# Umbilical veins in dichorionic twins. A morpho-functional assessment

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*Summary:* Investigations on singleton and twin pregnancies show different functional behaviour on maternal-fetal relationship. In some ways twin pregnancies may be considered at risk and they may develop associated pathologies such as hypertension. The aim of this work was to evaluate the morpho-functional bethaviours of umbilical cord veins in twin and singleton gestations to better understand the role of these extra-embryonic tissues in the regulation of pregnancies.

The umbilical cords were studied from singleton pregnancies and from dichorionic twin pregnancies. Biochemical and morphological investigations were carried out.

A significant decrease in the anisotropy values was observed in endothelial cells from twins compared with singletons. Our ultrastructural data show immaturity features at the vein vessel wall level in twins. Furthermore, immunohistochemical investigations showed a lower degree of expressivity concerning adhesion molecules such as ICAM-1 and ELAM. Morphogenetic extracellular glycoproteins like fibronectin and tenascin seem over-expressed in twin pregnancies.

Our morpho-functional data well testify the lower maturation degree of umbilical cord veins in twins with respect to singletons.

*Key-words:* Umbilical vein; Endothelium; Pregnancy; Twin; Ultrastructure.

## INTRODUCTION

Twin pregnancies are characterized by a higher occurrence of gestational hypertension and preeclampsia and a higher rate of fetal growth retardation that singleton pregnancies ([1, 2]).

Utero-placental flow velocity waveform assessment can predict singleton pregnancy complications, such as gestational hypertension and preeclampsia ([3]). On the contrary, twin pregnancies complicated by gestational hypertension and preeclampsia showed normal resistance indices from the uterine artery at 20-24 weeks of gestation ([4]). Moreover, uncomplicated twin pregnancies have shown resistance index values in the uterine artery lower than singleton pregnancies ([4]).

Furthermore, twin pregnancies are charaterized by a fetal growth retardation and sometimes by a discordant growth between the twins.

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Umbilical flow velocimetry may prove relevant for early identification of twin pregnancies with discordant growth ([5]).

Abnormal umbilical artery waveforms are associated with a decrease in the number of small arterial vessels present in the tertiary stem villi of the placenta from the twin presenting a growth reduction ([6]).

This data suggests an utero-placental and fetus-placental vascular reactivity different in twin pregnancies with respect to singleton ones.

Previous studies reported morphological modifications of twin placentas with respect to the singleton, including a lesser maturity and a cellular hyperplasia without hypertrophy ([7, 9]).

There have been no studies of a characterization of the vessel wall.

So the aim of the present work was to investigate dichorionic twin umbilical veins in comparison to singleton ones. The present study was performed as follows: 1) A physico-chemical evaluation of the endothelial cell plasma membrane; ([2, 3]). A vessel wall and smooth muscle cell morpho-structural investigation.

## MATERIALS AND METHODS

The umbilical cords were obtained from singleton pregnancies (5 cases) and from dichorionic twin pregnancies (4 cases). In all cases the pregnancies were uncomplicated, maternal age (singletons 28±5 years, twins 27±6 years) and gestational age at delivery matched (singletons 38.9±0.7 weeks, twins 38.2±0.4 weeks). The fetal weight was 3,375±124 grams for singleton pregnancies while in twins the weight was 2,970±320 grams for the heavier twin and 2,610±230 grams for the lighter one.

Umbilical cords were cut from the placentas soon after birth and treated as described below.

### *Cell preparation*

The cells were prepared by an adaptation of the method of Jaffe *et al.* ([10]). The cords were cut from the placenta soon after birth and placed in a sterile container filled with cord buffer (0.14 M NaCl, 0.004 M KCl, 0.001 M phosphate buffer, pH 7.4, 0.011 M glucose) at 4° C. The cord was inspected, and all areas with clamp marks were cut off. The umbilical vein was cannulated with a blunt 14 gauge needle, perfused with 100 ml of cord buffer to washout the blood, and allowed to drain. Ten ml of 0.2% collagenase (type CLS, Worthington Biochemical Corp., Freehold, N.Y.) of cord buffer was then infused into the umbilical vein. After incubation in a water bath at 37° C for 15 minutes, the collagenase solution containing the endothelial cells was flushed from the cord by perfusion with 30 ml of cord buffer. The effluent was collected in a sterile 50 ml conical centrifuge tube (2070, Falcon Plastics, Oxnard, Ca.) containing 10 ml of Medium 199 (TC 199). The cells were sedimented at 990 rpm in ALC 4225 centrifuge for 10 minutes and washed once with 20 ml of TC 199. The cell button was resuspended into 2 ml of fresh culture medium. Cell vitality (almost 90%) was texted with an in vivo staining.

### *Fluorescence measurements*

A fluorescent probe 1-4-trimethylaminophenyl-1,3,5 -hexatriene (TMA-DPH) was used. Cell incubation with the proble was performed as described by Sheridan and Block ([11]). Briefly, 3 µl of TMA-DPH ($10^{-3}$M) were incubated at room temperature (23° C) with 2 ml of freshly prepared endothelial cells ($2\times10^6$ cells per ml) in 50 mM TrisHCl buffer solution, pH 7.4. Fluorescence intensities (100 readings each) of the vertical and horizontal components of the emitted light were measured on a Perkin-Elmer MPF66 spectrofluorometer equipped with two glass prism polarizers (excitation wavelength 365 nm emission wavelength 430 nm). Steady-state fluorescence anisotropy (r) of TMA-DPH was calculated by using the following equation:

$$r = \frac{Iv - GIh}{Iv + 2GIh}$$

where G is the instrumental factor which corrects the (r) value for an unequal detection of vertically (Iv) and horizontally (Ih) polarized light, respectively. Fluorescence anisotropy varies inversely with the fluidity of the membrane surrounding the proble ([11]).

Results were expressed as mean ± standard deviation (SD) and tested with the student t test for unpaired data; statistical significance was set at $p<0.05$.

### *Electron microscopy*

For transmission electron microscopy (TEM) the samples were fixed in glutaraldehyde 2% in cacodylate buffer 0.1 M post-fixed in osmium tetroxide and embedded in araldite. The thin sections were examined with a Philips CM 10 transmission electron microscope. For scanning

Table 1. – *Antibodies used in the study.*

| Antibody | Specificity | Diluition | Source |
|---|---|---|---|
| *Monoclonal* | | | |
| FN15 | Fibronectin | 1:10 | Sigma Imm. |
| 84H10 | ICAM-1 (inter- cellular adhesion molecule) leukocytes, endothelial cells (CD54) | 1:20 | Immunotech |
| CD12B6 | ELAM (endothelial leukocyte adhesion molecule) | 1:10 | Immunotech |
| OK-HLADR | HLA-DR | 1:5 | Ortho Diagnostic |
| *Polyclonal* | | | |
| Anti-Tenascin | Tenascin | 1:30 | D.B.A. Italia |

electron microscopy (SEM) the specimens were fixed in glutaraldehyde, post-fixed in osmium tetroxide and then dehydrated in graded alcohols and critical point dried (CPD) with liquid $CO_2$. The specimens were then mounted on aluminium stubs, covered with a thin gold film and observed with a Philips 505 scanning electron microscope.

*Immunohistochemistry*

Cord samples were immediately frozen in liquid nitrogen and stored at −70° C. Six μm cryostat sections were dried and fixed in acetone at 4° C. For each sample a section was stained with hematoxylin and eosin and sequential sections were immunohistochemically processed with monoclonal and polyclonal antibodies (Table 1).

For the immunohistochemical evaluation we employed the avidin-biotin-peroxidase complex technique according to Sternberger [12]. Sections were incubated with specific monoclonal antibodies for 1 hour at room temperature and with specific polyclonal antibodies overnight at 4° C (dilutions reported in Table 1). After incubation with the monoclonal antibodies, cord sections were rinsed in a tris-buffered saline (TBS) and incubated for 30 minutes with goat anti-mouse serum associated with peroxidase. A streptavidin-biotin-peroxidase complex was used for immunostaining (Dakopatts). The immuno histochemical reaction was detected by incubation for 5-15 minutes with the coloured substrate 3 amino-9-ethyl carbazole (AEC), that precipitates giving a red-orange colour.

Incubation with polyclonal antibodies was followed, by rinsing in TBS, and incubation with biotin-conjugated goat anti-rabbit serum for 30 minutes at room temperature and afterwards with an avidin-biotin-peroxidase complex for 30 minutes (Histostain-SP, Kit Zymed Laboratories).

Peroxidase activity was developed with a solution of 3'-3' diaminobenzidine (DAB, Sigma chemicals) in TBS containing 0.3% $H_2O_2$.

All cord sections were subsequently stained with Meyer's Hematoxylin.

## RESULTS

### I) *Membrane fluidity*

A significant decease in the anisotropy values of TMA-DPH was observed in endothelial cells from twins compared with singletons (singletons: $r = 0.335 \pm 0.005$; twins: $r = 0.287 \pm 0.006$). The reduction in r reflects reduced membrane lipid order, i.e. decreased membrane microviscosity (Fig. 1).

### II) *Transmission Electron Microscopy (TEM)*

#### 1) *Singletons*

*Endothelium* (Fig. 2a)

In the umbilical veins obtained from singleton pregnancies the endothelial cells appeared fully differentiated and presented the typical feature of moderately flat elements with cytoplasmic protrusions extending into the vascular lumen, pinocytotic and cytoplasmic vesicles, polyribosomes, scarce rough endoplasmic reticulum (RER) and areas rich in filaments. In addition, the cytoplasm contained Weibel and Palade bodies, especially in the

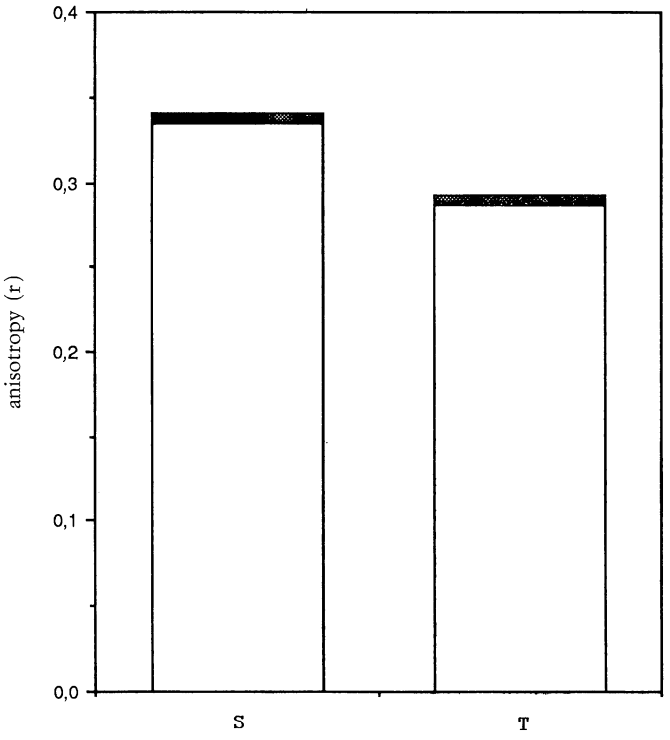


Fig. 1. — TMA-DPH anisotropy r parameter in endothelial cells obtained from umbilical cord of singleton ,S) and twin (T) pregnancies. * $p < 0.01$.

proximity of the luminal side and a fair number of predominantly rod-shaped mitochondria. The nucleus generally showed a lenticular shape with frequent invaginations, an evident nucleolus and a larger amount of euchromatin than heterochromatin thickened in peripheral clusters. Lateral junctions regularly linked adjacent cells resting on a continuous basal lamina.

Underneath endothelial cells, and adjacent to the basal lamina, we observed a rather thick, regularly structured, inner elastic lamina with a characteristic wavy pattern.

*Muscle cells* (Fig. 2b)

The tunica media appeared rich in adherent elongated, smooth muscle cells, rich in filaments, indicating a cotractile rather than synthetic cytotype. The surrounding stromal connective network appeared loosely structured, consisting of bundles of collagen fibers and rare fibroblast-like cells. The smooth muscle cells showed a round central nucleus with an evident nucleolus.

Mitochondria, free ribosomes and RER were observed in the paranuclear region. The rest of the cell appeared occupied by microfilaments, denser in focal points of aggregation, and by glycogen granules. The plasma membrane showed numerous invaginations and typical caveolae. The muscle cell basal lamina extended into the basal membrane-like fibrillar stromal component that linked these cells to collagen bundles.

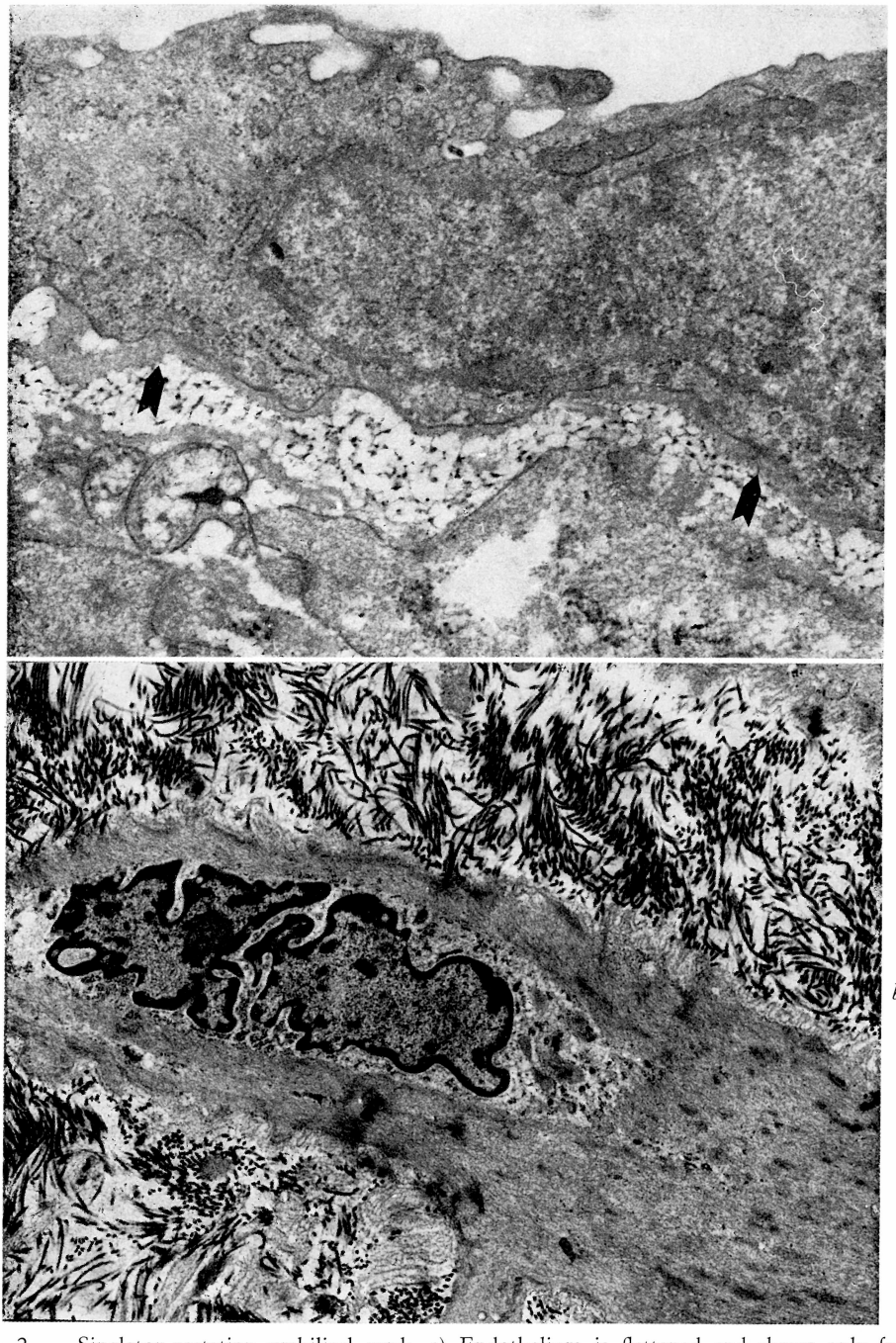

Fig. 2. — Singleton-gestation umbilical cord: *a*) Endothelium is flattened and shows only few cytoplasmic organelles. Lamina elastica underlying endothelial cell is also evident (↗) (TEM, × 32000); *b*) Smooth muscle cell rich in microfilaments and surrounded by small bundles of collagen fibers (TEM, × 12000).

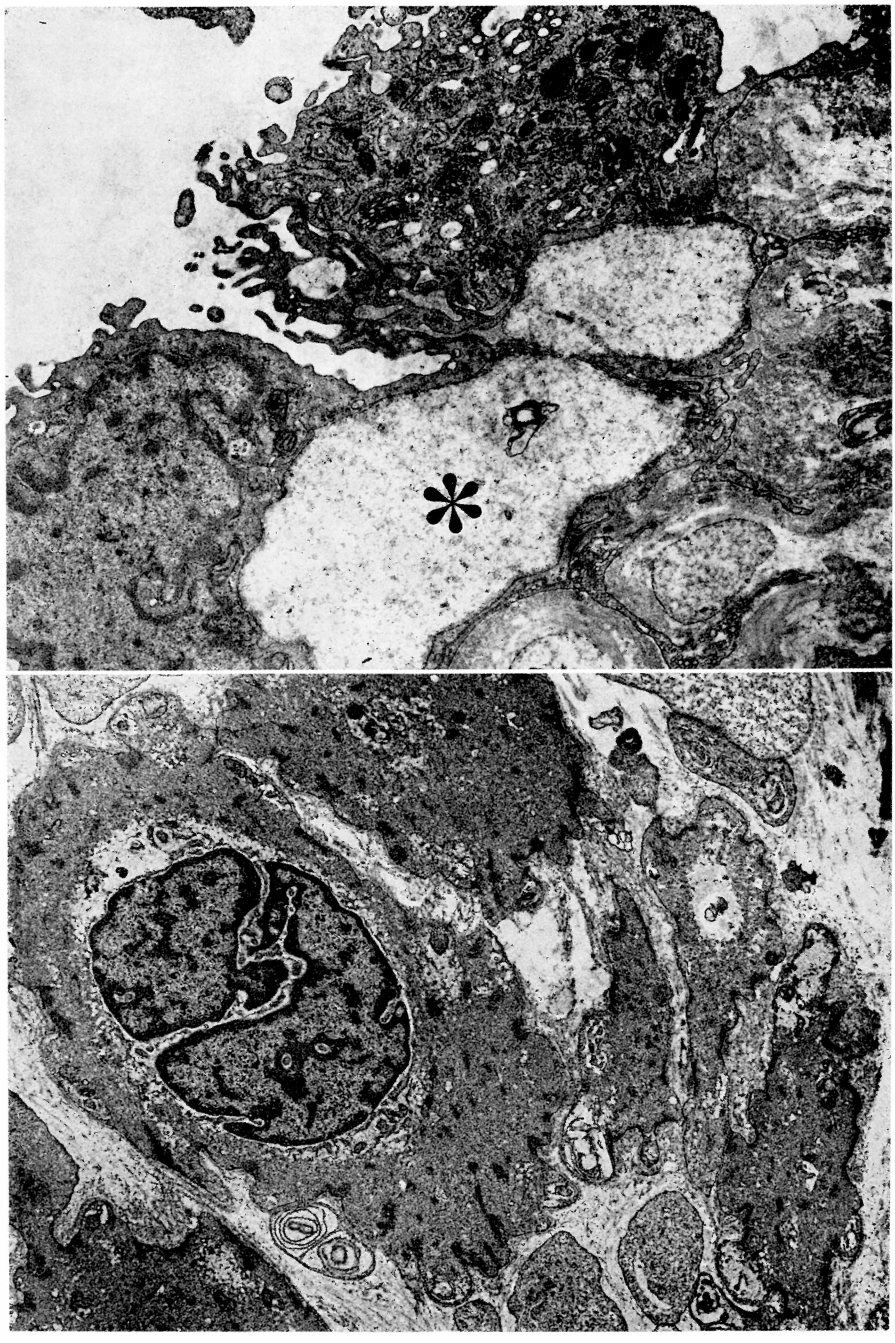

Fig. 3. — Twin-gestation umbilical cord: *a*) Endothelial cells show a more globular morphology if compared with singleton ones. Furthermore, their cytoplasm displays several organelles as mitochondria and rough endoplasmic reticulum cisternae. Under endothelial cells large electron-lucent areas are evident (❊) (TEM, × 11700); *b*) Smooth muscle cells, with a rounded shape, show an indented nucleous surrounded by a cytoplasmic halo poor in microfilaments (TEM, × 9500).

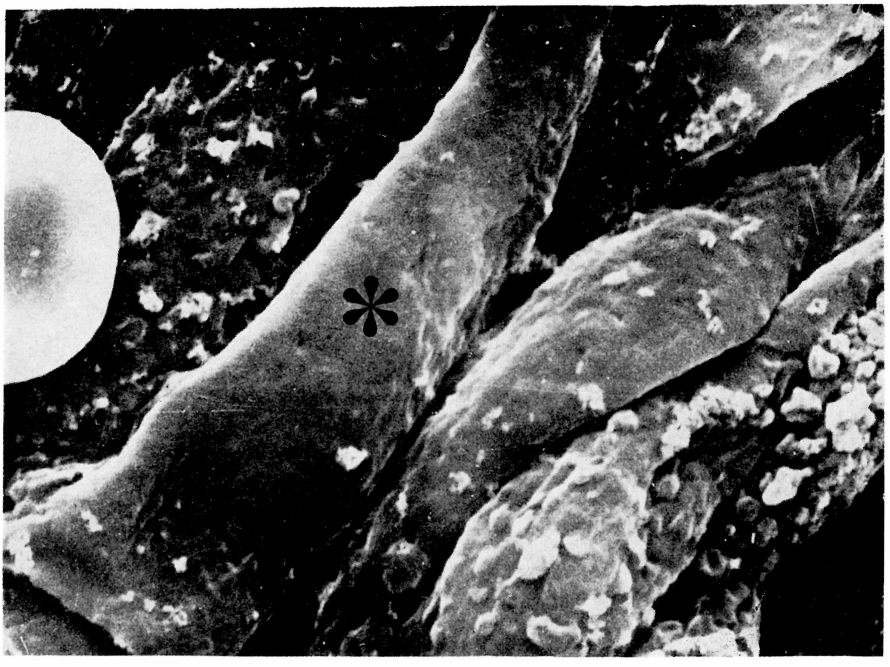

Fig. 4. — Singleton-gestation umbilical cord: elongated endothelial cells with a relatively smooth surface (❊) intermixed with endothelial elements showing superficial blebs and plicae (SEM, × 5000).

The presence of a loose mesenchymal tissue, Wharton's jelly, was observed externally.

2) *Twins*

The umbilical vein wall of twin cords did not show a marked difference in thickness versus the singletons, even though both the endothelium and the muscle cells showed cytotypes that partially differentiated them from those of umbilical cords from singleton pregnancies.

*Endothelium* (Fig. 3a)

The endothelial cells generally appeared globular and protruding in the lumen. Aspects of cell activation, such as richness of dilated RER cisternae, evident Golgi apparatus, mitochondria and pinocytotic vesicle abundance, supported the presence of active metabolic functions.

*Muscle cells* (Fig. 3b)

Also the muscle cells appeared rich in RER and with a synthetic rather than contractile phenotype.

The overall cell shape was basically globular, with frequent and peripheral invaginations and mitochondria-associated sarcolemmal caveolae. Along the microfilament bundles course there were more numerous focal densities. The nuclei were surrounded by abundant, dilated RER that occupied most of cytoplasm and the intercellular connections did not appear very tight. The stromal connecting network that surrounded the muscle wall was loosely organized, with the presence of a basal membrane-like fibrillar component and scarcity of organized collagen bundles. This observation, together with the above-mentioned aspects, suggests a

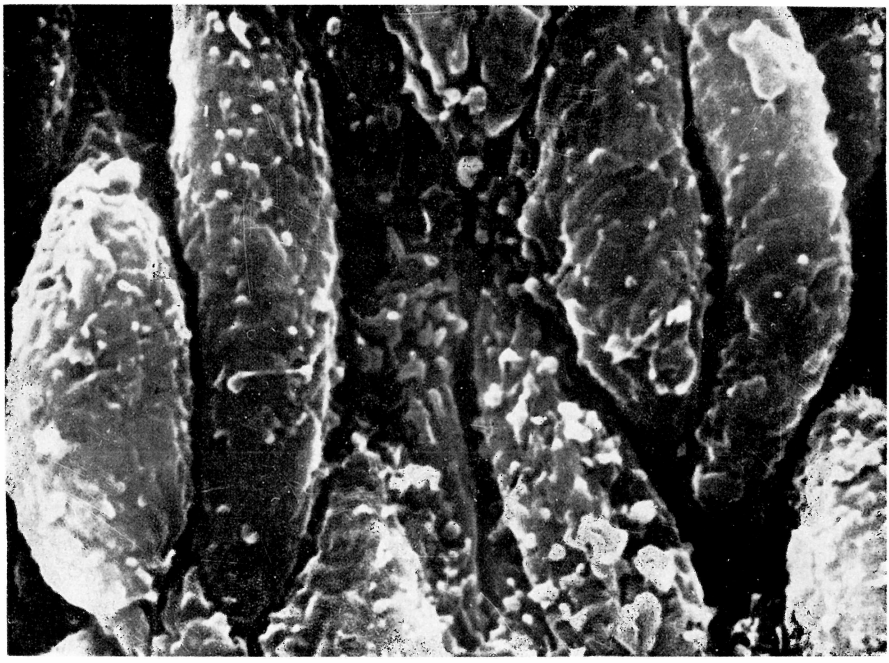

Fig. 5. — Twin-gestation umbilical cord: endothelial cells with a luminal plasmalemma rich in irregular protrusions (SEM, × 5000).

"young" tissue, even though already differentiated.

III) *Scanning Electron Microsopy (SEM)*

1) *Singletons* (Fig. 4)

In the endothelium of singleton umbilical veins, the cells, though clearly elongated, had a rather globular central portion.

Their surface was scarcely folded or vesiculated and next to cells which had almost a smooth surface, others with small superficial vesicles were observed.

The overall cell orientation in this condition had a scarcely parallel arrangement and the main axis of single endothelial elements did not always lie in one direction, perhaps also due to a state of mild contraction maintained during sample processing for observation with SEM.

2) *Twins* (Fig. 5)

The SEM analysis showed cellular elements that also had an elongated shape but with a more obvious globular central portion.

The cells showed a more uniform surface morphology, even though two main cytotypes could be identified.

We observed cellular elements with a slightly uneven surface, due to thin folds mainly located perpendicularly to the cell main axis. These cells were interspersed with elongated elements even more globular at the central portion, with surface protrusions of an irregularly round or pseudo worm-like shape.

In this gravid condition the cells were arranged in quite an orderly manner, with a prevalent orientation of their main axis.

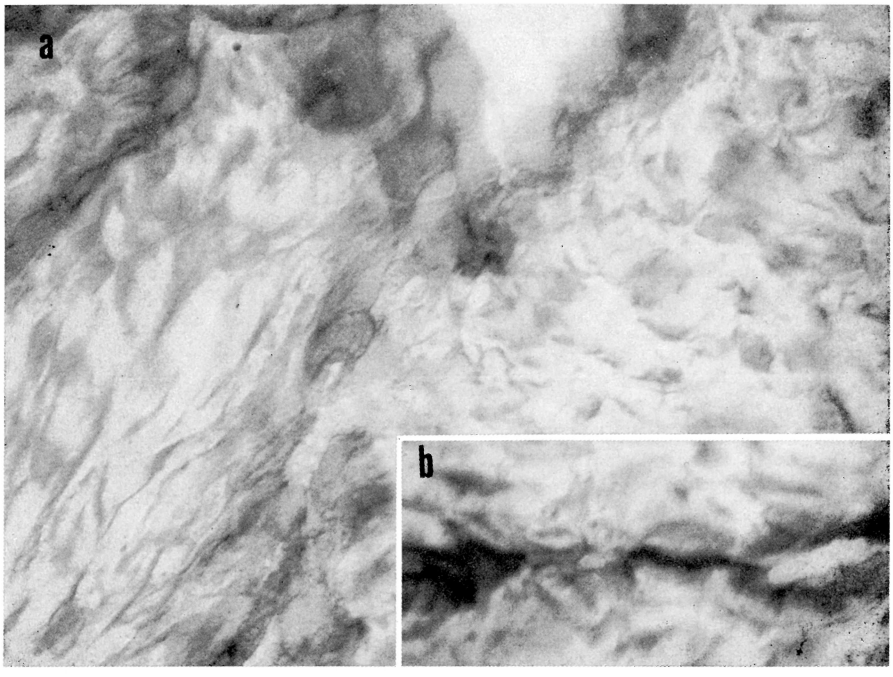


Fig. 6. — *a*) Singleton-gestation umbilical cord: vein endothelial cells show a more intense ICAM-1 positivity compared to the twin ones (*b*) (immunoperoxidase, × 630).

IV) *Immunohistochemistry*

*Singletons*

*Fibronectin*: The extracellular matrix of the umbilical cord veins showed a limited positivity for the anti-fibronectin antibody, most evident at the subendothelial basement membrane level (Fig. 6a).

*Tenascin*: This glycoprotein appeared to be located mainly in intercellular sites (Fig. 7a).

*ICAM-1*: Immunohistochemical reaction for the antigen ICAM-1 showed a fair positivity of the umbilical vein endothelial cells (Fig. 8a).

*ELAM*: Overall, umbilical vein endothelial cells tested positive for the anti-ELAM antibody (Fig. 9a).

*HLA-DR*: The vein endothelial cells did not display a positivity for the HLA-DR superior to a minimal background.

*Twins*

*Fibronectin*: Anti-fibronectin antibody indicated the presence of this glycoprotein in the extracellular matrix of the umbilical vein.

A weak positivity seemed to be present also in the vein endothelial cells. This glycoprotein seemed to be expressed with greater intensity than in the singleton (Fig. 6b).

*Tenascin*: The immunohistochemical reaction was more intense than in the singletons. This glycoprotein was located especially in extracellular areas of the vessel wall (Fig. 7b).

*ICAM-1*: The adhesion molecule ICAM-1 was expressed by the vein endothelial cells in a way similar to that of the endothelial cells of the singleton umbilical cords, even though sometimes the reaction seemed less intense (Fig. 8b).

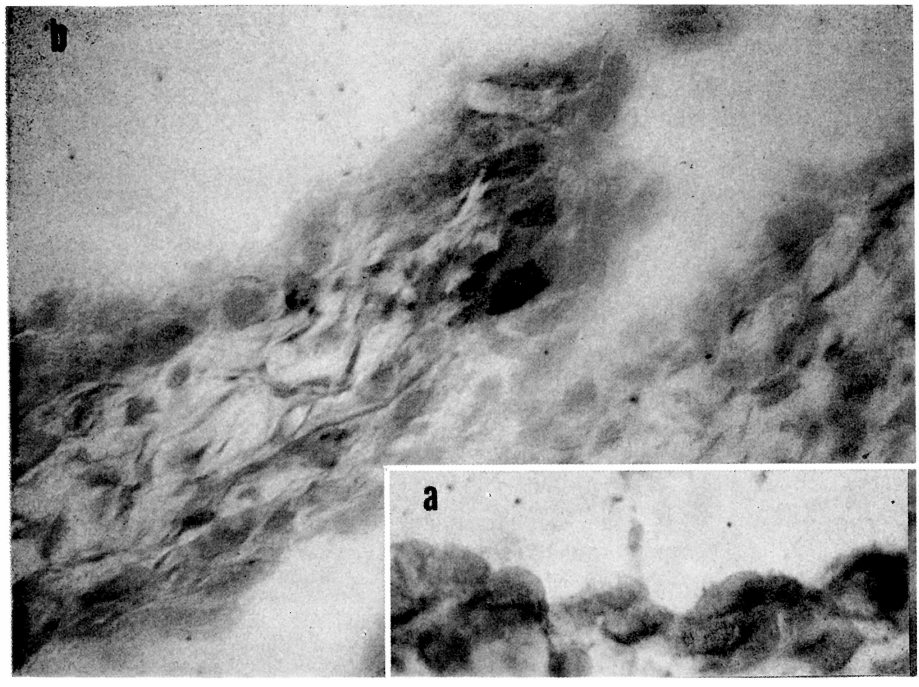


Fig. 7. — *a*) Singleton-gestation umbilical cord: the vein endothelial cells show and enhanced ELAM positivity in comparison with the twin vein endothelial cells (*b*) (immunoperoxidase, × 630).

*ELAM*: Endothelial cells exhibited aspects of immunopositivity to this adhesion molecule. However, overall the reaction appeared less intense versus the singletons (Fig. 9b).

*HLA-DR*: Endothelial cells of the vein resulted HLA-DR negative, as did those from singleton cords.

## DISCUSSION

The endothelial cell plasma membranes obtained from twin cords were more fluid than those from singletons.

It is well known that the main functions of the cell membranes is strictly dependent upon the physical state of its lipid bilayer, which in turn is strictly related to lipid/protein interactions, cholesterol/phospholipid ratio, unsaturated/saturated fatty acid ratio. In fact alterations in the lipid fluidity affect a wide number of fundamental cellular and membrane functions, such as cell cycling, differentiation, proliferation, permeability, transmembrane transduction, activity and kinetics of membrane bound enzymes and carriers ([13, 15]). As far as the endothelial cells are concerned a marked decrease in cellular membrane fluidity was associated with enhanced binding of monocytes and there is evidence that the physical state of the central and midacyl chains within the lipid bilayer of the pulmonary artery endothelial cell plasma membrane modulates transmembrane transport of serotonin by these cells ([16, 17]).

It can be assumed that endothelial membrane physical state alterations may also modify other cellular functions and activity. This aspect is important becau-

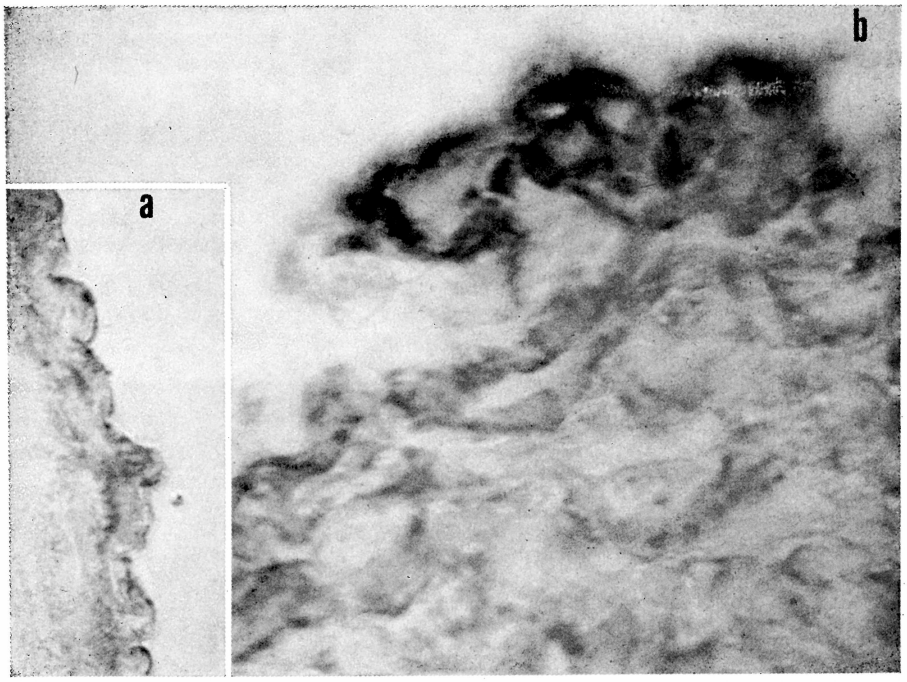


Fig. 8. — Cord stained with mAb against fibronectin. Note twin (*b*) extracellular matrix and vein endothelial cells showing a more intense positivity compared to the singleton-gestation (*a*) (immunoperoxidase, × 250 *b*, × 630 *a*).

se endothelial cells produce vasodilator substances (prostacyclin and endothelium-derived relaxing factor EDRF), release a vasoconstrictor endothelin-1 and stimulate the conversion of angiotensin I to II. There is also evidence that endothelial cells may play an important role in the pathogenesis of the preeclampsia ([18]).

In endothelial cells obtained from Pregnancy Induced Hypertension (PIH) affected women, a decrease of the membrane microviscosity was observed ([19]). As far as the umbilical cord is concerned, our ultrastructural and histochemical observations of the umbilical vein also appear to underline a different behaviour of the venous vessel from twin pregnancies, versus the singleton ones.

Both endothelial and smooth muscle cell types in the twin cords show greater cytoplasmic organelle content, related to a synthetic-active condition rather than a differentiated-stable one, and are often a fairly rounded cell shape.

From an overall impression, the immunohistochemical characterization, shows a less intense expression of the adhesion molecules ICAM-1 and ELAM in twin cords, versus the singletons.

Such phenotypic modulation would appear to indicate the existence of a lesser reserve of structural expressivities of cord endothelial cells in multiple pregnancies. These molecules are known to represent structural-functional moments of the endothelial barrier that have neither an ubiquitous nor a homogeneous distribution ([20]). Therefore, they tend to express, rather than to have constitutive aspects, a specific metabolic state of such a cellular

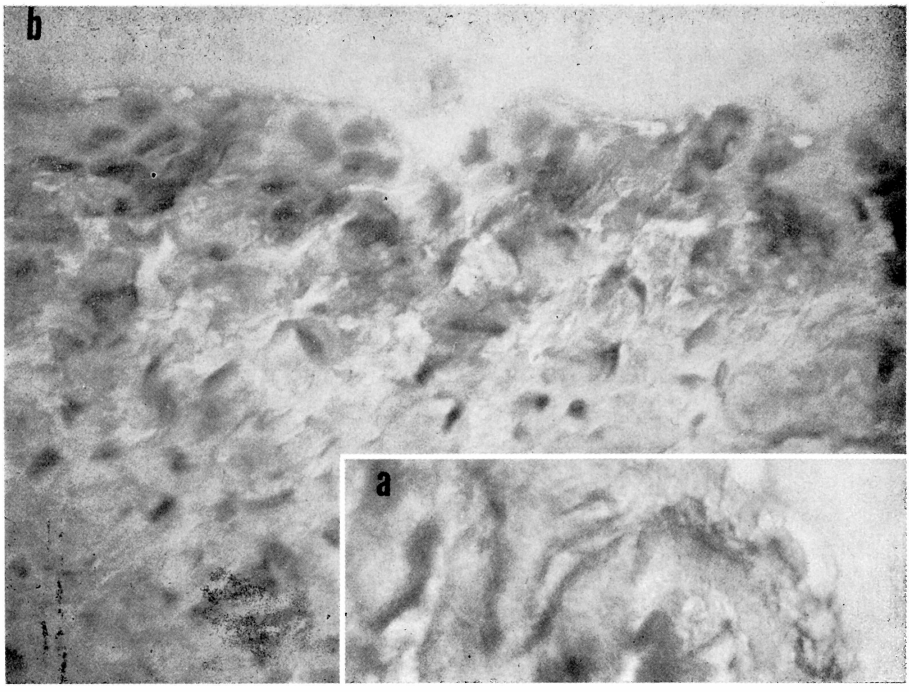


Fig. 9. — Tenascin expression in umbilical cord veins. This glycoprotein shows a lesser level of expression in the extracellular matrix and in vein endothelial cells of singleton gestation (*a*) if compared to the twin one (*b*) (immunoperoxidase, × 630).

barrier, that modulates the adhesion to and transmigration through the endothelium of leukocytes ([20-22]). In addition, ELAM usually appears in the endothelial capillaries only when these structures are activated by the presence of abnormal stimuli ([23]).

These observations are in agreement with concepts put forward by various authors that, in a fully structured vessel wall, endothelium and muscle are maintained in a quiescent and differentiated state, but that those two cell types can express phenotypes sensitive to changes in the surrounding microenvironment (e.g. humoral and mechanical factors) ([24]).

Even if autocrine and/or paracrine factors are able to influence both endothelial and muscle cell behaviour, it is also known that the behaviour of such cell elements can be modulated by extracellular matrix and cell-matrix interactions, in assuming both a particular function and a particular shape ([25, 26]).

Our submicroscopic investigations revealed the presence of a decreased compactness of the venous wall, an increase in intercellular spaces among the smooth muscle cells and a reduced presence of collagen fibrillae in twin cords, versus the singletons.

By immunohistochemistry we also noticed the presence of a greater positivity for the anti-fibronectin and tenascin antibodies in twin cords, versus the singletons.

Fibronectin is a glycoprotein of the stromal matrix. It is secreted by various cell types, including endothelial and muscle cells ([27]). It appears to play an important role in tissue organization during

both the adult healing processes and the morphogenesis of tissues and organs during embryonic development ([28, 29]).

Its increased presence in the venous wall of twin cords versus the singletons might evidence a state of reduced structural stability reached by such adnexal components than that observed in singleton pregnancies.

Tenascin is another extracellular matrix molecule involved in the process of cell development and differentiation, even if its presence appears less prolonged and more spatially restricted than fibronectin ([30, 31]).

Its action seems to interfere with, rather than support, that of fibronectin, therefore their simultaneous presence and relative proportions can influence cell differentiation ([32]).

Tenascin can modify cell shape and when added to substrates in vitro tends to make the cells into a rounded shape, in contrast with fibronectin that promotes cell spreading ([33]).

Both these stromal proteins could mediate their action through interactions with receptor complexes which in turn are known to interact with cytoskeletal filaments. The extracellular matrix, on the other hand, also seems to be influenced in its formation by various factors, i. e. cellular, humoral and mechanic ([34]). In simple systems in which cells adhere but are not subject to great mechanical stress, there is a predominant presence of an amorphous intercellular matrix, while the application of significant stretching forces promotes the development of collagen and elastic fibers ([34]).

So, the increased presence of amorphous intercellular matrix among smooth muscle cells of the venous wall of twin cords, associated with an often fairly rounded cell shape of contractile and endothelial elements, supports the notion of a limited morpho-structural development and a reduced functional efficiency of such a vascular structure.

## CONCLUSION

The present data seem to underline a peculiar vascular condition of both the endothelial plasma membrane function and the vein vessel wall structure. At the present state of our research it is impossible to identify the precise origin of such structural behaviour, which means that it comes from intrinsic factors of the vessel wall or from hemorheological or humoral factors of fetal blood refluing from placenta. In effect, twin placentas showed signs of cellular hyperplasia and immaturity ([9, 7]). As far as the endothelial membrane is concerned an increased fluidity was observed in PIH and in Insulin-Dependent Diabetes Mellitus which shows a more frequent association with hypertension. Twin pregnancy itself presents a higher risk of PIH, as well as an increased incidence of underdeveloped fetuses because of a placental defect, probably due to placental-fetal factors with a reduction of villus tree circulation ([1, 6]). It can be assumed that vessel modifications of the umbilical cord vein observed by us, could be the sign of different vascular reactivity in twin pregnancies.

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