Research Paper

Abnormal proinflammatory and stressor environmental with increased the regulatory cellular IGF-1/PAPP-A/STC and Wnt-1/β-Catenin canonical pathway in placenta of women with Chronic venous Disease during Pregnancy

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Abstract

Lower limbs venous insufficiency refers to a wide variety of venous disorders grouped by the term of chronic venous disease (CVD). Hemodynamic and hormonal changes related to pregnancy period, may promote the development of CVD affecting approximately 1 in 3 women. It has been shown that the presence of this condition is associated with damage and placental suffering. Thus, taking IGF-1/PAPP-A/STC-2, inflammatory cytokines production, PI3K/Akt and Wnt/β-catenin pathways as a part of the alterations that occurs in the placenta due to CVD, the aim of this study will be to examine the main components of these pathways. Genic and protein expression of PAPP-A, STC-2, IGF-1, IRS-4, Wnt-1, β-catenin, c-myc, Cyclin D1, IL-4/IL-6 and PI3K/Akt/mTOR pathway will be analysed through RT-qPCR and immunohistochemical techniques in women with CVD (n=62) and pregnant women without this condition (HC) (n=52). PAPP-A, IGF-1, IL-4, IL-6, IRS-4, PI3K, Akt, mTOR, Wnt-1, β-catenin, c-myc and Cyclin D1 expression were found to be increased in women with CVD, whereas STC-2 were decreased in this group, compared to non-affected women. Our study has demonstrated that IGF-1/PAPP-A/STC-2 axis, PI3K/Akt and Wnt/β-catenin pathways, along with c-myc, Cyclin D1 and inflammatory cytokines are altered in placenta women with CVD. These results extent the knowledge that CVD is associated to a placenta damage with abnormal tissue environment and cellular regulation.

Key words: Venous insufficiency; Chronic Venous Disease; Pregnancy; IGF-1/PAPP-A/STC-2 pathway; Wnt/β-catenin canonical pathway
1. Introduction

Lower limbs venous insufficiency refers to a plethora of venous disorders, grouped under the term of Chronic Venous Disease (CVD), being the varicose vein its most important clinical manifestations [1]. It is a condition with a high prevalence in western societies, where approximately 60-70% of adults may present CVD [2, 3]. Annual incidence varies from 2.6% in women to 1.9% in men, according to the Framingham study [4]. Numerous risk factors could promote the onset of CVD, such as female gender, genetic predisposition, smoking, obesity, or pregnancy [5, 6]. Women’s body undergoes a wide range of changes during pregnancy which are crucial for the fetal development, mainly due to hormonal and hemodynamic alterations, importantly affecting the vascular system [7-9]. It has been described that up to a 28% of women could present CVD during gestation, generally in the third trimester [7, 10-12].

Recent studies have demonstrated how pregnancy related CVD is associated with the presence of placental suffering and damage markers, finding an increase in processes of angiogenesis and lymphangiogenesis, hypoxia and oxidative stress [12-15]. Worthy of note is the fact that gestational CVD has been linked with higher levels of systemic lipid peroxidation in these women, along with a fetal pH acidification in the newborns [15]. Equally, these alterations have also been described in severe vascular pathologies such as preeclampsia [16-18] or fetal growth restriction [19], thus showing the importance of continue deepening in the pathophysiological mechanisms of placental damage in women with CVD.

In this sense, it is known that placenta is an essential organ for the maternofoetal exchange of nutrients and waste products, with important functions in the metabolism and in the release of many substances [20, 21]. In this line, the expression of pregnancy-associated plasmatic protein A (PAPP-A) has been shown to be of great relevance in the placental tissue, where it is synthesised, depending its serum levels of a broad variety of factors such as age, ethnic, weight and physiological or pathological status of the mother [22]. PAPP-A, also known as pappalysin 1 is a metalloprotease belonging to the superfamily of metzincins, and its activity plays a key role during pregnancy, increasing the bioavailability of insulin-like growth factors (IGF) 1 and 2 [23]. PAPP-A degrades the IGF binding proteins (IGFBP) 4 and 5, being IGFBP-4 its main target. Consequently, raise free IGF levels within the tissue, binding to the IGF-1 receptor (IGF-1R), hence leading to a signalling cascade mediated by IGF [24].

It has been shown how PAPP-A overactivation may induce an inflammatory response, mostly conducting to a cytokine production and release through the PI3K/Akt pathways, regulated by IGF signalling [25]. In addition, binding of IGF to its receptor, triggers the activation of downstream products like insulin receptor substrate 4 (IRS-4), Wnt/β-catenin canonical pathway, c-myc or Cyclin D1, with mitogenic effects [26-28]. On the other hand, stanniocalcin-2 (STC-2) is an important component of IGF axis, acting as a negative regulator of the proteolytic activity of PAPP-A in the tissue [29]. Proper homeostasis of trophic factors such as IGFs are crucial for the development and functions of the placenta, and their dysregulation have been shown to be related with the pathogenesis of some pregnancy complications [30].

Many authors have revealed the alterations of PAPP-A expression levels and all its cascade in vascular pathologies during pregnancy like preeclampsia, with serious implications in the maternofoetal health [31, 32]. Recently, Wang et al. [33] have showed how genic and protein expression of Wnt/β-catenin components is altered in the placental tissue of women with this condition. Likewise, PI3K/Akt pathway have also been observed to play an important role of the pathogenesis of preeclampsia [34]. For that reason, it is necessary to evaluate the status of PAPP-A and their subsequent cell signalling through the analysis of the genic and protein expression in the placenta of women presenting gestational CVD.

2. Patients and Methods

2.1. Study population

An observational, analytical and prospective cohort study was accomplished, comprising 114 women in the third trimester of pregnancy (32 weeks). 62 were clinically diagnosed with CVD, being median age 33[22 to 40] years old, and gestational period 40.5 [39 to 41.5] weeks. Other 52 patients were studied in parallel as controls, being median age 34[27 to 40] years old, and gestational period 41[39 to 42] weeks. This study has been performed according to basic ethical principles: autonomy, beneficence, nonmaleficence and distributive justice. Its development pursued Good Clinical Practice guidelines, principles announced in Declaration of Helsinki (2013) and Oviedo Convention (1997). Patients were informed and each one of them signed the pertinent informed consent. The Project was approved by Clinical Research Ethics Committee from Central Hospital of Defense Gómez Ulla – UAH (37/17) in March of 2017. During consultation in third trimester it is conducted a medical history, general physical examination and exploration of the lower limbs by Eco-Doppler
samples included multiple cotyledons. These fragments were introduced in two different sterile tubes: one containing Minimum Essential Medium (MEM) with 1% antibiotic/antimycotic (both from ThermoFisherScientific, Waltham, MA, EEUU) and the other one containing RNAlater® solution (Ambion, Austin, TX, EEUU). In the laboratory, the samples were processed in a laminar flow bench II Telstar AV 30/70 Müller 220 V 50 M Hz (Telstar SA Group, Terrassa, Spain), in a sterile environment. The preserved samples were maintained in 1 mL RNAlater®, -80°C until gene expression analysis were accomplished. Samples preserved in MEM were destined to histological and immunodetection studies. Samples preserved in MEM were washed and hydrated multiple times with medium without antibiotic to eliminate blood cells and were cut in fragments that were fixed in F13 (60% ethanol, 20% methanol, 7% polyethylene glycol, 13% distilled H2O), following established protocols (15). Once included, paraffin embedded tissue blocks are prepared with molds. When paraffin is solidified, samples from placental tissue were used as negative control, whose incubation with primary antibody was substituted for incubation with blocking solution (PBS). Chromogenic substrate was prepared immediately before exposure (5mL distilled H2O, 2 drops of buffer, 4 drops of DAB, 2 drops of hydrogen peroxide). This technique allows a brown dyeing. In all immunohistochemistry studies, sections from the same tissue were used as negative control, whose incubation with primary antibody was substituted for incubation with blocking solution (PBS).

2.5. Statistical analysis and results interpretation

For statistical analysis, software GraphPadPrism® 6.0 was used. Mann-Whitney U statistical test was applied. Data are expressed as median with interquartile range (IQR). Significance p-value defined was p<0,05 (*), p <0,01 (**), p <0,001 (**). For every patient in every established group, 5 sections and 10 fields per section were examined by random choice.

Patients were described as positives when marked mean area in the sample was greater or equal to 5% from total, following anatopathological protocol by Cristóbal et al. [40]. Slides were examined under Zeiss Axioshot (Carl Zeiss, Germany) optical microscope.
Table 1. Primers utilized in RT-qPCR and temperature (Tm).

| Gene    | Seq. Fwd (5’→3’) | Seq. Rev (5’→3’) | Tm |
|---------|------------------|------------------|----|
| TBP     | TGCACTAGGAGCCAAGATGAA | CATATCGAGTCTCCCAACCA | 60°C |
| IGF-1   | GCCTTCAGTGGCCGCGTGG | GGATATCGAGGCACGG | 69°C |
| STC-2   | GCCCTGCTGGCCGCTGAC | GCGCATGCTGCATTGAC | 51°C |
| PAPP-A  | CCCAGCAGTCAGATCATCCTT | AGCTGCGCTTCACCTCAG | 52°C |
| Wnt-1   | CGATGTGGGCTATATTGAAC | CGGATTTGCGCATACAG | 60°C |
| β-catenin | TGGGGCCTACTAGTGGACTAC | GCAGACAACGAAACTGCATTA | 59°C |
| Cyclin D1 | CAGAAGTGGGGACCGTTCGCTT | GTCAGAGGATAGCCAGCG | 60°C |
| c-myc   | GCTGCTGGGCAAAAGGTCA | TGCGGATGTTGCTGATGT | 57°C |
| IRS-4   | CTCCAGCCACTGACAGAAGAGA | CTGACGCTGGGTCACAG | 61°C |
| PISK    | CTGCGCTTCGCTCACCACCCCTCT | GCCCTTAATCTCTCCCTCCCTGTT | 60°C |
| Akt     | TGTCTCTGTAGCCCTGGTTT | CCGTATACTGAGCTGCTG | 60°C |
| mTOR    | ATCCAGACCTGACACCAACCT | TCACACACACCAAACCATC | 60°C |
| IL-4    | ACAGCCTCAGACAGGAGAGAG | TGTCCTGGAGGCACAAAGA | 85°C |
| IL-6    | AGTAGTGAGGACCAAGCCAGG | TGCCATTGTTGGTCCCTGA | 60°C |

Table 2. Primary antibodies (A) and secondary (B) employed in immunochemistry studies, showing dilutions used and protocol specifications.

| A Antigen | Species | Dilution | Dealer | Protocol specifications |
|-----------|---------|----------|--------|-------------------------|
| IGF-1     | Mouse   | 1:100    | Abcam  | Sodium citrate 10 mM pH 6 before incubation with blocking solution. |
| PAPP-A    | Rabbit  | 1:250    | Abcam  | Triton 1% in PBS, 10 minutes, before incubation with blocking solution. |
| STC-2     | Mouse   | 1:100    | Abcam  | Triton 1% in PBS, 10 minutes, before incubation with blocking solution. |
| Wnt-1     | Rabbit  | 1:100    | Abcam  | Triton 1% in PBS, 10 minutes, before incubation with blocking solution. |
| β-catenin | Rabbit  | 1:1250   | Abcam  | Triton 1% in PBS, 10 minutes, before incubation with blocking solution. |
| Cyclin D1 | Rabbit  | 1:100    | Abcam  | Sodium citrate 10 mM pH 6 before incubation with blocking solution. |
| c-myc     | Rabbit  | 1:100    | Abcam  | Sodium citrate 10 mM pH 6 before incubation with blocking solution. |
| IRS-4     | Rabbit  | 1:100    | Abcam  | Sodium citrate 10 mM pH 6 before incubation with blocking solution. |
| PISK      | Mouse   | 1:100    | Abcam  | Sodium citrate 10 mM pH 6 before incubation with blocking solution. |
| Akt       | Rabbit  | 1:100    | Abcam  | Sodium citrate 10 mM pH 6 before incubation with blocking solution. |
| mTOR      | Rabbit  | 1:100    | Abcam  | Sodium citrate 10 mM pH 6 before incubation with blocking solution. |
| IL-4      | Mouse   | 1:250    | Abcam  | Sodium citrate 10 mM pH 6 before incubation with blocking solution. |
| IL-6      | Mouse   | 1:200    | Abcam  | Sodium citrate 10 mM pH 6 before incubation with blocking solution. |

| B Antigen | Species | Dilution | Dealer | Protocol specifications |
|-----------|---------|----------|--------|-------------------------|
| IgG (Mouse) | Goat   | 1:300    | Sigma  | Sodium citrate 10 mM pH 6 before incubation with blocking solution. |
| IgG (Rabbit) | Mouse | 1:1000   | Sigma  | Sodium citrate 10 mM pH 6 before incubation with blocking solution. |

3. Results

3.1. Patients with gestational CVD show an increase in PAPP-A and IGF-1 expression in placental villi

First we analyzed the IGF-1/PAPP-A/SCT-2 pathway expression in placentas of women gestational CVD and in controls HC by using RT-qPCR and immunochemistry. Our results showed a significant increase in PAPP-A gene expression in placentas of patients with CVD, expressed in arbitrary units (CVD=36.213 [18.563-40.890], HC=32.438 [15.203-39.895], ***p=0.003, Figure 1.A). Protein expression analysis showed a significant increase in placental villi percentage positive for PAPP-A in patients with CVD (CVD=55.000 [12.000-99.000], HC=40.000 [10.000-86.000] ***p=0.0003, Figures 1.B and D-F). Besides, decidual cells percentage with positive expression for such protein was significantly higher in placentas of women with gestational CVD [CVD=55.000 [20.000-88.000], HC=42.000 [19.000-84.000], *p=0.0297, Figures 1.C and E-G].

Our study showed a significant decrease in SCT-2 gene expression in placenta of women gestational CVD in comparison to HC (CVD=33.992 [19.068-49.124], HC=36.389 [20.560-40.256], *p=0.0376, Figure 2.A). Immunochemistry study showed a decrease in the percentage of positive villi for SCT-2 in placentas of women with gestational CVD (CVD=33.000 [13.000-55.000], HC=50.000 [20.000-85.000], ***p<0.0001, Figure 2.B and D-F), just like decidual cells percentage (CVD=53.500 [12.000-91.000], HC=68.500 [22.000-98.000], ***p=0.0002, Figures 2.C and E-G).

The study of IGF-1 gene expression showed a significant increase in placenta of women gestational CVD in comparison to HC (CVD=30.902 [19.068-49.134], HC=28.394 [7.759-39.889], *p=0.0188, Figure 3.A). In parallel, a significant increase was observed in IGF-1 protein expression in placental villi of women with gestational CVD (CVD=52.000 [13.000-99.000], HC=34.500 [9.000-97.000], *p=0.0210, Figure 3.B and D-F), as well as in decidual cells of placenta of women with gestational CVD (CVD
3.2. Increase in IRS-4 expression in placentas of women with gestational CVD

Next, we investigated cell transduction signal related to IGF-1 and PAPP-A stimulation. We observed a significant increase in IRS-4 gene expression in placentas of women with gestational CVD in comparison to HC (CVD =34.986 [18.826-45.852], HC=30.386 [11.969-44.028], *p=0.0120, Figure 4.A). Immunochemistry studies showed how placental villi percentage with positive expression for IRS-4 was significantly enhanced in patients with gestational CVD (CVD =75.500 [32.000-99.000], HC=36.000 [14.000-81.000], ***p<0.0001, Figure 4.B and D-F), just like percentage of decidual cells for these patients (CVD =74.500 [34.000-99.000], HC=34.000 [14.000-72.000], ***p<0.0001, Figure 4.C and E-G).

3.3. Placenta of women with gestational CVD show an activation of PI3K/Akt/mTOR pathway.

The gene expression study showed a significant increase in PI3K / Akt / mTOR in placentas of women with gestational CVD [(PI3K=CVD=27.729 [12.966-43.915], HC=24.536[10.190-43.915], *p=0.0236, Figure 5.A); (Akt=CVD=34.063[17.542-46.616], HC=24.723 [10.566-44.562], **p=0.0015, Figure 6.A); (mTOR=CVD=31.925 [13.033-47.592], HC=25.079 [10.490-43.069], ***p=0.0040, Figure 7.A)].

Figure 1. A. RNA expression levels for PAPP-A by RT-qPCR. B. Percentage of placental villi with positive protein expression for PAPP-A by using immunochemistry techniques. D-G. Images where immunoexpression of PAPP-A is showed in placental villi (D and F) and in decidual cells (E and G). CVD=Women with diagnosed gestational chronic venous disease. HC= Venous control p<0.05 (*), p<0.01 (**), and p<0.001 (***). Arrow=shows positive expression of the protein.

Figure 2. A. mRNA expression levels for STC-2 by RT-qPCR. B. Percentage of placental villi with positive protein expression for STC-2 by using immunochemistry techniques. D-G. Images where immunoexpression of STC-2 is showed in placental villi (D and F) and in decidual cells (E and G). CVD=Women with diagnosed gestational chronic venous disease. HC= Venous control p<0.05 (*), p<0.01 (**), and p<0.001 (***). Arrow=shows positive expression of the protein.
Figure 3. A. mRNA expression levels for IGF-1 by RT-qPCR. B. Percentage of placental villi with positive protein expression for IGF-1 by using immunohistochemistry techniques. D-G. Images where immunohistoexpression of IGF-1 is showed in placental villi (D and F) and in decidual cells (E and G). CVD=Women with diagnosed gestational chronic venous disease. HC= Venous control. *p<0.05 (*), **p<0.01 (**), and ***p<0.001 (***) Arrow shows positive expression of the protein.

Figure 4. A. Expression levels of mRNA for IRS-4 by RT-qPCR. B. Percentage of placental villi with positive protein expression for IRS-4 by using immunohistochemistry techniques. D-G. Images where immunohistoexpression of IRS-4 is showed in placental villi (D and F) and in decidual cells (E and G). CVD=Women with diagnosed gestational chronic venous disease. HC= Venous control. *p<0.05 (*), **p<0.01 (**), and ***p<0.001 (***) Arrow shows positive expression of the protein.

Figure 5. A. mRNA expression levels for PI3K by RT-qPCR. B. Percentage of placental villi with positive protein expression for PI3K by using immunohistochemistry techniques. D-G. Images where immunohistoexpression of PI3K is showed in placental villi (D and F) and in decidual cells (E and G). CVD=Women with diagnosed gestational chronic venous disease. HC= Venous control. *p<0.05 (*). Arrow shows positive expression of the protein.
Immunohistochemical studies show a significant increase in placental villi with positive protein expression for PI3K in the placentas of women with CVD (CVD = 55,000 [15,000-94,000], HC = 48,500 [16,000-87,000], * p = 0.0333, Figure 5.C and D). PI3K expression in decidual cells was also significantly increased (CVD = 58,000 [26,000-87,000], HC = 52,000 [21,000-86,000], p = 0.0378, Figure 5.C).

Furthermore, Akt protein expression showed a significant increase in the placental villi of patients with gestational CVD (CVD = 64,500 [21,000-97,000], HC = 51,500 [14,000-87,000], ** p = 0.0035, Figure 6.B and D). Similarly, Akt protein expression in decidual cells was significantly higher compared to HC (CVD = 61,000 [31,000-89,000], HC = 47,000 [13,000-81,000], ** p = 0.0023, Figure 6.C and E).

Furthermore, mTOR showed an increase in protein expression in placental villi with CVD (CVD = 62,500 [18,000-97,000], HC = 45,000 [12,000-94,000], ** p = 0.0016, Figure 7.B and D). It was similarly observed in the decidual cells of CVD placentas (CVD = 63,000 [21,000-98,000], HC = 45,500 [14,000-86,000], ** p = 0.0015, Figure 7.C and E).

3.4. Placentas of women with gestational CVD show an increase in Wnt-1/β-catenin pathway.

In parallel, we also studied the Wnt-1/β-catenin canonic pathway in placenta of women with gestational CVD and HC. The genic expression analysis for Wnt-1 showed a significant increase in placentas of women with gestational CVD.
comparison to HC patients (CVD=37.549 [21.987-49.585], HC=35.540 [5.865-39.678], **p=0.0026, Figure 8.A). In this sense, placental villi percentage positive for Wnt-1 expression was observed as significantly raised (CVD=75.000 [33.000-98.000], HC=24.000 [6.000-63.000], ***p<0.0001, Figure 8.B and D-F). There were not significant differences observed in percentages of Wnt-1 expression in decidual cells from the study groups (CVD=56.000 [21.000-97.000], HC=52.000 [10.000-96.000], p=0.1762, Figure 8.C and E-G).

In addition, a significant increase in gene expression of β-catenin was observed in placentas of women with gestational CVD in comparison to HC (CVD=31.464 [28.811-45.655], HC=30.967 [20.572-39.710], *p=0.0143, Figure 9.A). Protein expression analysis showed how the percentage of placental villi with positive expression for β-catenin was significantly higher in placentas of women with gestational CVD (CVD=59.000 [25.000-81.000], H=54.000 [21.000-79.000], *p=0.0288, Figure 9.B and D-F). There were no significant differences observed in percentage of decidual cells with positive expression for β-catenin among the established study groups (CVD=65.500 [22.000-99.000], HC=61.000 [15.000-85.000], p=0.0900, Figure 9.C and E-G).

The study of c-myc genic expression by RT-qPCR showed a significant increase in placentas of women with gestational CVD comparing to HC (CVD=32.836 [21.233-49.982], HC=32.103 [20.710-37.950], *p=0.0360, Figure 10.A). There were a significant differences observed in the percentage of placental villi (CVD=53.000 [9.000-96.000], HC=46.000 [7.000-82.000], *p=0.0463, Figure 10.B and D-F), and decidual cells positive for c-myc expression in placentas of women with gestational CVD in comparison to the HC group (CVD=65.000 [34.000-99.000], HC=61.000 [21.000-85.000], *p=0.0284, Figure 10.C and E-G).

![Figure 8. A. mRNA expression levels for Wnt-1 by RT-qPCR. B. Percentage of placental villi with positive protein expression for Wnt-1 by using immunochemistry techniques. D-G. Images where immunoexpression of Wnt-1 is showed in placental villi (D and F) and in decidual cells (E and G). CVD=Women with diagnosed gestational chronic venous disease. HC=Venous control p<0.05 (*), p<0.01 (**), and p<0.001 (***). Arrow=shows positive expression of the protein.](image)

![Figure 9. A. mRNA expression levels for β-catenin by RT-qPCR. B. Percentage of placental villi with positive protein expression for β-catenin by using immunochemistry techniques. D-G. Images where immunoexpression of β-catenin is showed in placental villi (D and F) and in decidual cells (E and G). CVD=Women with diagnosed gestational chronic venous disease. HC=Venous control p<0.05 (*), p<0.01 (**), and p<0.001 (***). Arrow=shows positive expression of the protein.](image)
Cyclin D1 showed a significant increase in its gene expression in placentas of women with gestational CVD in comparison to placentas of HC women (CVD=32.390 [29.145-49.170], HC=30.375 [5.812-38.770], *p=0.0028, Figure 11.A). The immunohistochemistry studies showed how Cyclin D1 expression was significantly greater in terms of expression percentage in placental villi of women with diagnosed gestational CVD in comparison to HC group (CVD=54.000 [28.000-91.000], HC=45.000 [10.000-77.000], **p=0.0010, Figure 11.B and D-F). In parallel, the increase in the percentage of Cyclin D1 protein expression was observed in decidual cells from placentas of women with CVD (CVD=77.000 [25.000-97.000], HC=34.500 [7.000-63.000], ***p<0.0001, Figure 11.C and E-G).

3.5. High expression of IL-4/IL-6 in placenta of women with gestational CVD

Gene expression studies using RT-qPCR showed an increase in IL-4 / IL-6 in CVD placentas [(IL-4 = CVD = 32,058 [21,987-49,369], HC = 26,974 [19,700-41,569], *** p <0.0001, Figure 12.A); (IL-6 = CVD = 30,016 [17,557-49,670], HC = 23,537 [12,489-43,894], * p = 0.0241, Figure 13.A)]. The study of protein expression in placental villi similarly showed an increase in IL-4 / IL-6 in women with gestational CVD [(IL-4 = CVD = 65,000 [21,000-99,000], HC = 45,000 [19,000-86,000], *** p <0.0001, Figure 12.B and D); (IL-6 = CVD = 56,000 [17,000-89,000], HC = 46,500 [19,000-81,000], ** p = 0.0031, Figure 13.B and D)]. This significant trend of
increased protein expression was also shown in the decidual cells of placentas from women with gestational CVD [(IL-4 = CVD = 58,500 [29,000-97,000], HC = 54,000 [23,000-87,000], * p = 0.0483, Figure 12.C and E); (IL-6 = CVD = 64,500 [35,000-99,000], HC = 52,000 [12,000-98,000], *** p <0.0001, Figure 13.C and E)].

4. Discussion

In this paper, we have demonstrated that the placenta of CVD women show an abnormal villous cells behaviour with increased expression of the IGF-1/PAPP-A/STC-2 along with IRS-4, PI3K/Akt/mTOR activation pathways as well as the Wnt/β-catenin canonical pathway accompanied by a proinflammatory environment.

Pregnancy is associated to development of CVD in a relevant group of previously healthy women. In the placenta of these women, there is evidence of cellular damage and tissue hypoxemic damage [13]. However, the pathogenic mechanisms involved in alteration of the placenta villous cells of CVD remains undefined. It has been shown that the proliferation and differentiation of villous cells is regulated by different signals including IGF-1/PAPP-A/STC-2 pathway. IGF bioavailability is regulated in a paracrine manner in human placenta, controlling fetal and maternal tissue growth where it is bind to cells membrane glycosaminoglycans in placental villi,
In placenta villous. Interestingly, a ECM matrix (ECM) is a critical regulatory signal for certain pathological conditions including those of the placenta tissue [44, 45]. Interestingly, a ECM remodelling has been observed in placenta of women with CVD [46]. Thus, it is possible to suggest that in the placenta of women with CVD, ECM alteration might contribute to the induction of the observed PAPP-A overexpression. In addition, further mechanisms may play a pathogenic role in the induction of the IGF-1/PAPP-A/STC-2 pathway abnormalities found in placenta villi and decidual cells. Decrease in STC-2 has been related with ectopic calcifications in a broad range of tissues [47]. Furthermore, the binding of calcium ions to the C-terminal domain PAPP-A play an important role in the interaction with IGFBP-4 and its proteolysis [48].

An augmentation in placental villi calcifications has been described in CVD women [14]. Thus, this excessive deposit of calcium in the placenta might also favour the proteolytic activity of PAPP-A. In addition, PAPP-A expression and activation is also regulated by the immunoinflammatory environment in damaged tissues. IL-6 appears to be a cytokine relevant for the IGF-1/PAPP-A/STC-2 regulation. [49] A direct association between PAPP-A levels and IL-6 has been described in fluid from inflammatory lesions, elucidating the link between inflammation and IGF-1 system. IL-4 also induce the expression of PAPP-A, acting synergistically with TGF-β [50]. In this work, we have demonstrated an increased expression of IL-6 and IL-4 in villous placenta of CVD women supporting a potential additional mechanism for the observed increased IGF-1/PAPP-A activity. Interestingly, cytokine abnormal expression have been described in vascular damage of the placenta [51]. Furthermore, PAPP-A has been associated to the induction of cytokines expression by the activation of IGF-1/PI3K pathways [25]. Interestingly, it is possible to suggest that a pathogenic positive feedback between the cytokines IL6 and IL4 and IGF-1/PAPP-A pathway may be operating in the placenta villous.

IGF-1 plays a critical role in the activation of the PI3K/Akt/mTOR pathway. A key component in the IGF-1 signalling is the adaptor protein IRS-4. When IGF-1 binds to its receptor, IGF-1R, a tyrosine kinase receptor, phosphorylates itself and IRS-4, thus initiating the process of cell transduction [52]. Afterwards, many researches have shown that IRS4 phosphorylates PI3K, therefore being a direct inductor of PI3K/Akt/mTOR pathway [53, 54]. The overexpression of IGF-1 and PI3K/Akt/mTOR has been involved in the pathogenesis of vascular wall inflammation and aging [55, 56]. Interestingly, in human placenta, enhanced IGF-1 levels have been associated to increased PI3K/Akt/mTOR pathway activity and subsequent alterations in placental protein synthesis, mitochondrial function and nutrient transport [57, 58]. Our findings show a marked overexpression of phosphorylated IRS-4 and PI3K/Akt/mTOR pathway in placenta villous of CVD women. These findings support that the observed increased levels of IGF1 induce an enhanced transduction signal of the IGF-1R receptor and of the PI3K/Akt/mTOR pathway in the villi cells. Thus, it is possible to suggest that this pathway activation may contribute to the cellular placenta damage and accelerated aging observed in CVD women. [59]. In addition, the overexpression of hypoxia inducible factor 1- α (HIF-1α) found in placenta from CVD women may also be involved in the induction of this pathogenic PI3K/Akt/mTOR pathway in placenta villi. Moreover, PI3K/Akt/mTOR pathway is equally related with other pathological and vascular endothelial growing factor (VEGF) [60].

The development and normal functioning of placenta cells is complex and several regulatory pathways are involved. There evidence that in addition to PI3K/Akt/mTOR, Wnt/β-catenin pathway plays a critical role in the regulation of villous palcenta cells [61]. Pathological placenta conditions such as those observed in women with preeclampsia are associated to alteration in Wnt/β-catenin pathway [33]. Our results clearly show an increase in Wnt-1 and β-catenin expression in placenta villous cells of women with CVD. Wnt-1 is an important activator of canonical Wnt pathway that is physiologically highly expressed in trophoblasts in the early pregnancy, reducing its levels in the term, thus denoting the importance of this pathway in the initial stages of gestation [61]. The overexpression of Wnt-1 may be explained by the stage of villous cellular damage observed in placenta of women with CVD (human pathology). It is possible to suggest that the observed Wnt-1 overexpression may support an involution of the placenta cells from CVD women. In addition, it has been well-established the interaction
between IGF-1/PI3K signalling and canonical Wnt/β-catenin pathway at different levels [62]. After its activation in the cytoplasm, β-catenin translocates to the nucleus, forming a complex with other molecules and controlling the expression of a wide variety of genes such as c-myc and Cyclin D1, which will be essential in the cell fate determination [33]. Our results show an important increase of both, Cyclin D1 and c-Myc in the placental tissue of women with CVD, in comparison with healthy subjects. The overexpression of both genes support the relevance of the described Wnt-1 and β-catenin expression in placenta villous cells from CVD women [63]. It has been proposed a coordinated role of IGF-1/PI3K and β-catenin in promoting the expression of Cyclin D1 [28]. Interestingly, cyclin D1 promotes the regulation of placenta angiogenesis during the third trimester of gestation, a process increased in placenta of women with CVD [12, 64]. c-Myc plays a critical role in the regulation of the proliferation and differentiation of trophoblast cells in physiological or pathophysiological conditions [65, 66]. Taken together our results show that the villous cells of CVD women suffer an abnormal environment with increased signalization of the critical PI3K/Akt/mTOR and Wnt/β-catenin pathways. The abnormal placenta tissue homeostasis is explained by an increases in IGF-1/PAPP-A/STC-2 stressor stimulation as well as in a local immune mediated proinflammatory ambiance. The described extracellular signalizations and intracellular abnormal regulatory events give lights for the understanding of the increased cellular damage and apoptosis and tissue remodellation observed in placenta from CVD women. An interesting point in future studies is to measure the systemic levels of these parameters to check their effects on the organism.

5. Conclusions

Our study is the first to describe that villous placenta cells of women presenting CVD show an alteration in the genic and protein expression of IGF-1/PAPP-A/STC-2 axis as well as its downstream signalling components such as IRS-4 PI3K/Akt/mTOR and Wnt/β-catenin pathways. The abnormal placenta tissue homeostasis is explained by an increases in IGF-1/PAPP-A/STC-2 stressor stimulation as well as in a local immune mediated proinflammatory ambiance. The described extracellular signalizations and intracellular abnormal regulatory events give lights for the understanding of the increased cellular damage and apoptosis and tissue remodellation observed in placenta from CVD women. An interesting point in future studies is to measure the systemic levels of these parameters to check their effects on the organism.
Competing Interests

The authors have declared that no competing interest exists.

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