Remodelling of collagen fibres in the placentas of women with venous insufficiency during pregnancy

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Summary. Haemodynamic changes produced during pregnancy lead to elevated venous pressure in the legs and an increased resting consumption of oxygen. These events can cause varicose veins, or venous insufficiency (VI), which by creating an environment of hypoxia could affect the structure and function of the placental barrier. This study assesses the remodelling state of the placental villi by examining differences in collagens with a known role in villus structure and in placental barrier permeability between patients with and without VI.

Samples of 67 placentas from women with VI (n=24) and without VI (n=43) during their pregnancy were processed for gene and protein expression analysis of COL-I, COL-III, MMP-2 and MMP-9 by RT-qPCR and immunohistochemistry.

While no differences in COL-I expression levels were detected in the samples from women with and without VI, significant differences did emerge in both gene and protein expression levels of COL-III. Importantly, COL-I/III ratios were reduced in the VI group compared to controls. MMP-2 activity was similar in the two groups while MMP-9 levels were significantly elevated in VI with greatest expression differences observed at the level of the decidual cells.

Mothers who developed VI during pregnancy showed significantly higher COL-III and MMP-9 levels consistent with a state of remodelling of the placental villi.

Key words: Venous insufficiency, Placenta, Pregnancy, Collagen, Metalloproteinase

Introduction

Venous insufficiency (VI) is the most common syndrome of all venous circulatory disorders (Meissner et al., 2007; Nicolaides et al., 2008). VI is defined as a peripheral venous system abnormality in which venous return is reduced or altered because of modifications of the vein itself (its wall and valves) and also of the muscle pump and capillary bed (Thulesius, 1993). The manifestations of VI are most frequent in the legs as they are furthest from the heart and veins have to withstand greater return pressures. Persistent venous hypertension produced by VI causes several clinical signs such as pain, swelling, skin colour changes and ulcers (Eberhardt and Raffetto, 2014). VI may affect one or more of the three vein systems in the legs (superficial, deep and perforator) (Uhl et al., 2012). Epidemiological studies across the world have revealed that VI shows high variation in terms of its incidence and prevalence (Robertson et al., 2008). During pregnancy, women carry a higher risk of developing venous insufficiency, otherwise known as varicose veins. This is because of the increased abdominal pressure, increased blood
volume and hormone changes that occur throughout pregnancy (Beebe-Dimmer et al., 2005). The prevalence of varicose veins during gestation is 60–73% in multiparous and 40–60% in primiparous women (Benirschke et al., 2012; Asbeutah et al., 2015).

The placenta is a specialized organ which along with the foetal membranes and amniotic fluid supports the normal growth and development of the foetus. This organ can be affected by modifications in blood flow (Jennifer and Gudrun, 2010; Abou-Kheir et al., 2015), or by insufficient venous return. When blood flow is reduced, structural modifications in the placenta have been described such as those affecting numbers of functional villi or normal numbers of villi (Benirschke et al., 2012). The extracellular matrix (ECM) is an essential component of the placenta that supports and maintains the structure of stem and mesenchymal villi, and the exchange function of terminal villi (Smith et al., 2016). If the fibrillar component of the matrix is damaged this could affect villus behaviour. The fibrillar protein collagen forms macroaggregates arranged as a scaffold with an important role in ECM support and tone (Vizza et al., 2001; Sati et al., 2008). The distribution of collagen fibres in the stroma and placental vessels has been well established. In the human placenta, scarce quantities of collagen type I (COL-I) and collagen type III (COL-III) compared with other collagen types have been reported (Rukosuev et al., 1990; Sati et al., 2008).

Abnormal blood flow in the intervillus space can affect the diffusing function of the placental barrier creating a state of hypoxia. Offsetting mechanisms develop at the level of the ECM (Niknejad et al., 2013) and these could induce COL-I and COL-III remodelling as occurs in other tissues (Balasubramanian et al., 2013; Asgari et al., 2017). In this remodelation the role of metalloproteinases 2 (MMP-2) and 9 (MMP-9) is important. These MMPs have been described by many authors in such settings of a modified ECM (Fu et al., 2017; Martinez-Fierro et al., 2017). MMPs play an important role in the synthesis and degradation of the extracellular matrix under physiological and pathological conditions, any alterations in this balance may lead to a degradation or accumulation of the matrix with degenerative and structural changes in placental barrier (Xu et al., 2002; Raffetto et al., 2008; MacColl and Khalil., 2015).

Our study hypothesis was that pregnant women with VI could develop a hypoxic state inducing remodelling of the fibrillar collagen component of the placental ECM.

Materials and methods

Patients and tissue specimens

Samples were taken from 67 placentas in women who had developed VI (n=24) or not developed VI (n=43) during pregnancy. Mean participant age was 34.3±5.3 years (mean±SD) in those without VI and 33.0±3.7 years in those with VI. These participants were recruited among patients attending the Gynaecology and Obstetrics Dept. of the University Hospital of Henares, Madrid (Spain) in the second term of pregnancy.

Inclusion criteria were women at the second trimester of pregnancy with clinical evidence of VI. Exclusion criteria were presence of diabetes mellitus, blood hypertension, systemic and organ specific autoimmune disease, active infectious diseases, venous malformations, heart, kidney and lung insufficiency, preeclampsia and Hellp syndrome. As controls we included pregnant women without clinical evidence of VI. Venous insufficiency was diagnosed using a portable colour Eco-Doppler M Turbo (Fujifilm Sonosite Inc, Washington, USA) 7.5 Mz transducer by an expert physician according to the Classification System for Chronic Venous Disorders (CEAP). With four hours of labour after delivery with standard protocols, a sample of placenta was harvested by this attending physician. Samples were transferred refrigerated to the laboratory in minimum essential medium (MEM) containing antibiotics/antimycotic at 1% (both from Thermo Fisher Scientific, Waltham, MA, USA) or RNAlater® solution (Ambion, Austin, TX, USA). This study was performed adhering to the four basic principles of ethics: autonomy, beneficence, non maleficence and justice, and following guidelines for Good Clinical Practice, and the tenets of the Declaration of Helsinki (2013) and Oviedo Agreement (1997). Written informed consent for donation of their placenta was obtained from each patient. The study protocol was approved by the Ethics Clinical Research Committee of the Hospital Universitario del Henares (A16-11).

Processing of tissues

Samples of placenta were processed in a sterile environment under a class II laminar flow hood Telstar AV 30/70 Müller 220 V 50 MHz (Grupo Telstar SA, Terrassa, Spain). For gene expression analysis, tissue specimens in RNaLater® were kept in 1 mL of this solution at -80°C until analysis. The samples kept in MEM were processed for histology. Tissues were washed/hydrated several times in antibiotic-free MEM to eliminate blood cells, and cut into fragments to be fixed in F13 fluid (60% ethanol, 20% methanol, 7% polyethylglycol, 13% distilled H2O). Once fixed, the dehydrated tissue fragments were embedded in paraffin and 5 µm sections cut using a rotation microtome HM 350 S (Thermo Fisher Scientific). These thin sections were collected onto glass slides impregnated with a 10% polylysine solution.

RT-qPCR

By real time quantitative polymerase chain reaction (RT-qPCR), the amount of cDNA in each sample was quantified for the genes of interest: COL-I, COL-III, MMP-2 and MMP-9. Results were normalized to those
of the constitutively expressed gene TBP (TATA box binding protein). For all the genes examined, specific primers were designed (Table 1) using the online applications Primer-BLAST (Ye et al., 2012) and AutoDimer (Vallone and Butler, 2004). RNA was extracted using the isothiocyanate guanidine-phenol-chloroform procedure of Chomczynski and Sacchi (1987). RT-qPCR was conducted in a StepOnePlus™ system (Applied Biosystems-Life Technologies, ThermoFischer Scientific, Carlsbad, USA) using the relative standard curve method. To this end, 5 μL of each sample diluted 1/20 in nuclease-free distilled water were mixed with 10 μL of iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, California, USA), 1 μL of forward primer, 1 μL of reverse primer and 3 μL of DNase- and RNase-free water in a MicroAmp ® 96-well plate (Applied Biosystems-Life Technologies) in a total reaction volume of 20 μL.

Picrosirius red

Tissue sections were deparaffinized for 30 minutes in xylol (PanReac AppliChem, Barcelona, Spain) and then rehydrated in a decreasing series of alcohol until their complete hydration in water. The steps of the staining procedure were: 1. Sirius red (30 min); 2. Rinsing in running water (10 min); 3. Dehydration in 100% alcohol (5 min); 4. Clearing sections in xylol (10 min); and 5. Mounting with Cytoseal™. Picrosirius red-stained sections were examined under bright-field illumination for general collagen, or polarized light for fibrillar collagen. With this stain, collagen I appears red-orange and collagen III yellow-green (Coquand-Gandit et al., 2017).

Immunohistochemistry

The avidin-biotin complex (ABC) method was used to immunohistochemically label the antigens of interest using the chromogen peroxidase as tracer. Sections were washed and equilibrated in phosphate buffered saline (PBS). Non-specific binding sites were blocked by incubating for 45 min at room temperature in blocking solution: 10% foetal bovine serum (FBS), 1% bovine serum albumin (BSA), and 0.05% Tween®20 in PBS. Sections were incubated overnight at 4°C with the primary antibody diluted in blocking solution according to the antibody (Table 2). Incubation with the secondary antibody bound to biotin was 1 h and 30 min at room temperature diluted according to the antibody: IgG(rabbit) biotinated in PBS (1:1000) (Sigma, San Luis, MO). Sections were then incubated with the avidin-peroxidase conjugate (ExtrAvidin®-Peroxidase, Sigma-Aldrich) for 1 h at room temperature diluted 1/200 in PBS. The chromogenic substrate diaminobenzidine was used to develop the sections (Kit DAB, SK-4100) (Vector, Burlingame, CA, USA), controlling the appearance of labelling under the microscope.

Evaluation of expression

Tissue observations were made using a Zeiss Axiophot light microscope (Carl Zeiss, Germany) equipped with a digital camera AxioCam HRC (Carl Zeiss, Germany). Samples were scored positive when labelling was equal to or more than 5% over the total, five histologic sections were selected at random. Immunostaining in the tissue was assessed by two independent histologists (J.B and MA.O) blinded to the outcome measure, the same form to Rodríguez-Berruguete et al. (2015).

Statistical analysis

All statistical tests were performed using the programme GraphPad Prism® 5.1. (San Diego, California, USA). Data are provided as the mean ± standard deviation and compared using the Mann Whitney U test. Significance was set at p<0.05 (*).

Results

Collagen type I

The gene expression of collagen I showed a non-significant tendency towards higher levels in the placentas of women with VI (Fig. 1). Mean expression

![Fig. 1. Levels of mRNA for the genes COL-I and COL-III quantified by RT-qPCR. Results were normalized to that of the reference gene TBP and are provided in arbitrary units. C: control, VI: venous insufficiency. *p<0.05.]
levels were $158.863\pm 30.825$ RQ in the control group and $170.818\pm 33.916$ RQ in the VI group.

The protein expression patterns of collagen I in the placentas differed according to the presence or absence of VI. In women without VI during pregnancy, this protein was mainly localized around large arteries or veins and labelling diminished closer to the terminal villi (Fig. 2A,B). The placentas of women with VI showed high collagen I protein expression throughout the ECM and labelling was more intense than in controls. In particular, villi showed large deposits of this collagen type (Fig. 2C,D).

**Collagen Type III**

The gene expression of collagen III was significantly greater in the placentas of women with VI ($p<0.05$) (Fig. 1). Mean expression levels of this gene were $82.349\pm 15.012$ RQ in women without VI and $135.134\pm 31.258$ RQ in women with VI.

The protein expression of collagen III was detected

### Table 1. Primers used for RT-qPCR: sequences and binding temperatures (Temp).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5'→3')</th>
<th>Temp.</th>
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<tr>
<td>TBP</td>
<td>Fwd TGC ACA GGA GCC AAG AGT GAA</td>
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</tr>
<tr>
<td></td>
<td>Rev CAC ATC ACA GCT CCC CAC CA</td>
<td></td>
</tr>
<tr>
<td>COL I</td>
<td>Fwd CCA TGT GAA ATT GTG TCC CA</td>
<td>60.0°C</td>
</tr>
<tr>
<td></td>
<td>Rev GGG GCA AGA CAG TGA TTG AA</td>
<td></td>
</tr>
<tr>
<td>COL III</td>
<td>Fwd GAC TTC CAA GAC CTC CTC TTT</td>
<td>62.0°C</td>
</tr>
<tr>
<td></td>
<td>Rev CCA CAA GGA TTA CAA GGC TTG</td>
<td></td>
</tr>
<tr>
<td>MMP2</td>
<td>Fwd ATA ACC TGG ATG CCG TCG TG</td>
<td>60.0°C</td>
</tr>
<tr>
<td></td>
<td>Rev CTT CAC GCT CTT CAG ACT TTG G</td>
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</tr>
<tr>
<td>MMP9</td>
<td>Fwd CTT TGA GTC CGG TGG ACG ATG</td>
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</tr>
<tr>
<td></td>
<td>Rev CGC CAG TAC TTC CCA TCC TTG</td>
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Fig. 2. Immunodetection of COL-I. **A** and **B**, slight labelling for protein expression detected in control subjects. **C** and **D**, elevated protein expression in the placentas of women with venous insufficiency around vessels (arrow).
in the placental ECM. In the control group, this expression was visible in zones corresponding to the muscle layers of the great vessels of the villi, and diminished expression was observed towards the terminal villi (Fig. 3A,B).

In the placentas of the women with VI, greater collagen III protein expression was detected than in the control group (Fig. 3B,C). Immunostaining was observed throughout the vessel walls and rest of the villus stroma. In zones of terminal villi, levels of expression were similar.

**Ratio collagen type I /III**

Col I/III colocalization was assessed through Sirius red staining. Both collagens were in disequilibrium and

<table>
<thead>
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<th>Antigen</th>
<th>Species</th>
<th>Clone</th>
<th>Dilution</th>
<th>Provider</th>
<th>Protocol Specifications</th>
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<tr>
<td>COL- I</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>1:400</td>
<td>Sigma-Aldrich (C 2456)</td>
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<tr>
<td>COL-III</td>
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<td>Monoclonal</td>
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<td>MMP-2</td>
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<td>Monoclonal</td>
<td>1:1000</td>
<td>NeoMarkers (MS-567)</td>
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<tr>
<td>MMP-9</td>
<td>Mouse</td>
<td>Monoclonal</td>
<td>1:500</td>
<td>Abcam (ab119906)</td>
<td>10mM Sodium citrate pH=6 before incubation with blocking solution</td>
</tr>
</tbody>
</table>

**Fig. 3.** Immunodetection of COL-III. **A and B.** Protein expression in control subjects. **C and D.** Elevated protein expression observed around large vessels and in the villi of the placentas of women with venous insufficiency (arrow).
collagen III was more abundant than collagen I in the villi of placentas in the VI group. Around vessels and villi a lower Col I/III ratio was detected in the VI group (Fig. 4).

**Metalloproteinase 2**

Expression for the gene MMP-2 was similar in both study groups (Fig. 5): 88.898±12.889 RQ in the control group and 114.821±17.558 RQ in the VI group, although a trend was observed towards higher expression levels in the placentas of women with VI.

At the protein level, immunolabelling for MMP-2 expression was also similar in the two groups in terms of both its localization and intensity. This metalloproteinase was detected in the muscle component of the large vessels of the villi and in the syncytiotrophoblast. It should be highlighted that decidual cells showed an abundance of this protein and labelling was more intense in the VI group (Fig. 6).

**Metalloproteinase 9**

MMP-9 gene expression was significantly higher in the placentas of women with VI, p<0.05 (Fig. 5): 91.807±13.243 RQ for controls versus 144.658±17.155 RQ for the VI group.

Immunohistochemical staining revealed the presence of cells positive for this metalloproteinase both in foetal vessels and in fibrinoid deposit. No significant differences in staining patterns were observed between the study groups (Fig. 7A-F). In VI specimens, elevated and intense activity for this MMP was detected at the decidual cell level with minor activity observed in control placental specimens (Fig. 7C,F).

**Discussion**

This study revealed that women with VI during pregnancy show histological alterations at the level of the placenta. The normal ratio of the collagen
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Fig. 6. Immunodetection of MMP-2. A and B. Labelling for protein expression around large vessels and in the villi of control placentas (A) and decidual cells in control placentas (B). C and D. Elevated protein expression observed around great vessels, in villi (C) and in decidual cells (D) in the placentas of women with venous insufficiency.

Fig. 7. Immunodetection of MMP-9. A-C. Reduced protein expression detected around the capillaries of villi (arrows) and slight intensity labelling observed in decidual cells (C) in control placentas. D-F. Major protein expression detected around large vessels (D), in the extracellular matrix of a villous (E) and in the decidual cells (F) in the placentas of women with venous insufficiency.
component varied the expression of enzymes able to remodel collagen was compromised. The important role of collagen in a large number of disorders has been highlighted (Pascual et al., 2008; Tas et al., 2016; Sotomayor et al., 2017). Several authors have mentioned the role played by collagen in the structure of the placental villus and how its dysregulation can affect this structure (Rukousuev, 1992; Khoshnoodi et al., 2008). Oefner et al. (2015) described the importance of collagen in placental diseases. Much evidence exists for the placental coexpression of collagen I and III (Vizza et al., 2005; Sati et al., 2008), though the presence of collagens in the human placenta has not yet been related to venous insufficiency. Both collagen types are similar in composition (Balasubramanian et al., 2013), though the presence of type III could indicate different physiological processes. Elevated collagen expression has been described in settings of a placental pathology such as preeclampsia (Jiang et al., 2014).

In our study, the different proteins of the collagen component (COL I and COL III) showed different gene and protein expression profiles. Asgari et al. (2017) observed by atomic force microscopy the important role of COL III for collagen I fibrogenesis. This author mentioned how COL III was present in distensible organs conferring reduced mechanical rigidity. Iwahashi et al. (1997) described that modification in level of COL I in the placentas might be closely associated with disturbance in trophoblastic cell functions and the supply of nutrients to the developing fetus. Furthermore, it has been described the deposition of COL I in uteroplacental matrix in relation with Hypertension in Pregnancy (Li et al., 2017). In earlier studies it was shown that this reduction in the COL-I/III ratio is indicative of the formation of heterotypical fibres, changing their diameter and dimensions (Stevenson et al., 2006). Volk et al. (2011) mentioned that this could affect organ functionality and might suggest a distended placenta in women with VI.

These data suggest that the capacity of reaction of the placental villi will be greater in women with VI than without VI. The collagen activity observed here could be a mechanism triggered by a possible hypoxic state caused by altered arterio-venous flow in the placental chamber. Increased levels of collagen proteins will lead to increased levels of extracellular matrix fibres, and this will impair exchange between the foetal and maternal bloodstream. This would be considered as a stronger vasculosyncytial barrier which would contribute to the reduced passage of oxygen towards the foetal blood. Our findings indicate another implication of matrix components added to those described by Chen and Aplin (2003) as exacerbations induced by hypoxia.

Matrix-fibrillar protein activity is understood by researchers as a balance between their synthesis and degradation directly linked to the activity of metalloproteinases. More specifically, the gelatinases MMP-2 (gelatinase A) and MMP-9 (gelatinase B) capable of degrading collagen have been attributed important roles in remodelling processes (Burrows et al., 1996; Bujan et al., 2000; Bellón et al., 2001; Xu et al., 2001; Chen and Aplin, 2003). MMP-2 and MMP-9 are produced both in vascular cells and inflammatory cells (Jacob et al., 2001; Sansisvelst-Morell et al., 2007). In our study, no differences were detected between our study groups of women with and without VI in MMP-2 expression or the cell types involved. This may be because this protein has been related to acute remodelling events and our samples would reflect a slower, more persistent altered state of blood flow (Hu et al., 2007). Our MMP-9 results essentially indicate its presence at the level of the white blood cells both in the maternal and foetal blood. This is consistent with reports of the secretion of MMP-9 by placental leukocytes at term as a mechanism directed at promoting labour (Flores-Pliego et al., 2015). Unlike other authors, we found no significant activity of this metalloproteinase at the level of the trophoblast cells (Bo et al., 2013). However, our findings point to the participation of decidual cells in secreting this enzyme as these showed significantly more MMP-9 activity in the placentas of women with VI. A role of this cell population could be related to the activity of white blood cells in promoting the mechanisms of child birth.

Conclusions

This is the first study to examine the possible relationship between having VI in pregnancy and the fibrillar collagen composition of the placental extracellular matrix. Our observations were the increased presence of COL III in the placentas of women with VI along with greater MMP-9 activity in decidual cells. Further studies are needed to confirm if indeed the venous return abnormalities seen in pregnant women could induce alterations at the level of the placenta.

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