecular Biology* **165** 202–211. (https://doi.org/10.1016/j.jsbmb.2016.06.005)

Trincot CE, Xu W, Zhang H, Kulikauskas MR, Caranasos TG, Jensen BC, Sabine A, Petrova TV & Caron KM 2019 Adrenomedullin induces cardiac lymphangiogenesis after myocardial infarction and regulates cardiac edema via connexin 43. *Circulation Research* **124** 101–113. (https://doi.org/10.1161/CIRCRESAHA.118.313835)

Valenzuela-Sánchez F, Valenzuela-Méndez B, Rodríguez-Gutiérrez JF, Estella-García Á & González-García MÁ 2016 New role of biomarkers: mid-regional pro-adrenomedullin, the biomarker of organ failure. *Annals of Translational Medicine* **4** 329. (https://doi.org/10.21037/atm.2016.08.65)

Viengchareun S, Kamenicky P, Teixeira M, Butlen D, Meduri G, Blanchard-Gutton N, Kurschat C, Lanel A, Martinerie L, Sztal-Mazer S, *et al.* 2009 Osmotic stress regulates mineralocorticoid receptor expression in a novel aldosterone-sensitive cortical collecting duct cell line. *Molecular Endocrinology* **23** 1948–1962. (https://doi.org/10.1210/me.2009-0095)

Vigué B, Leblanc PE, Moati F, Pussard E, Foufa H, Rodrigues A, Figueiredo S, Harrois A, Mazoit JX, Rafi H, *et al*. 2016 Mid-regional pro-adrenomedullin (MR-proADM), a marker of positive fluid balance in critically ill patients: results of the ENVOL study. *Critical Care* **20** 363. (https://doi.org/10.1186/s13054-016-1540-x)

Wetzel-Strong SE, Li M, Klein KR, Nishikimi T & Caron KM 2014 Epicardial-derived adrenomedullin drives cardiac hyperplasia during embryogenesis. *Developmental Dynamics* **243** 243–256. (https://doi.org/10.1002/dvdy.24065)

Wils J, Duparc C, Cailleux AF, Lopez AG, Guiheneuf C, Boutelet I, Boyer HG, Dubessy C, Cherifi S, Cauliez B, *et al*. 2020 The neuropeptide substance P regulates aldosterone secretion in human adrenals. *Nature Communications* **11** 2673. (https://doi.org/10.1038/s41467-020-16470-8)

Yamaguchi T, Baba K, Doi Y & Yano K 1995 Effect of adrenomedullin on aldosterone secretion by dispersed rat adrenal zona glomerulosa cells. *Life Sciences* **56** 379–387. (https://doi.org/10.1016/0024-3205(94)00903-1)

Yamaguchi T, Baba K, Doi Y, Yano K, Kitamura K & Eto T 1996 Inhibition of aldosterone production by adrenomedullin: a hypotensive peptide, in the rat. *Hypertension* **28** 308–314. (https://doi.org/10.1161/01.hyp.28.2.308)

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