# AUTHOR QUERY FORM

| | | |
|---|---|---|
| ELSEVIER | **Journal:** YPLAC<br><br>**Article Number:** 2681 | **Please e-mail or fax your responses and any corrections to:**<br><br>**E-mail: corrections.eseo@elsevier.tnq.co.in**<br><br>**Fax: +31 2048 52789** |

Dear Author,

Please check your proof carefully and mark all corrections at the appropriate place in the proof (e.g., by using on-screen annotation in the PDF file) or compile them in a separate list. Note: if you opt to annotate the file with software other than Adobe Reader then please also highlight the appropriate place in the PDF file. To ensure fast publication of your paper please return your corrections within 48 hours.

For correction or revision of any artwork, please consult http://www.elsevier.com/artworkinstructions.

Any queries or remarks that have arisen during the processing of your manuscript are listed below and highlighted by flags in the proof.

| Location in article | Query / Remark: Click on the Q link to find the query's location in text<br>Please insert your reply or correction at the corresponding line in the proof |
|---|---|
| **Q1** | Please list all names for up to 6 authors. For more than 6 authors, use 'et al.' after the first 6 authors in Refs. [12,13,15,18]. |
| **Q2** | Please check the sentence 'Each plotted corresponds to…' for clarity, and correct if necessary. |
| **Q3** | Please confirm that given names and surnames have been identified correctly. |
| | Please check this box if you have no corrections to make to the PDF file ☐ |

Thank you for your assistance.

Placenta xxx (2012) 1–4

Contents lists available at SciVerse ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Short communication

# Expression of the epithelial sodium channel sensitive to amiloride (ENaC) in normal and preeclamptic human placenta

Q3 G.I. Marino, B.A. Kotsias*

*Laboratorio de Canales Iónicos, Inst. Inv. Médicas A. Lanari, UBA, IDIM-CONICET, C. de Malvinas 3150, 1427 Buenos Aires, Argentina*

ARTICLE INFO

*Article history:*
Accepted 11 November 2012

*Keywords:*
ENaC
Human placenta
Preeclampsia
Syncytiotrophoblast
Immunostaining
Western blot

ABSTRACT

Aldosterone modulates the activity of the epithelial sodium channel (ENaC) through changes in its trafficking, membrane expression and open probability. Plasma levels of aldosterone are decreased in preeclampsia. Herein we postulated that if aldosterone regulates ENaC expression then its expression should be decreased in preeclampsia. We found a diminished expression of the three subunits of the ENaC in the membranes of preeclamptic placentas in comparison with the normal ones. Although the role of ENaC in placental tissues is poorly understood, these differences may have consequences for the ion transport involved in the pathophysiology of preeclampsia.



## 1. Introduction

The epithelial sodium channel (ENaC) is highly regulated by aldosterone which is controlled by a negative-feedback by the renin–angiotensin–aldosterone system with aldosterone as the final hormone, essential for $Na^+$ balance. Aldosterone modulates ENaC through changes in its three subunits trafficking, membrane expression and open probability ([1,2] for references). Other hormones and paracrine factors such as vasopressin and angiotensin II modulate in parallel the activity of ENaC [3,4].

$Na^+$ currents are involved in cell migration, documented in tumor cell proliferation [5], epithelial and vascular cells [6,7] and BeWo trophoblastic cells [8]. One of the major extra-renal renin–angiotensin systems during pregnancy is in the placenta, which is altered in preeclampsia [9]. The interaction of genetic, immunological and endocrine factors is involved in the physiopathology of preeclampsia with inadequate trophoblastic invasion and maternal endothelial dysfunction (for references [10–13]). In preeclampsia there is a lower plasma volume expansion, lower cardiac output and an increase in total peripheral vascular resistance. Plasma levels of renin, angiotensin II and aldosterone increase during normal pregnancy although in preeclampsia for reasons that are unclear, renin activity, angiotensin II, and aldosterone decrease and the ratio aldosterone/renin is increased [10]. We tested the hypothesis that if aldosterone regulates ENaC expression, the amount of ENaC should be decreased in preeclamptic placentas.

## 2. Methods

Membrane vesicles and fixed explants were obtained from normal and preeclamptic placentas at full term, stored at the Cátedra de Biología Celular, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (see [14] for a detailed description of the cases). ENaC protein was assessed by Western blot or immunohistochemistry [8]. 75 μg of membrane protein from placentas and 100 μg of total proteins from BeWo and T84 cells (positive control) (ATCC, USA) were used for immunoblot studies. We employed rabbit polyclonal antibodies of human αENaC and gENaC (dilution 1:1000 overnight), a mouse monoclonal antibody of human bENaC (dilution 1:500 overnight) and a normal rabbit IgG (Santa Cruz Biotechnology Inc, CA, USA) to detect unspecific bands in ENaC blotting from normal placentas. To confirm equal loading, each membrane was stripped and analyzed for actin protein expression, demonstrating that the band intensities did not present significant changes between the samples studied. Densitometry was performed and after normalization with actin, the values were plotted as ENaC/actin relative ratio. For localization studies (see Ref. [8] for details) samples were incubated overnight with the primary antibodies (1:100). Immunofluorescence was performed using anti-rabbit-FITC for detection of α and γENaC subunits and anti-mouse-FITC for βENaC (Jackson Immunoresearch Lab, West Grove, PA, USA) (1:2500, 1 h). Mounted slices were analyzed on a fluorescence microscope (Axiostar HAL 100; Carl Zeiss, Oberkochen, Germany). Control samples were performed by omitting the primary antibody and an additional control for α and γENaC was carried out with normal rabbit IgG (data not shown).

## 3. Results and discussion

Protein fractions of apical membrane of normal and preeclamptic syncytiotrophoblast were probed with anti-α, β and γENaC antibodies. The abundance of the subunits of ENaC protein (ENaC/actin relative ratio) from 6 to 8 samples was decreased in preeclamptic placental explants when compared with the normal ones. Similar results were obtained in 6–12 placentas using staining with Ponceau Red to estimate the relative amounts of total protein (data not

* Corresponding author. Tel.: +54 11 4514 8701.
*E-mail addresses:* kotsias@retina.ar, kotsias@yahoo.com (B.A. Kotsias).

0143-4004/$ – see front matter © 2012 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.placenta.2012.11.008

shown). Fig. 1 shows the proteolytic processed bands of the expected size of ENaC subunits in the cell membrane [8,15]: ~40 kDa for αENaC, and ~75 kDa for γENaC, and ~100 kDa for βENaC. A normal rabbit IgG in normal placentas recognized a ~55–60 kDa polypeptide corresponding to a non-specific band. T84 and trophoblastic BeWo cell lines protein extracts were analyzed as positive controls. In addition, we found that the three ENaC subunits are present in the polarized syncytium (Fig. 2A, D, G) but the intensity of the channel protein staining in preeclampsia is lower compared with that in normal samples (B, E, H).

There are reports on the alteration in the expression and properties of ion channels in animal models and human preeclampsia: ENaC [16], potassium and voltage-gated sodium channel [17–19], CFTR [20] and a genetic variant of the βENaC subunit [21]. ENaCs are activated in a complex manner in response to factors from the external environment, among them a proteolytic processing. Plasmin-dependent cleavage of the γENaC subunit [22] may be particularly relevant in disorders associated with proteinuria and extracellular volume expansion because several years ago Chen et al. [23] found that urine from women with preeclampsia contains an altered plasmin activity depending on the severity of the disease. We detected only a band of 40 kDa of the αENaC subunit and a 75 kDa in the γ subunit but not the full-length polypeptides probably because we used only membrane proteins where the proteolytic processed forms of the proteins are necessary for full channel activity [8,15]. We do not have a clear explanation for the difference between the molecular weights of the αENaC subunits from samples from membranes of placental tissue (40 kDa) and BeWo cells maintained in culture medium (35 kDa).

The causes of the reduced aldosterone concentration in preeclampsia are not clear. Escher et al. [24] found in preeclamptic women a reduced activity of the synthase CYP11B2 which catalyzes aldosterone although the studies on the association of this gene polymorphism with gestational hypertension or preeclampsia

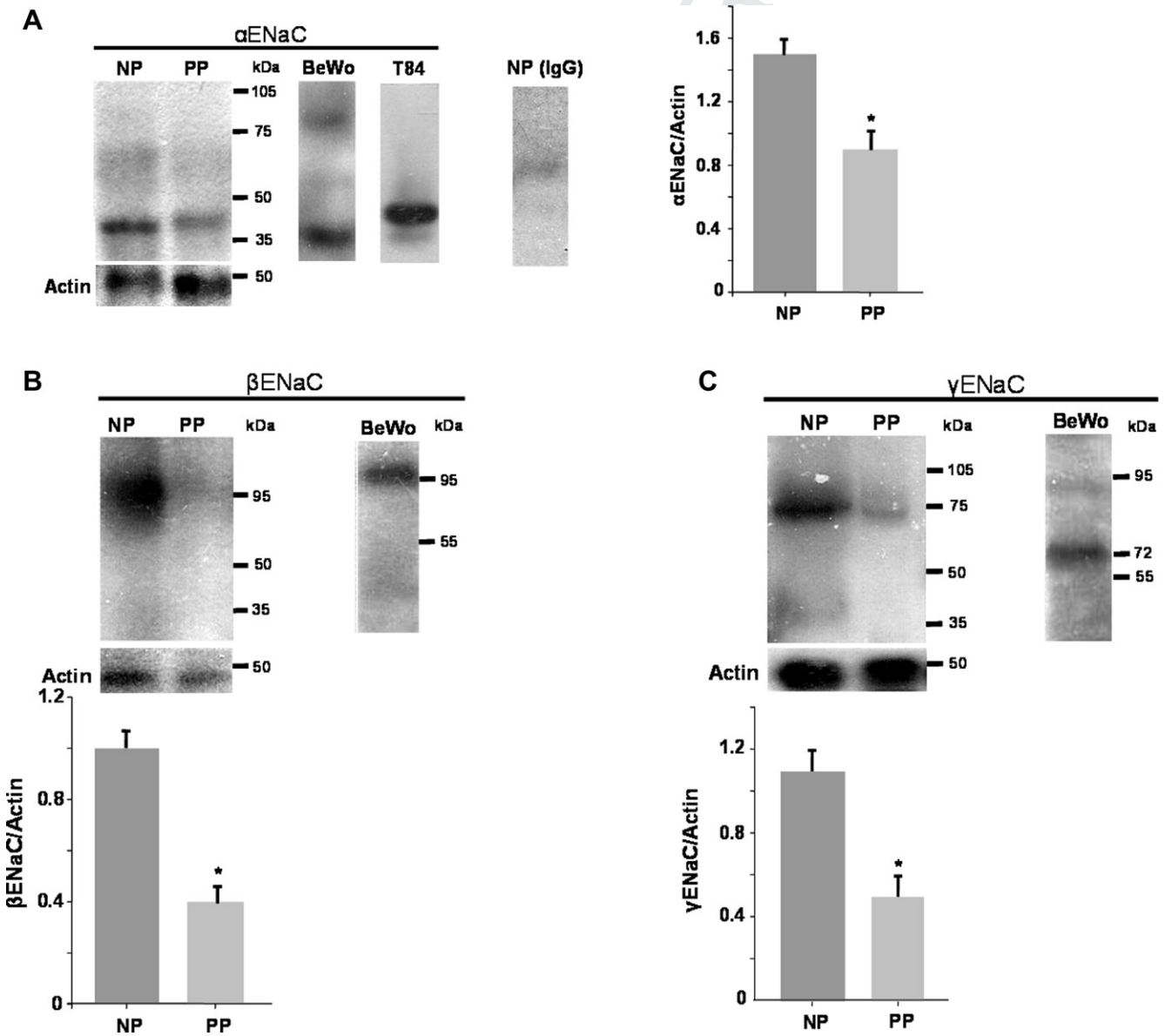


**Fig. 1.** Semi quantitative immunobloting analysis of α (A), β (B) and γ (C) ENaC membrane abundance in normal and preeclamptic placental explants. Representative immunoblots show that ENaC protein level expression from the three subunits was lower in preeclamptic placental explants (PP) than in normal placental explants (NP). Densitometry of immunoblots containing ENaC protein level expression was performed, and after normalization for actin in each lane, the values were plotted as ENaC/actin relative ratio, for all subunits. Each plotted corresponds to the mean ± SEM obtained from six to eight normal and preeclamptic placental explants ($p < 0.05$). SEM = standard error of mean. Q2

Please cite this article in press as: Marino GI, Kotsias BA, Expression of the epithelial sodium channel sensitive to amiloride (ENaC) in normal and preeclamptic human placenta, Placenta (2012), http://dx.doi.org/10.1016/j.placenta.2012.11.008

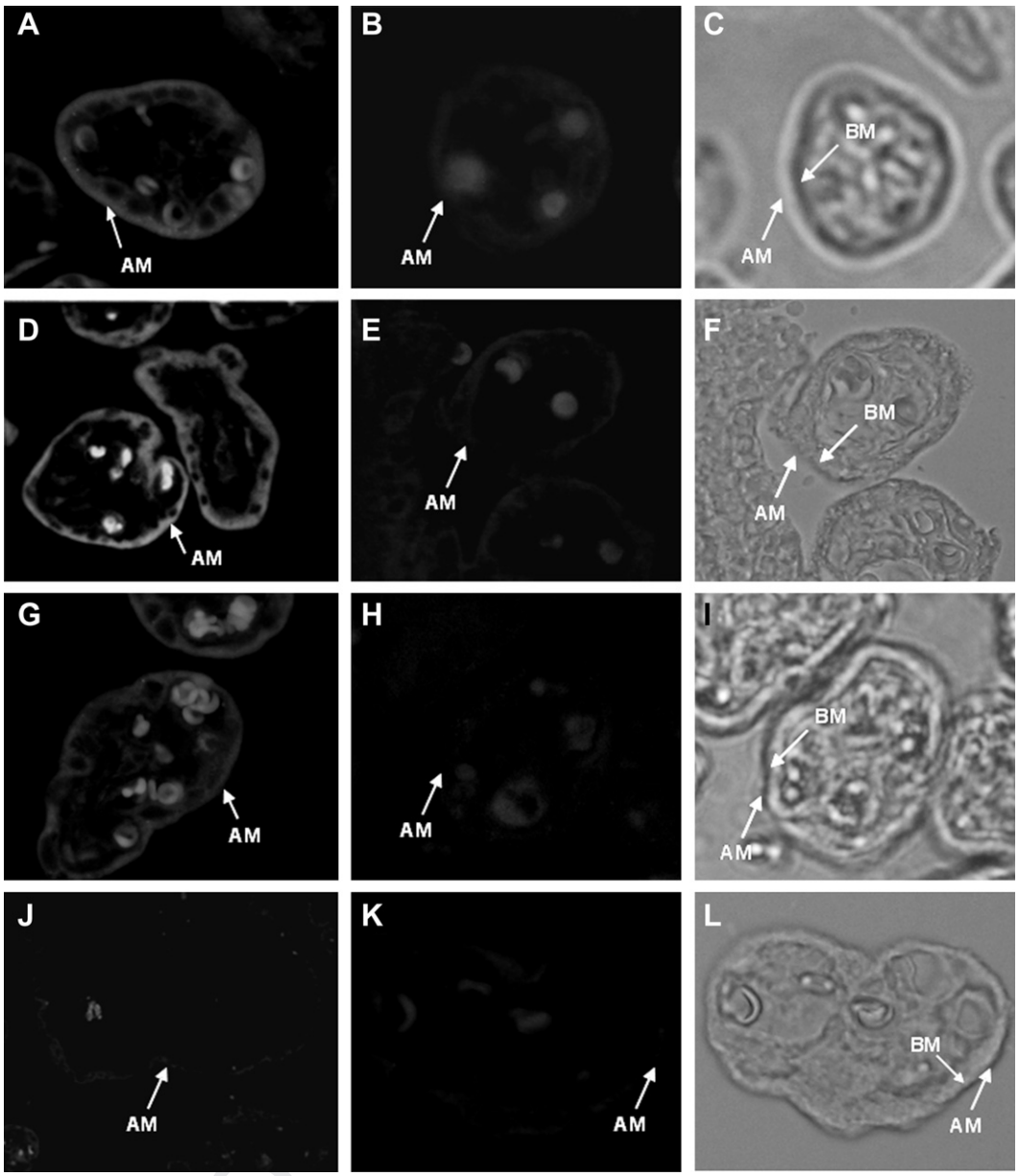


**Fig. 2.** Localization of ENaC proteins in normal and preeclamptic microvillous border of human syncytiotrophoblast (hST) with fluorescence microscopy. Immunostaining with a polyclonal antibody anti-aENaC (A and B), a monoclonal anti-βENaC (D and E) and a polyclonal antibody anti-γENaC (G and H), revealed specific labeling in the apical membrane of hST from normal placentas (arrows in A, D and G). In preeclamptic placentas (B, E and H) the three ENaC subunits abundance was much lower than in normal explants. Negative controls for the experiments of normal (J) and preeclamptic placentas (K) were performed by omitting the primary antibody and with anti-rabbit-FITC. Tissue integrity of B, E, H, and K was tested (C, F, I and L, respectively). The data are representative of three experiments under each condition. AM: Apical membrane; BM: basal membrane. All pictures were obtained at a 400× magnification.

remain controversial (see [25] for references). The trophoblast itself is a tissue involved in the transport of numerous ions, such as $Na^+$ through ENaC which are regulated by aldosterone and other factors and hormones as in the epithelia. One of the proposed mechanisms for preeclampsia could be impaired trophoblast invasiveness and defects in spiral arterial remodeling (see discussions in Refs. [10–13]). We showed that aldosterone and amiloride treatments influence wound healing in BeWo cells by their effects upon ENaCs and by reducing the expression of αENaC by antisense oligonucleotides producing a significant inhibition in wound healing and in current stimulation [8,16]. Although our studies are in vitro, the reduced expression of ENaC in preeclampsia may have consequences for ion and nutrient transport and cell migration, leading to a disturbed placental development.

## Acknowledgments

We thank Dr Alicia Damiano from the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires for giving us the placenta samples. This work was supported by grants from the University of Buenos Aires, National Research Council (CONICET) and Ministerio de Ciencia, Tecnología e Innovación Productiva.

## References

[1] Galizia L, Ojea A, Kotsias BA. Amiloride sensitive sodium channels (ENaC) and their regulation by proteases. Medicina (B Aires) 2011;71:179–82.
[2] Palmer LG, Patel A, Frindt G. Regulation and dysregulation of epithelial $Na^+$ channels. Clin Exp Nephrol 2012;16:35–43.

Please cite this article in press as: Marino GI, Kotsias BA, Expression of the epithelial sodium channel sensitive to amiloride (ENaC) in normal and preeclamptic human placenta, Placenta (2012), http://dx.doi.org/10.1016/j.placenta.2012.11.008

[3] Mironova E, Bugaj V, Roos KP, Kohan DE, Stockand JD. Aldosterone-independent regulation of the epithelial $Na^+$ channel (ENaC) by vasopressin in adrenalectomized mice. Proc Natl Acad Sci U S A 2012;109:10095–100.
[4] Sun P, Yue P, Wang WH. Angiotensin II stimulates epithelial sodium channels in the cortical collecting duct of the rat kidney. Am J Physiol Renal Physiol 2012;302:F679–87.
[5] Qadri YJ, Rooj AK, Fuller CM. ENaCs and ASICs as therapeutic targets. Am J Physiol Cell Physiol 2012;302:C943–65.
[6] Chifflet S, Hernández JA, Grasso S. A possible role for membrane depolarization in epithelial wound healing. Am J Physiol Cell Physiol 2005; 288:C1420–30.
[7] Grifoni SC, Gannon KP, Stec DE, Drummond HA. ENaC proteins contribute to VSMC migration. Am J Physiol Heart Circ Physiol 2006;291:H3076–86.
[8] del Mónaco SM, Marino GI, Assef YA, Damiano AE, Kotsias BA. Cell migration in BeWo cells and the role of ENaC channels. J Membr Biol 2009;232:1–13.
[9] Irani RA, Xia Y. The functional role of the renin–angiotensin system in pregnancy and preeclampsia. Placenta 2008;29:763–71.
[10] Brown MA, Zammit VC, Mitar DA, Whitworth JA. Renin–aldosterone relationships in pregnancy-induced hypertension. Am J Hypertens 1992;5: 366–71.
[11] Huppertz B. IFPA award in placentology lecture: biology of the placental syncytiotrophoblast—myths and facts. Placenta 2010;31(Suppl. ):S75–81.
[12] Orendi K, Kivity V, Sammar M, et al. Placental and trophoblastic in vitro models to study preventive and therapeutic agents for preeclampsia. Placenta 2011;(32 Suppl.):S49–54.
[13] Gennari-Moser C, Khankin EV, Schüller S, et al. Regulation of placental growth by aldosterone and cortisol. Endocrinology 2011;152:263–71.
[14] Marino GI, Castro-Parodi M, Dietrich V, Damiano AE. High levels of human chorionic gonadotropin (hCG) correlate with increased aquaporin-9 (AQP9) expression in explants from human preeclamptic placenta. Reprod Sci 2010; 17:444–53.
[15] Hughey RP, Mueller GM, Bruns JB, et al. Maturation of the epithelial $Na^+$ channel involves proteolytic processing of the alpha- and gamma-subunits. J Biol Chem 2003;278:37073–82.
[16] del Mónaco S, Assef Y, Damiano A, Zotta E, Ibarra C, Kotsias BA. Characterization of the epithelial sodium channel in human pre-eclampsia syncytiotrophoblast. Medicina (B Aires) 2006;66:31–5.
[17] Sharkey LC, McCune SA, Yuan O, Lange C, Fray J. Spontaneous pregnancy-induced hypertension and intrauterine growth restriction in rats. Am J Hypertens 2001;14:1058–66.
[18] Mistry HD, McCallum LA, Kurlak LO, et al. Novel expression and regulation of voltage-dependent potassium channels in placentas from women with preeclampsia. Hypertension 2011;58:497–504.
[19] Riquelme G, de Gregorio N, Vallejos C, Berrios M, Morales B. Differential expression of potassium channels in placentas from normal and pathological pregnancies: targeting of the K(ir) 2.1 channel to lipid rafts. J Membr Biol 2012;245:141–50.
[20] Castro-Parodi M, Levi L, Dietrich V, Zotta E, Damiano AE. CFTR may modulate AQP9 functionality in preeclamptic placentas. Placenta 2009;30:642–8.
[21] Dhanjal MK, Owen EP, Anthony JA, Davidson JS, Rayner BL. Association of pre-eclampsia with the R563Q mutation of the beta-subunit of the epithelial sodium channel. BJOG 2006;113:595–8.
[22] Kleyman TR, Carattino MD, Hughey RP. ENaC at the cutting edge: regulation of epithelial sodium channels by proteases. J Biol Chem 2009;284:20447–51.
[23] Chen HF, Nakabayashi M, Satoh K, Sakamoto S. Urinary fibrinolysis in toxemia of pregnancy. Acta Obstet Gynecol Scand 1980;59:499–504.
[24] Escher G, Cristiano M, Causevic M, Baumann M, Frey FJ, Surbek D, et al. High aldosterone-to-renin variants of CYP11B2 and pregnancy outcome. Nephrol Dial Transplant 2009;24:1870–5.
[25] Ramírez-Salazar M, Romero-Gutiérrez G, Zaina S, Malacara JM, Kornhauser C, Pérez-Luque E. Relationship of aldosterone synthase gene (C-344T) and mineralocorticoid receptor (S810L) polymorphisms with gestational hypertension. J Hum Hypertens 2011;25:320–6.



Please cite this article in press as: Marino GI, Kotsias BA, Expression of the epithelial sodium channel sensitive to amiloride (ENaC) in normal and preeclamptic human placenta, Placenta (2012), http://dx.doi.org/10.1016/j.placenta.2012.11.008