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Pyogenic granuloma, an impaired wound healing process, linked to vascular growth driven by FLT4 and the nitric oxide pathway

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Pyogenic granuloma, also called lobular capillary hemangioma, is a condition usually occurring in skin or mucosa and often related to prior local trauma or pregnancy. However, the etiopathogenesis of pyogenic granuloma is poorly understood and whether pyogenic granuloma being a reactive process or a tumor is unknown. In an attempt to clarify this issue, we performed genome-wide transcriptional profiling of laser-captured vessels from pyogenic granuloma and from a richly vascularized tissue, placenta, as well as, from proliferative and involutive hemangiomas. Our study identified a gene signature specific to pyogenic granuloma. In the serial analysis of gene expression (SAGE) database, this signature was linked to 'white blood cells monocytes'. It also demonstrated high enrichment for gene ontology terms corresponding to 'vasculature development' and 'regulation of blood pressure'. This signature included genes of the nitric oxide pathway alongside genes related to hypoxia-induced angiogenesis and vascular injury, three conditions biologically interconnected. Finally, one of the genes specifically associated with pyogenic granuloma was FLT4, a tyrosine-kinase receptor related to pathological angiogenesis. All together, these data advocate for pyogenic granuloma to be a reactive lesion resulting from tissue injury, followed by an impaired wound healing response, during which vascular growth is driven by *FLT4* and the nitric oxide pathway.
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Vascular lesions remain a challenging area for pathologists. It is not uncommon to see many types of vascular lesions reported under the generic term of 'hemangioma'. It is possibly a residual influence from an early classification published by Virchow[1] that categorized vascular lesions according to the type of vessels they are constituted of. Currently, the International Society for the Study of Vascular Anomalies aims at classifying them according to their clinico-pathological characteristics and as such, divides them into vascular malformations and vascular tumors, a suggestion initially made by Mulliken and Glowacki.[2] However, this classification does not take into account vascular lesions presumed to be reactive such as reactive angioendotheliomatosis and possibly pyogenic granuloma, also known as lobular capillary hemangioma.

Identification of genetic abnormalities in various vascular malformations has resulted in a great improvement of their classification and in understanding their pathogenesis (for review, see Boon *et al*[3]). Comparable advances have not yet been made for pyogenic granuloma. This is attested to by the use of two different terms to define the same lesion: 'pyogenic granuloma', which implies a reactive condition, and 'lobular capillary hemangioma', which is indicative of a tumor. By comparing genome-wide transcriptional profiling of laser-captured, formalin-fixed and paraffin-embedded micro-dissected vessels of pyogenic granuloma, infantile hemangiomas and placenta, we attempted to better understand the pathogenesis of pyogenic granuloma.

Unsupervised statistical analyses demonstrated vessels of pyogenic granuloma to have a genetic profile distinct from those of infantile hemangiomas

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and placenta. Supervised analyses isolated a gene signature more specifically related to pyogenic granuloma. By analyzing gene ontology, terms this signature was linked to, serial analysis of gene expression tissues it was associated with, pathways it was affiliated with, and also the biological functions described for the genes constituting the signature. We advocate that pyogenic granuloma results from tissue injury, followed by an impaired wound healing process, during which vascular growth is driven by *FLT4* and the nitric oxide pathway.

Materials and methods

Samples

After an initial control step for RNA quantity and quality, capillaries from formalin-fixed and paraffin-embedded normal term placenta ($n=3$), proliferative infantile hemangioma ($n=3$), involuting infantile hemangioma ($n=4$) and pyogenic granuloma ($n=4$) were laser captured on the Veritas Microdissection Instrument (Applied Biosystems, Carlsbad, CA, USA), as previously described.[4]

Array Hybridization

RNA was extracted and amplified using the Paradise Reagent System Kit according to the manufacturer's recommendations (Paradise Reagent System Kit, Applied Biosystems). The amplified and purified cDNAs were then submitted to the Harvard–Partner Center for Genetics and Genomics Facility (www.hpcgg.org). Samples were labeled with biotinylated probes using Bioarray High Yield transcription kit following manufacturer's protocol (Enzo Biochemical, New York, NY, USA). Their concentration was determined by UV absorbance utilizing a Bio-Tek Plate Reader (Bio-Tek Instruments, Winooski, VT, USA). After fragmentation, 20 μg of each biotinylated cRNA preparation was hybridized to the Affymetrix Human X3P GeneChip Array (http://www.affymetrix.com/products/arrays/specific/x3p.affxArrays) according to the manufacturer's protocol. Microarrays were washed and stained on a Model 450 Fluidics station controlled by the Affymetrix GeneChip Operating System (GCOS). Images from the scanned chips were processed using an Affymetrix Model 7000 scanner with autoloader. The Affymetrix GCOS v1.3 operating system controls the Model 7000 scanner and data acquisition functions (www.affymetrix.com). Image files were downloaded, imported and analyzed.

Bioinformatics

Only transcripts present were considered for statistical analyses ($n=21\,318$). They were selected by excluding the transcripts that had an intensity value of <19, in less than nine out of the 14 samples studied. Those with a fold difference >2 were further studied. To identify these genes, a comparison was made between the mean of the intensity values from the transcripts of pyogenic granuloma and one of the other tissue/lesions tested. Additionally, the transcripts with a s.d. >1.8 were analysed. To isolate these, the median of the signal intensity of each transcript was defined for each group of lesions or tissue studied. The mean of these medians was then calculated, and subsequently used to divide the signal intensity of the individual transcript. Afterwards a log 2 conversion was performed, and the s.d. of each transcript was defined.

Transcripts with a s.d. >1.8 were submitted for further statistical analyses performed on the TIGR MultiExperiment Viewer platform (MeV4.6, http://www.tm4.org/mev/[5]). Correspondence analysis was performed. This analysis permits a statistical visualization of the relationship between transcript profiles and samples and was run using the three most informative χ^2-values. Analysis of variance was also employed, based on 5000 hazardous permutations. This allowed for the identification of genes more specifically associated to each one of the four tissue/lesions analyzed. To identify genes more specifically related to pyogenic granuloma, studies were made comparing two groups of transcripts: one including transcripts from pyogenic granuloma, and the other, from infantile hemangiomas and placenta combined. Between these two groups, a *t*-test was run on the MeV platform using 5000 hazardous permutations, a *P*-value of 0.001 and a Bonferroni adjusted correction. In-house filters were also designed to detect transcripts specifically over- and underexpressed in all pyogenic granuloma samples compared with the other groups.

Finally, the identified transcripts were annotated using the DAVID platform (http://david.abcc.ncifcrf.gov/), the UCSC Genome Bioinformatics database (http://genome.ucsc.edu/), the Standford Microarray database (http://smd.standford.edu/) and PubMed (http://ncbi.nlm.nih.gov/pubmed/).

Immunohistochemistry

Formalin-fixed and paraffin-embedded sections from pyogenic granulomas ($n=8$), placentas ($n=3$), proliferative infantile hemangiomas ($n=3$), involutive infantile hemangiomas ($n=6$), kaposiform hemangioendotheliomas ($n=2$), epithelioid hemangioendothelioma ($n=1$), reactive angioendotheliomatosis ($n=1$), venous malformation ($n=1$), spindle cell hemangioma ($n=1$) and inflammatory granulation tissue ($n=4$) were stained using the Ventana Discovery XT automated slide processor for an antibody directed against PLVAP (Rabbit Polyclonal, Sigma, dilution: 1/100) and the secondary OmniMap DAB anti-Rabbit detection kits (Ventana Medical Systems, Tucson, AZ, USA).

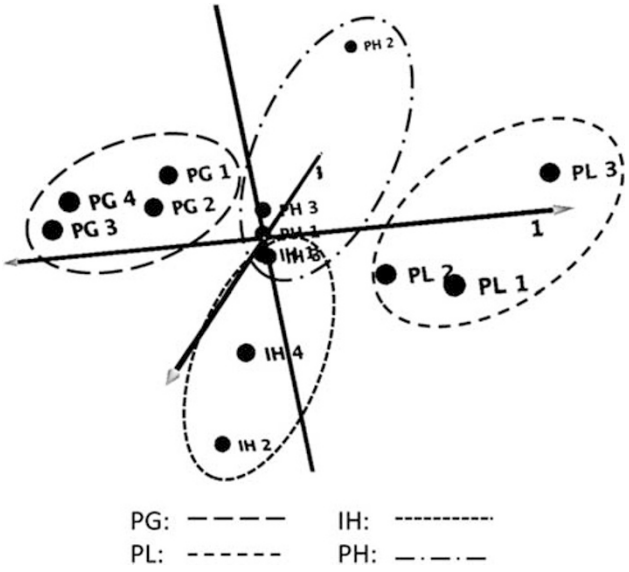

Figure 1 Correspondence analysis showing vessels dividing into four clusters with discrete overlap between proliferative and involutive infantile hemangiomas. PG: pyogenic granuloma, PL: placenta; IH: involutive hemangioma, PH: proliferative hemangioma.

Standard quality control procedures were undertaken to optimize antigen retrieval, primary antibody dilution, secondary antibody detection and other factors for both 'signal and noise'.

Results

Correspondence analysis was one of the studies performed based on the genome-wide transcriptional profiling of laser capture formalin-fixed and paraffin-embedded micro-dissected vessels of pyogenic granuloma, placenta, proliferative and involutive infantile hemangiomas. This allowed a better understanding of the relationship between the gene expression of the 14 analyzed samples of the four different types of lesions and tissue (Figure 1). This analysis revealed that the genes of similar lesions or tissue clustered together and thus, formed four groups, but with a discrete overlap between proliferative and involutive infantile hemangiomas.

A one-way analysis of variance was then carried out to identify the transcripts implicated in this partition into four groups. It identified 43 transcripts corresponding to 37 known genes. These were used for subsequent unsupervised hierarchical clustering (Figure 2). The derived phylogenetic tree demonstrated that the samples to divide first into two groups: one comprised of the placenta and the other consisting of pyogenic granuloma and infantile hemangiomas. A second division separated this latter group into pyogenic granuloma on one hand and hemangiomas on the other. A final split separated hemangiomas according to their phase,

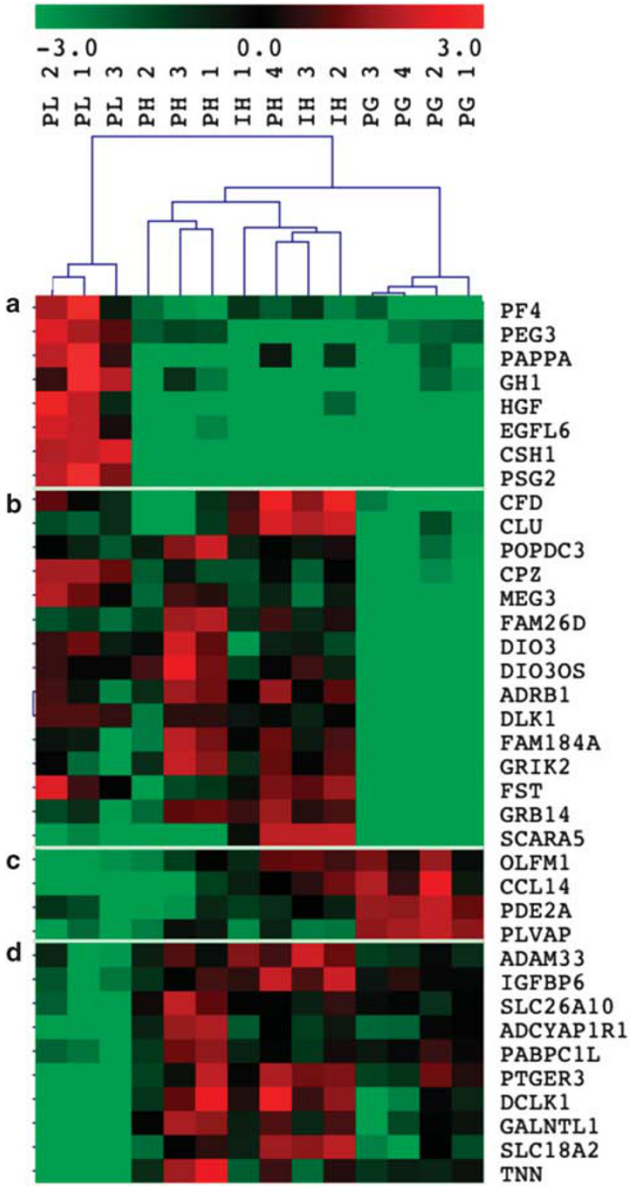

Figure 2 Heat-map of hierarchical clustering performed on genes identified by an analysis of variance. PG: pyogenic granuloma, PL: placenta, IH: involutive hemangioma and PH: proliferative hemangioma. (**a**) Genes specifically related to placenta. (**b**) Genes associated with placenta, proliferative and involutive infantile hemangiomas. (**c**) Genes more specifically linked to pyogenic granuloma. (**d**) Genes more specifically linked to proliferative and involutive infantile hemangiomas.

proliferative or involutive, with one proliferative hemangioma mis-clustered into involutive hemangiomas. For the genes identified by the analysis of variance, unsupervised hierarchical clustering assembled them into four groups: A–D (Figure 2). Group A contained eight genes highly upregulated in the placenta. They showed high enrichment scores for the Gene Ontology terms 'extracellular region' and 'female pregnancy'. An association with 'placenta normal' in Expressed Sequence Tags

database was detected. Group B, comprised 15 genes upregulated in placenta and infantile hemangiomas. They were linked to Gene Ontology terms including 'plasma membrane', 'regulation of system process' and 'cell surface receptor-linked signal transduction'. Genes of group C were related to pyogenic granuloma and to 'vascular, normal liver' in the Serial Analysis of Gene Expression database. The last 10 genes, forming group D, were common to vessels of proliferative and involutive infantile hemangiomas. They were associated with the Gene Ontology term corresponding to 'intrinsic to membrane'.

To identify genes specifically related to the vessels of pyogenic granuloma, a *t*-test was performed comparing the genes expressed in pyogenic granuloma to those of the placenta and hemangiomas combined. This analysis yielded a list of 31 differentially expressed genes (Figure 3a). According to the Serial Analysis of Gene Expression database, these genes were related to 'vascular hemangioma', 'placenta' and 'vascular normal liver'. In the Kegg pathways, they were associated with 'neuroactive ligand-receptor interaction' while in Gene Ontology, they were classified into 'vascular

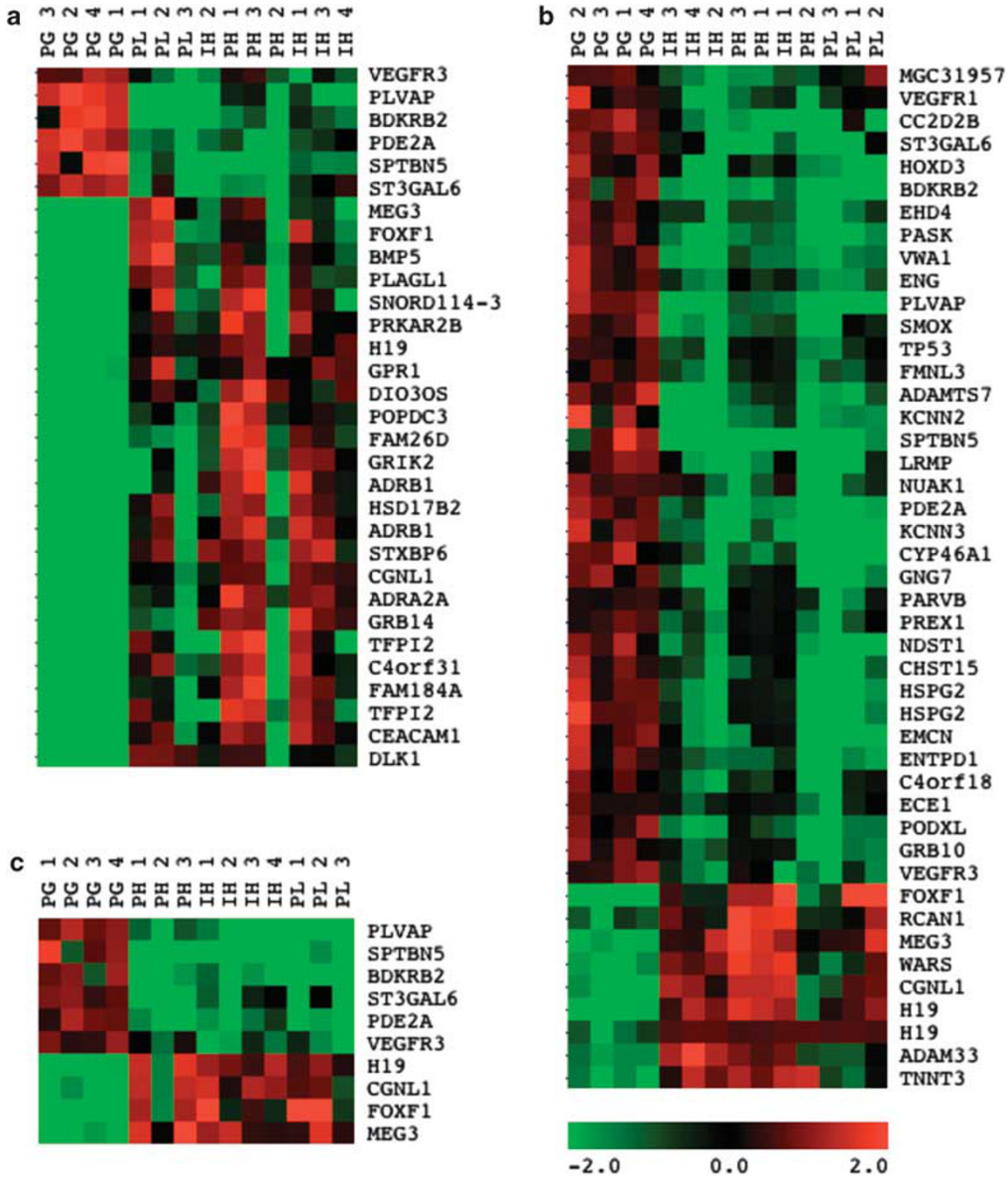

Figure 3 (**a**) Heat-map of significant genes isolated by a *t*-test comparing genes expressed in pyogenic granuloma to the ones in placenta and infantile hemangiomas combined. (**b**) Heat-map of significant genes isolated by in-house algorithms comparing genes expressed in pyogenic granuloma to those in placenta and infantile hemangiomas combined. (**c**) Heat-map of the 10 genes common to the *t*-test and in-house algorithm as specifically expressed in pyogenic granuloma compared with placenta and infantile hemangiomas combined.

process in circulatory system', 'negative regulation of blood pressure' and 'integral to membrane'. Additionally, two of the genes, *BDKRB2* and *PDE2A*, belong to nitric oxide pathway.

An in-house algorithm was designed in MS Excel with the intent to determine the genes specifically up- and downregulated in all pyogenic granulomas when compared with placenta and infantile hemangiomas combined. It recognized 45 such transcripts, corresponding to 43 genes with nine downregulated and 34 upregulated in pyogenic granuloma (Figure 3b). With the exception of one, *ECE1*, the genes selected by this method corresponded exactly to the ones isolated by a fold difference greater than two (Figure 4). In the Serial Analysis of Gene Expression database, these genes were associated with 'vascular endothelium', 'white blood cells monocytes', 'vascular hemangioma' and various cancers. They also showed high enrichment for Gene Ontology terms corresponding to 'embryonic morphogenesis', 'blood vessel morphogenesis', 'vasculature development', 'regulation of blood pressure', 'response to hypoxia' and 'cell surface receptor-linked signal transduction'. This gene signature was affiliated with five Kegg pathways: focal adhesion with *FLT-1*, *FLT4* and *PARVB*; purine metabolism with *ENTPD1* and *PDE2A*; endocytosis with *EHD4* and *FLT-1*; chemokine signaling pathway with *GNG7* and *PREX1*; and cytokine–cytokine receptor interaction with *FLT-1* and *FLT4*. In Biocarta, two genes were linked to 'actions of nitric oxide in the heart': *BDKRB2* and *PDE2A*. Finally, among these 43 genes, 10 were also identified by the *t*-test with six being upregulated in pyogenic granuloma (*FLT4, ST3GAL6, PDE2A, BDKRB2, PLVAP* and *SPTBN5*) and four downregulated (*H19, CGNL1, FOXF1* and *MEG3*) (Figure 3c). Their fold differences ranged from 3.2 to 12.7 and from 4.2 to 19.8, respectively (Figure 4).

Immunohistochemistry

All pyogenic granulomas showed moderate to strong staining for PLVAP in the endothelium of all capillaries ($n=8$, Figure 5a). Placentas show mild-to-moderate endothelial staining in a few capillaries in two cases and most capillaries in one case ($n=3$, Figure 5b). Proliferative infantile hemangiomas had very weak staining in 10–50% of capillaries ($n=3$, Figure 5c). Involutive infantile hemangiomas immuno-positivity ranged from negative to moderately positive from a few capillaries to as many as 30% ($n=6$, Figure 5d). Kaposiform hemangioendotheliomas were moderately to strongly positive in all capillaries ($n=2$). Epithelioid hemangioendothelioma was negative ($n=1$). Reactive angioendotheliomatosis was moderately positive ($n=1$). Venous malformation was focally weakly positive ($n=1$). Spindle cell hemangioma was moderately focally positive ($n=1$). Inflammatory granulation tissue showed moderate-to-strong staining in all capillaries ($n=4$). In all of the above, a variable number of arteries and veins stained weakly to strongly, the veins generally more than the arteries, as well as, in a survey of normal organs from an autopsy. In addition, most if not all capillaries stained strongly.

Discussion

Pyogenic granuloma, also called lobular capillary hemangioma, is a vascular proliferative lesion most commonly affecting skin or mucosa of children and young adults.[6] On rare occasions, it has been reported in organs such as liver[7] or central nervous system.[8] They have also been observed in the lumen of vessels[9] or in vascular lesions such as arteriovenous malformation[10] and capillary malformation.[11] Their pathogenesis is still poorly understood. Links between pyogenic granuloma development and pregnancy or trauma have been reported, and suggested as causative factor.[12,13] To improve our understanding of these lesions, we analyzed genome-wide transcriptional profiles of laser-captured micro-dissected vessels from pyogenic granuloma and from three richly vascularized entities, namely placenta, proliferative, and involutive infantile hemangiomas.

In the first part of our study, we attempted to better understand the relationship between the four tested entities. The different statistical analyses performed identified sets of genes specific for vessels of each tissue type, alongside sets of genes common to vessels of different tissue types. This could be expected considering the design of the

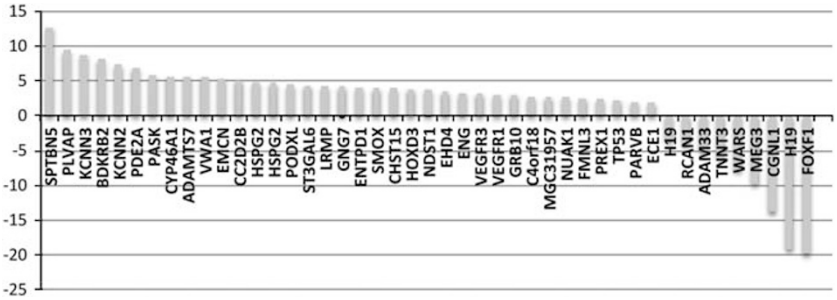

Figure 4 Fold differences >2 comparing genes expressed in pyogenic granuloma *versus* placenta and infantile hemangiomas combined.

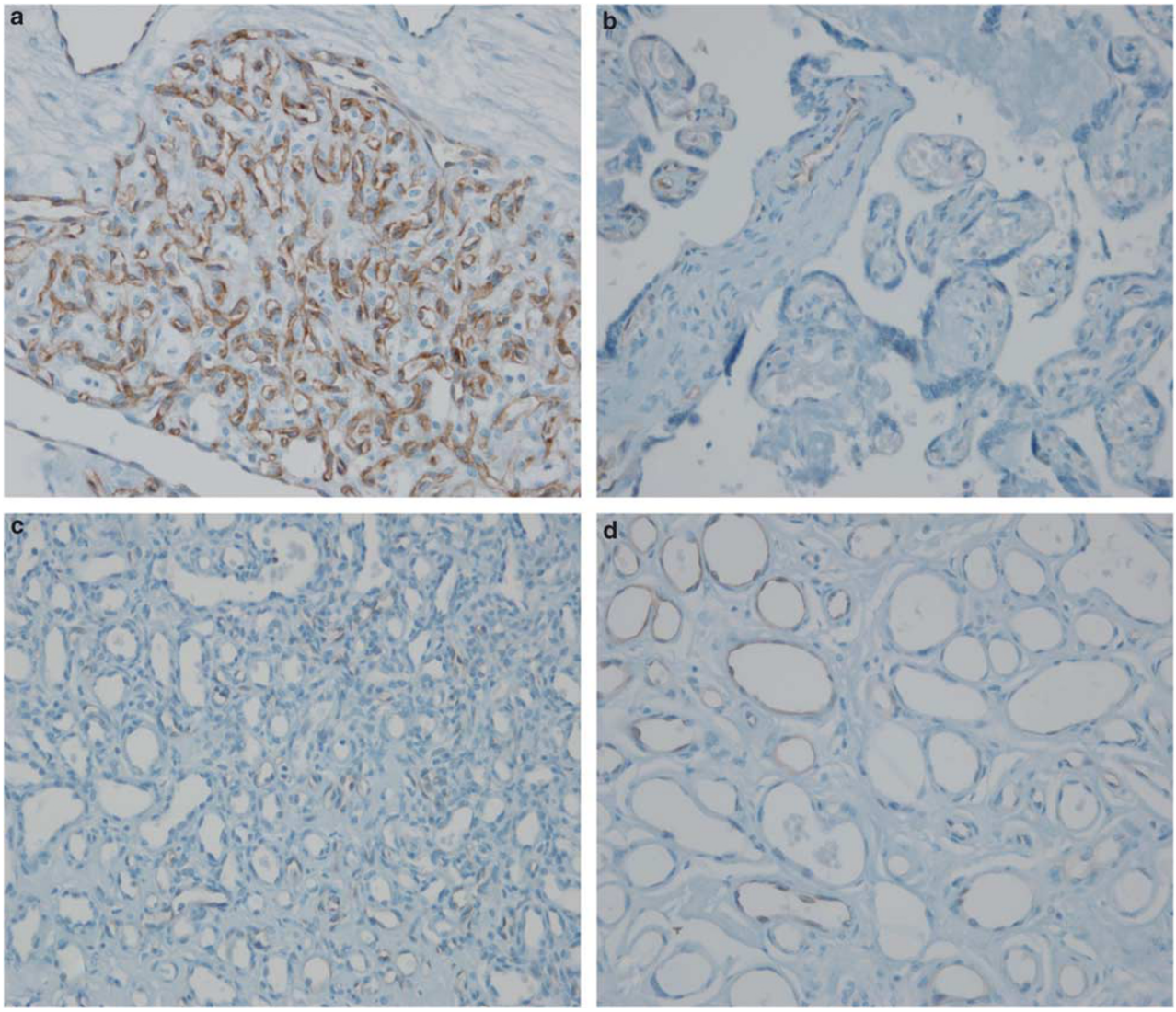

Figure 5 Immunohistochemical staining for PLVAP. (**a**) Intense staining of endothelium of all capillaries of pyogenic granuloma. (**b–d**) Weak staining of endothelium of rare capillaries in placenta, proliferative, and involutive infantile hemangiomas, respectively.

study that might schematically be phrased as: 'studying one tissue type, vessel, in various conditions'. Vessels of placenta and of infantile hemangiomas nicely illustrated this genetic similarity/dissimilarity. Indeed, correspondence analysis revealed vessels of the placenta to have a genetic signature distinct from that of all the other tested tissues. This finding was reinforced by the analysis of variance that identified a group of eight genes only upregulated in the placenta. Simultaneously, the latter test detected an additional set of 15 genes shared by the placenta and infantile hemangiomas, a likely finding as genetic similarities between infantile hemangioma and placenta are known.[14,15]

Among these 15 genes, one observed genes implicated in angiogenesis, *CLU*[16] and FST[17] as well as imprinted genes, such as *DIO3* and *DLK1*. The latter are part of the mir-379/mir-656 miRNA cluster, a chromosomal region that appears increasingly more relevant to normal placental development[17–19] and f*etal* growth,[20] stem cell differentiation[21] and various pathological conditions.[22–24] Further statistical analyses identified additional imprinted genes as specifically related to the placenta and hemangioma: *PLAGL1*, *DIO3OS*, *TFPI2*, *GRB14*, *H19* and *MEG3*. If the role of imprinted genes in placental development was already well established, it is, to our knowledge, the first assessment of them in infantile hemangiomas.

Between proliferative and involutive infantile hemangiomas, genetic similarity was recognized, with the identification by the analysis of variance of a cluster of 10 genes related to both of them. These genes were linked to the Gene Ontology term corresponding to 'intrinsic to membrane' and the two genes, *ADCYAP1R1* and *PTGER3*, are known to have a role in angiogenesis.[25,26] Finding some degree of genetic similarity between proliferative

and involutive infantile hemangiomas was not surprising as they are only two different time points in the natural evolution of these lesions. Our statistical analyses were not designed to unravel the differences between the stages of infantile hemangiomas but, studies that did, determined them to differ with regard to the genes of the chronic inflammatory response and of the NOTCH pathway.[4,27]

For pyogenic granuloma, correspondence analysis and analysis of variance revealed only dissimilarities with the other tissues tested. Such specific genetic behavior of vessels from pyogenic granuloma was not surprising, although not previously described. Indeed, pyogenic granuloma is clinically dissimilar from placenta and from infantile hemangiomas. For example, pyogenic granuloma almost never regresses spontaneously, in contrast to infantile hemangiomas.[6]

Because of these results, and to better understand the pathogenesis of pyogenic granuloma, we compared their profiles to those of placenta and hemangiomas combined, using the *t*-test, in-house algorithms and fold differences. It allowed identification of genes specifically related to pyogenic granuloma. *FLT-1* and *FLT4*, two tyrosine-kinase receptors, related to angiogenesis and vasculogenesis and to lymphangiogenesis, respectively. Interestingly, FLT4 has been recently implicated in angiogenesis of tumors and wounds[28] (for review see Bahram and Claesson-Welsh[29]). In this situation, and in contrast to FLT-1, signaling mediated through FLT4 does not require activation by any of its recognized ligands, VEGFB or VEGFC.[30] Finally, FLT4 missense somatic mutations have been found in infantile hemangiomas,[31] further implicating this gene in the pathogenesis of pyogenic granuloma.

ENG is specifically upregulated in pyogenic granuloma. Mutations in it cause hereditary hemorrhagic telangiectasia type 1, also known as Osler–Rendu–Weber disease, an autosomal dominant multisystemic vascular dysplasia (MIM: 187300, 108010). This gene codes for a major glycoprotein of the vascular endothelium implicated in both vasculogenesis and angiogenesis.

Other genes linked to pyogenic granuloma, *EMCN*, *PODXL* and *ENTPD1*, are related to hematopoietic stem cells,[32–34] and such would favor pyogenic granuloma to be linked to vasculogenesis. But, as discussed for infantile hemangiomas, pyogenic granuloma could similarly be viewed as the result of a vascular program concurrently associating with vasculogenesis and angiogenesis.[35] *ENTPD1* has been linked to hypoxia-induced angiogenesis as *BDKRB2* and *WARS*, two other genes upregulated in pyogenic granuloma.[36–38] The angiogenic action of BDKRB2 appears to result from primary intracellular production of nitric oxide.[37] *PDE2A* is another gene upregulated in pyogenic granuloma and implicated in the nitric oxide pathway[39] (Biocarta). Interestingly, the role of nitric oxide in the post-ischemic revascularization process has been well-documented.[40–42] Such findings highlight the role of the nitric oxide pathway in pyogenic granuloma development, possibly secondary to a hypoxic event.

In addition, the nitric oxide pathway has also been related to vascular injury.[43,44] Many genes specific to pyogenic granuloma are, at least by their functions, related to vascular injury, wound healing process or cancer angiogenesis: *BDKRB2*,[45–47] *NTPDase1*,[48] *ENG*,[49,50] *FLT4*,[28] *ST3GAL6*,[51] *NDST1*,[52] *HSPG2*[53] and *HOXD3*.[54] Moreover, all these genes have a fold difference >2 in pyogenic granuloma when compared with other tissue tested, further reinforcing the connection between pyogenic granuloma and vascular injury.

Immunohistochemistry directed against PLVAP was used to validate the array data. *PLVAP* was chosen because this gene was found to be specifically upregulated in pyogenic granuloma by all our statistical analyses, and having the second highest fold difference. Recently, the link between PLVAP and an antibody used for years as a vascular maker, the 'Pathologische Anatomie Leiden-endothelium' antibody, was described.[55] It was therefore not surprising to observe immuno-positivity of endothelial cells in various tissues, but the staining intensity and its frequency were highest in pyogenic granuloma, thus confirming the array data.

Finally, inflammatory granulation tissue, a known reactive lesion, was also tested for PLVAP reactivity. It demonstrated PLVAP positivity, but to a lesser extent in pyogenic granulomas. Interestingly, PLVAP has recently been demonstrated to play a role in leukocyte transendothelial migration.[55] All this reinforces the link between pyogenic granuloma and tissue insult.

In conclusion, genome-wide profiling analyses of micro-dissected vessels from formalin-fixed and paraffin-embedded placenta, infantile hemangiomas and pyogenic granuloma demonstrated genetic similarity/dissimilarity between the placenta, proliferative and involutive infantile hemangiomas, as well as dissimilarity with the vessels of pyogenic granuloma. Ascertainment of the genes specifically associated with pyogenic granuloma leads us to believe that it results from an injury followed by an impaired wound healing process associated with vascular growth driven by FLT4 and the nitric oxide pathway. Thus, this favors pyogenic granuloma to be a reactive lesion and not a tumor.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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