Proteomic Focus on the Alterations Occurring at the Human Atherosclerotic Coronary Intima

Initiation of atherosclerosis occurs within the intima where major molecular changes are produced during pathogenesis.

We aimed to study the intimal proteome from the human atherosclerotic coronary artery

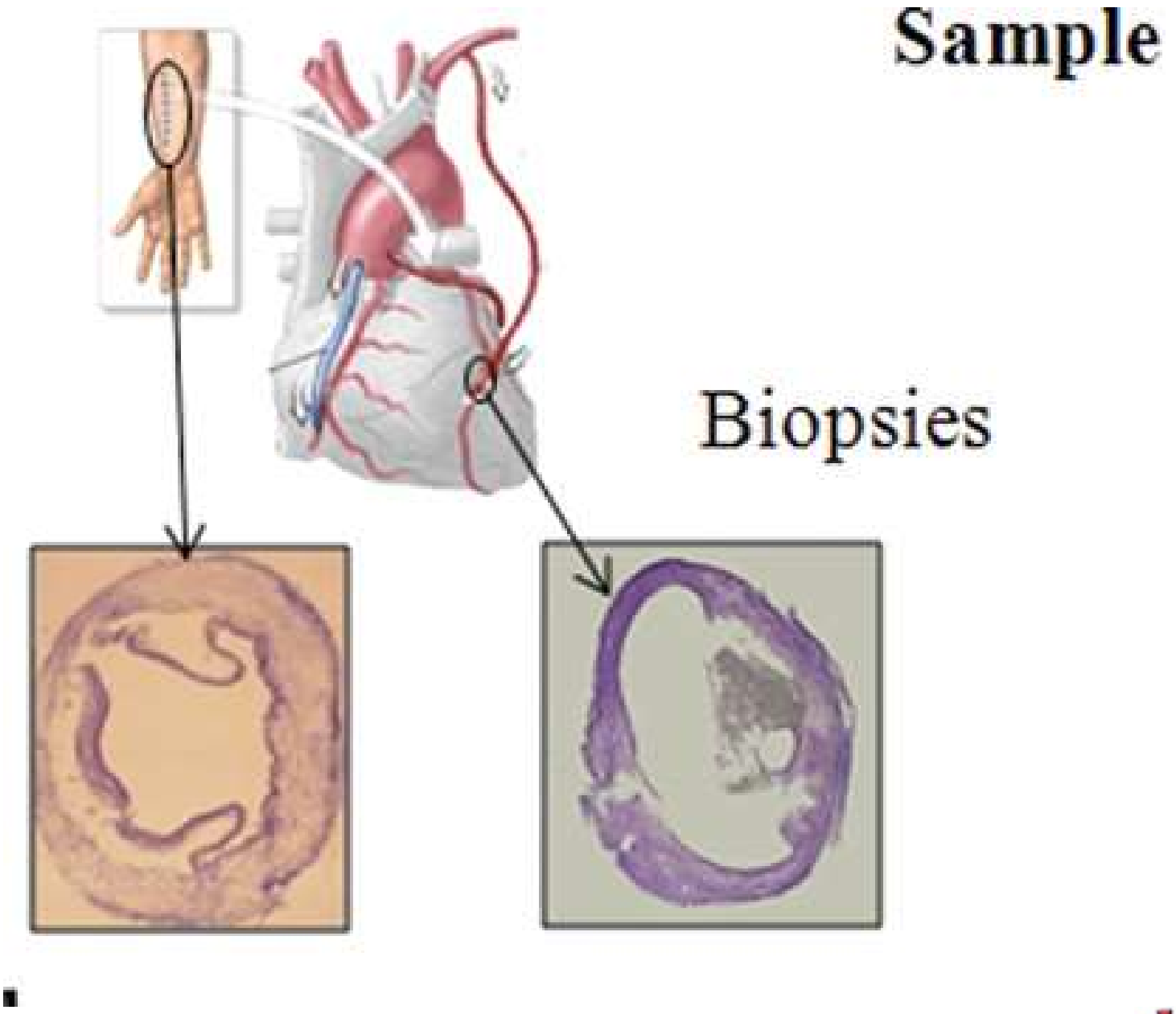
We used a two-dimensional Differential-In-Gel-Electrophoresis (DIGE) approach.

Proteomic Focus on the Alterations Occurring at the Human Atherosclerotic Coronary Intima

We aimed to study the intimal proteome from the human atherosclerotic coronary artery

We analyzed the intimal layer from human atherosclerotic coronaries, which were isolated by laser microdissection and compared with those from preatherosclerotic coronary

Workflow of the differential protein abundance analysis of atherosclerotic coronary intima.



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Human Artery Samples

Human arteries (*n* = 24-12 for two-dimensional DIGE) were from biopsy

Biopsies from coronary came from patients subjected to coronary bypass surgery and from heart transplant surgery.

Samples were immediately washed in saline, embedded in OCT, frozen with liquid nitrogen and stored at −80 °C until used.

Human Artery Samples

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Biopsies from coronary came from patients subjected to coronary bypass surgery and from heart transplant surgery.

Coronary necropsies were collected in a timeframe of 4–10 h.

Samples were immediately washed in saline, embedded in OCT, frozen with liquid nitrogen and stored at −80 °C until used.

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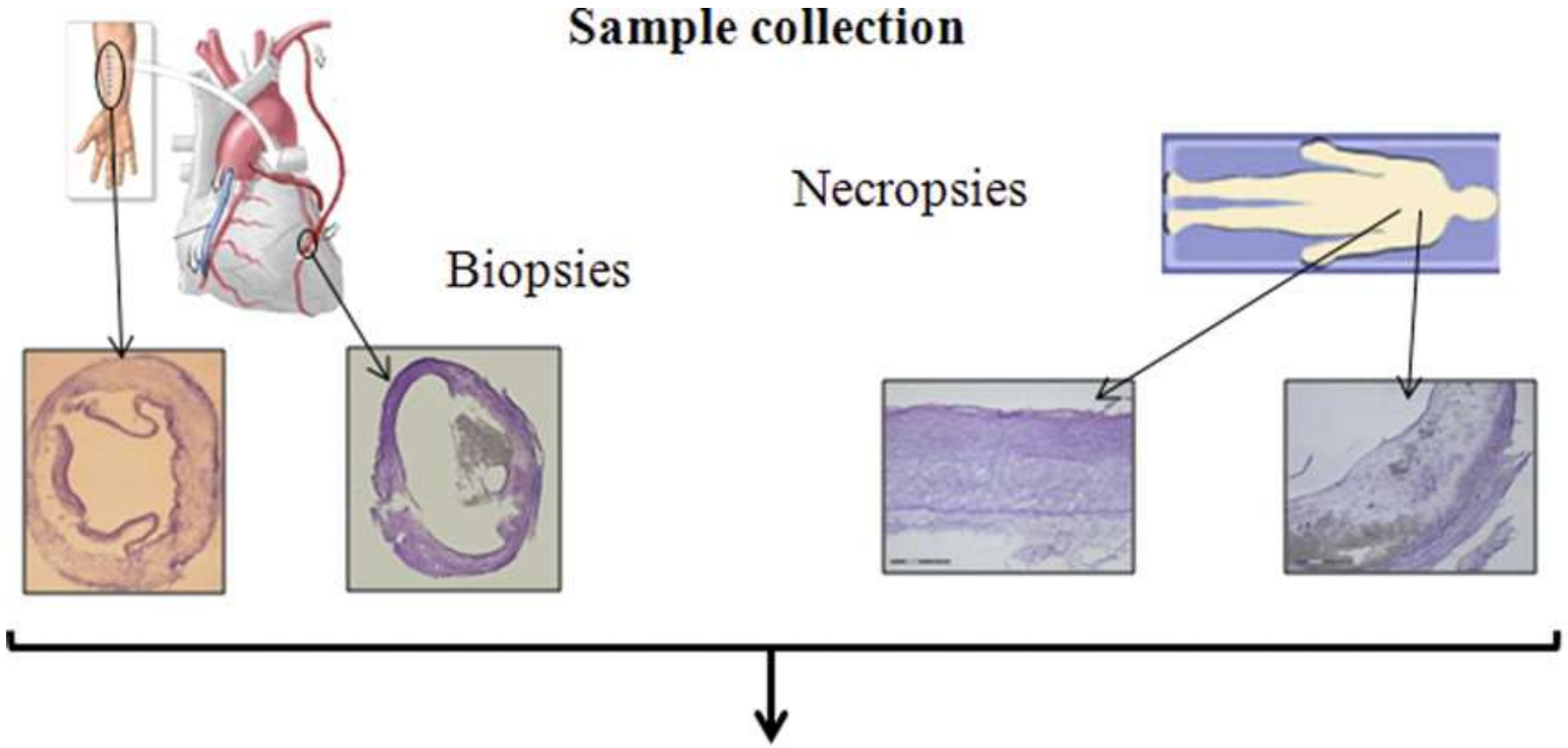

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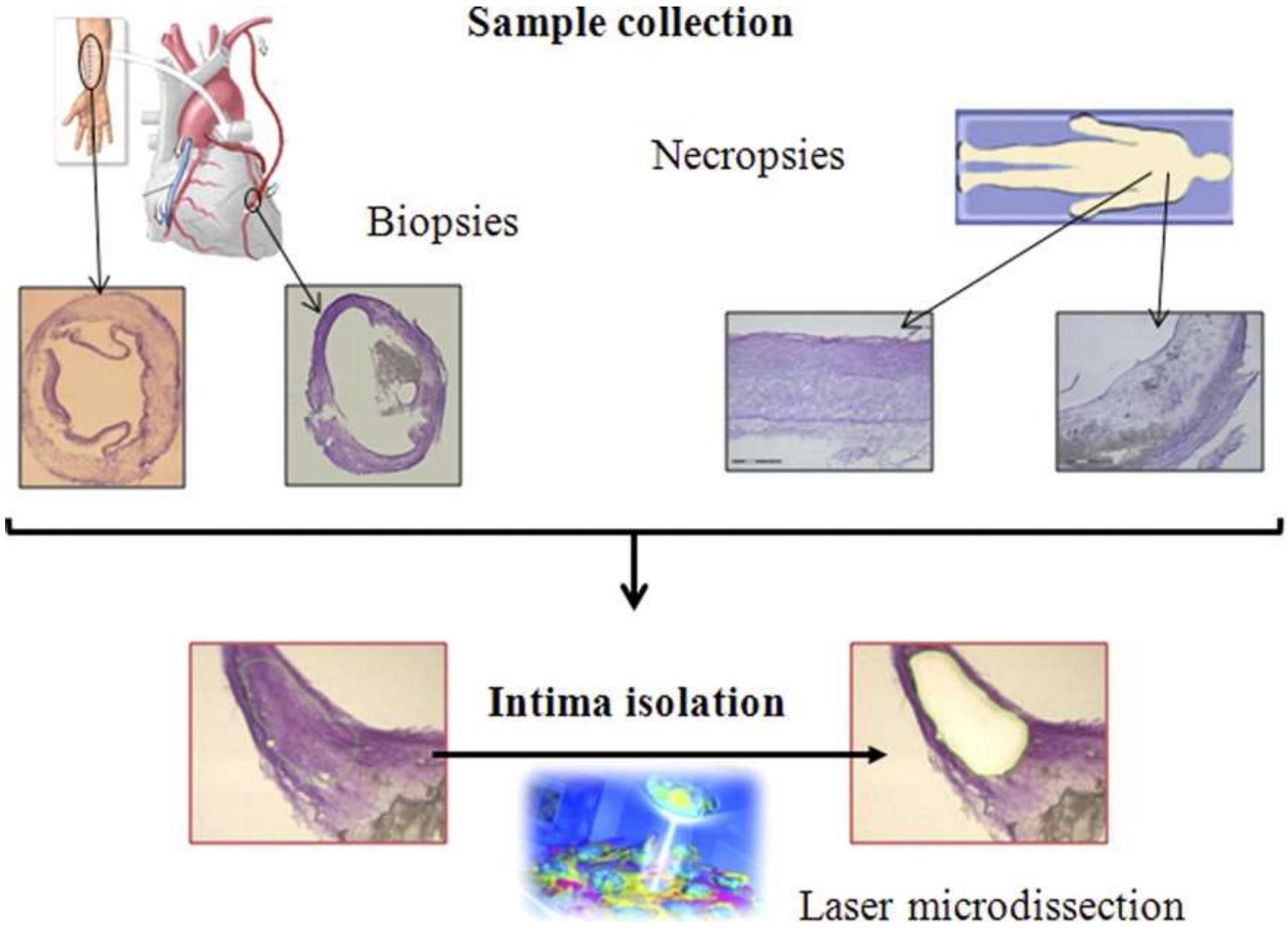

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Preparation of Histological Sections for Laser Microdissection

Serial 10-µm sections from OCT embedded arteries were cut and placed on Polyethylene membrane slides. Slides were not covered with coverslips as it would impede tissue microdissection

Tissue sections were fixed with −20 °C precooled 70% ethanol.

A fast staining method with cresyl violet dissolved in ethanol was performed on ice and with all solutions precooled (4 °C) to minimize proteases action.

staining solutions were supplemented with 0.01% protease inhibitor mixture, including fixation 70% ethanol

Slides were placed on ice until LMPC was carried out.

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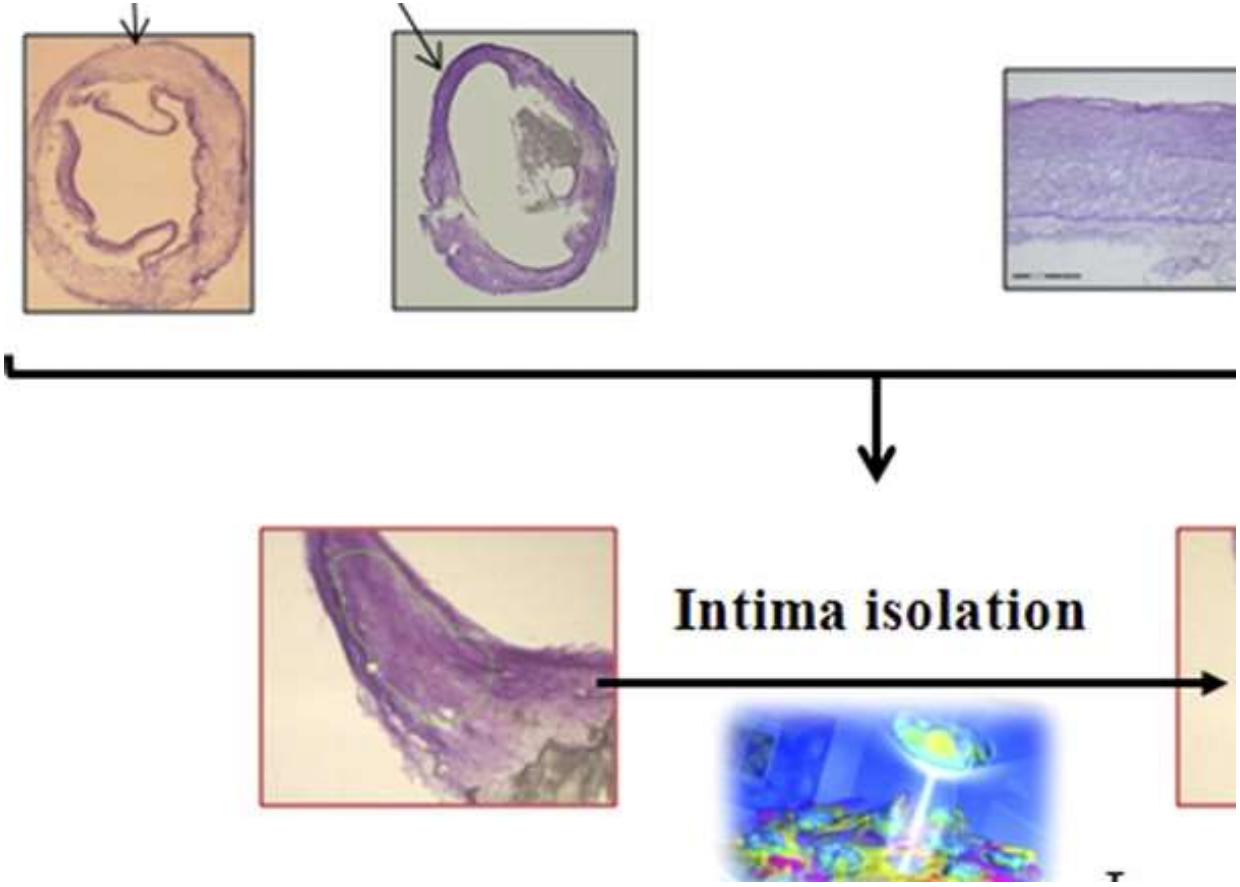

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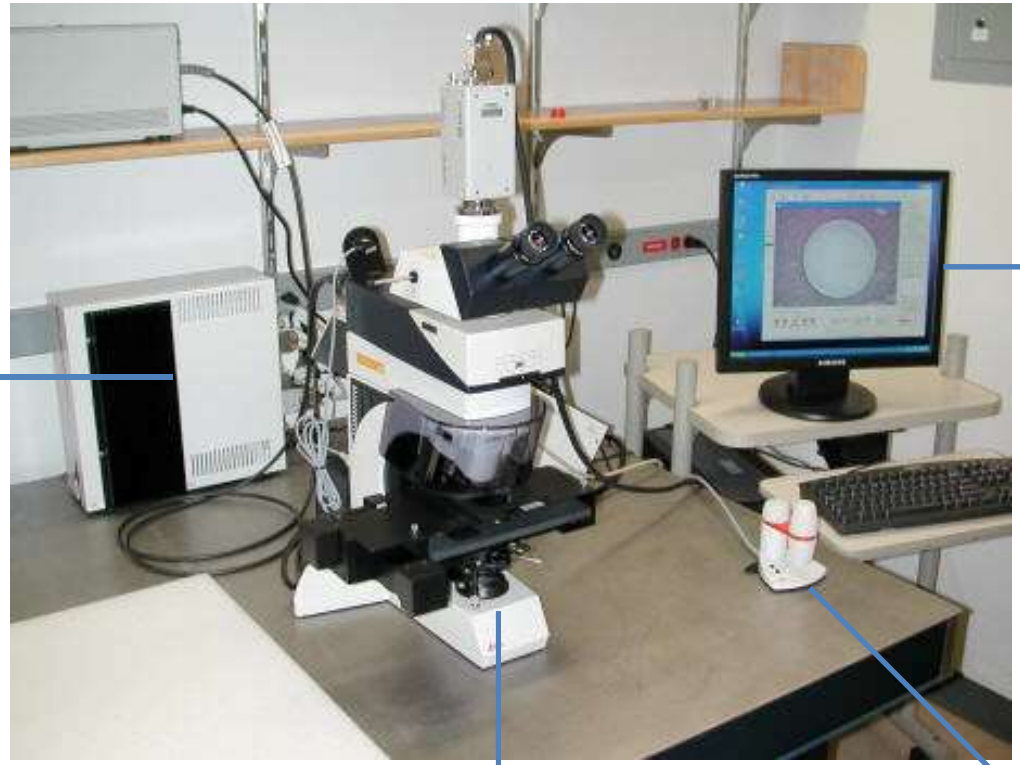
Isolation of Intima Layer by Laser Mediated Dissection

Intima layer from arteries addressed to DIGE analysis (*n* = 12) was isolated by Laser Mediated Dissection (LMPC technology in a MicroBeam system)

To select only the cells or tissue that we want

Laser Mediated Dissection



Overview of LMD

- Excise tissue (cells) from tissue
- Dissection under microscope
- Exclude contaminating cells/materials

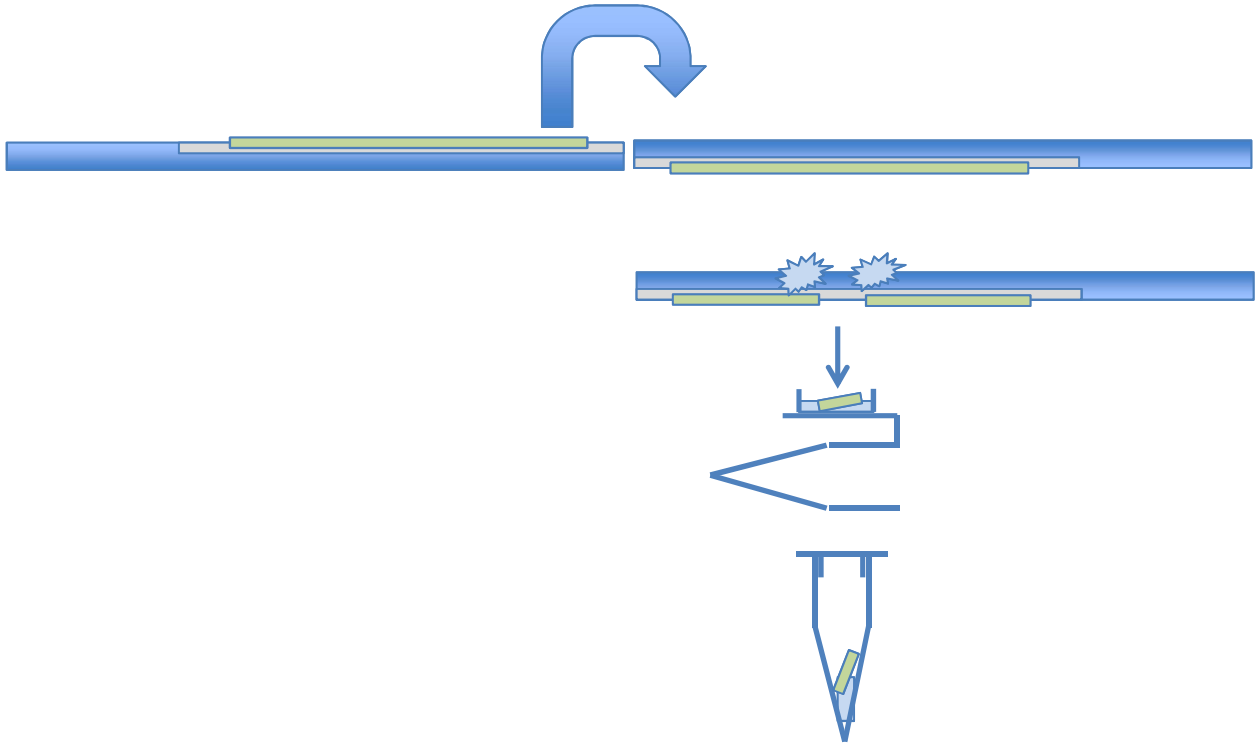
- Many potential tissue sources
- Accurate discrimination (staining, hi-mag)

Downstream applications

- QPCR, RNA-seq, microarray for gene expression analysis
- Protein isolation
- DNA sequencing for tissue specific sequences

Technical overview

- Attach tissue to membrane (PEN, etc.)
- Dissect out desired tissue with laser



buffer for processing.

LCM has several advantages over current tissue microdissection approaches: It is simple, requires no moving parts, involves no manual microdissection or manipulations, and enables one-step transfers. The transferred tissue on the film retains its original morphology, thereby allowing microscopic verification of the specificity of the captured material. LCM can be performed almost as quickly as photography of histologic tissue sections. The use of sterile, disposable transfer films minimizes potential contamination, which is particularly important for PCR-based analyses. The

Laser Capture Microdissection

Emmert-Buck *et al.*, 1996

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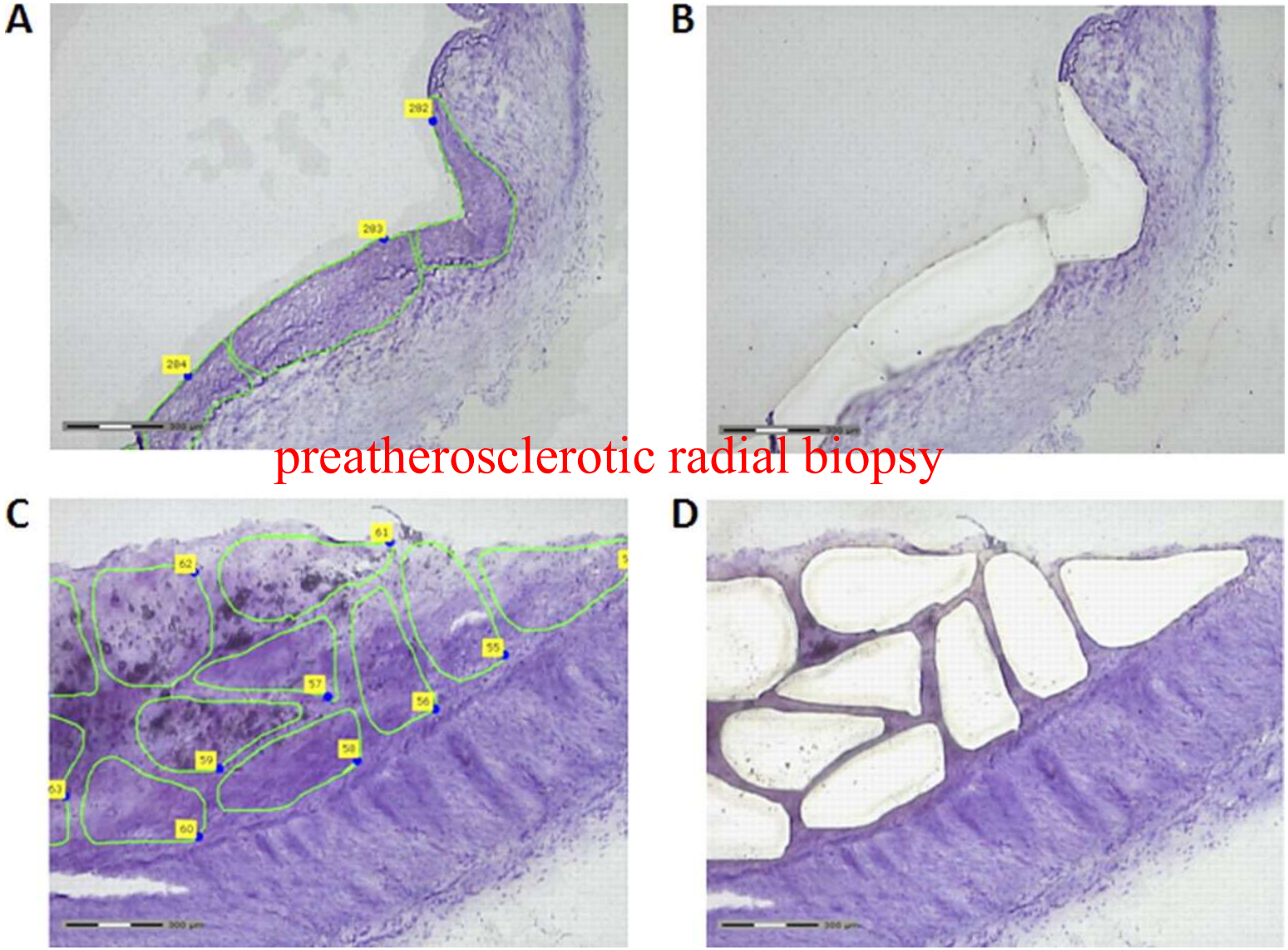
Selected elements for LMPC were cut and catapulted using the Robo LPC function with microdissection system software which allows both cutting and catapulting in a single step

In each case, 8 mm^2 of intimal tissue was microdissected and catapulted to a 500 µl adhesive cap tube

Maximum time of microdissection did not exceed in any case 2 h, in order to avoid possible protein degradation

Intima layer isolation by LMPC. An area of 8 mm2 was isolated by LMPC from the intima layer of all studied arteries for subsequent proteomic analysis.



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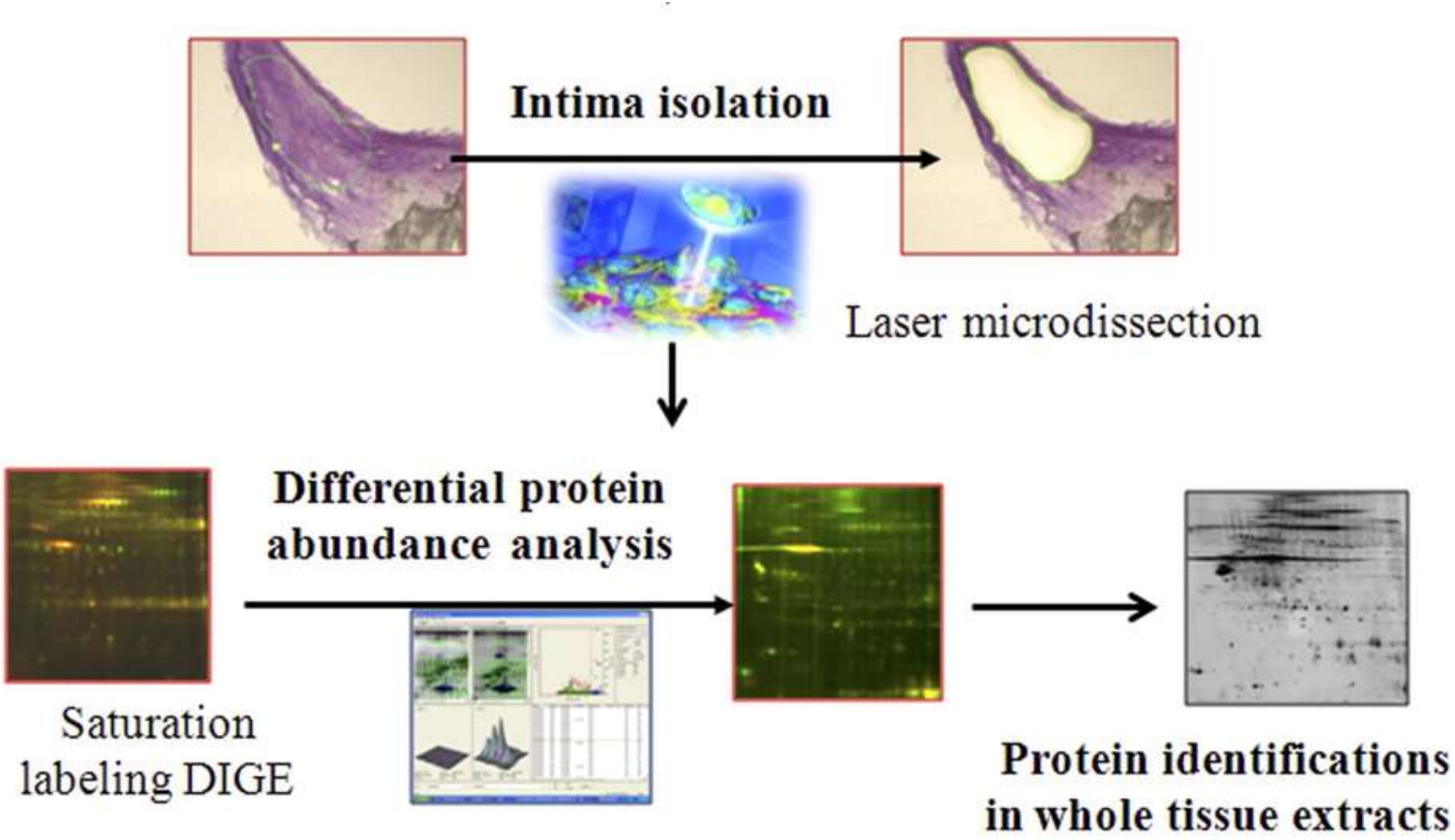
Proteins from isolated tissue were extracted in DIGE lysis buffer with the addition of dithiotreitol (DTT)

Workflow of the differential protein abundance analysis of atherosclerotic coronary intima.



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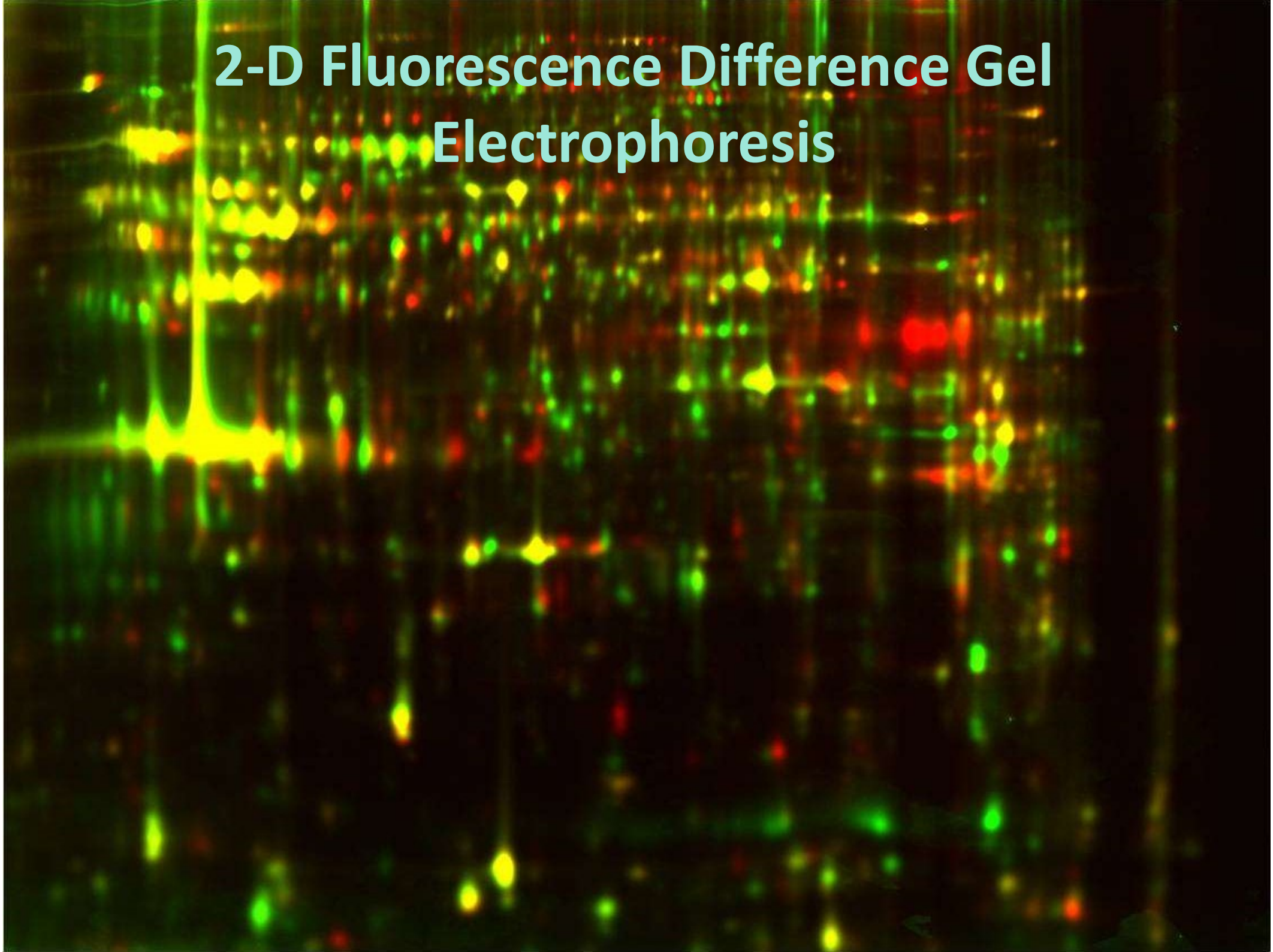
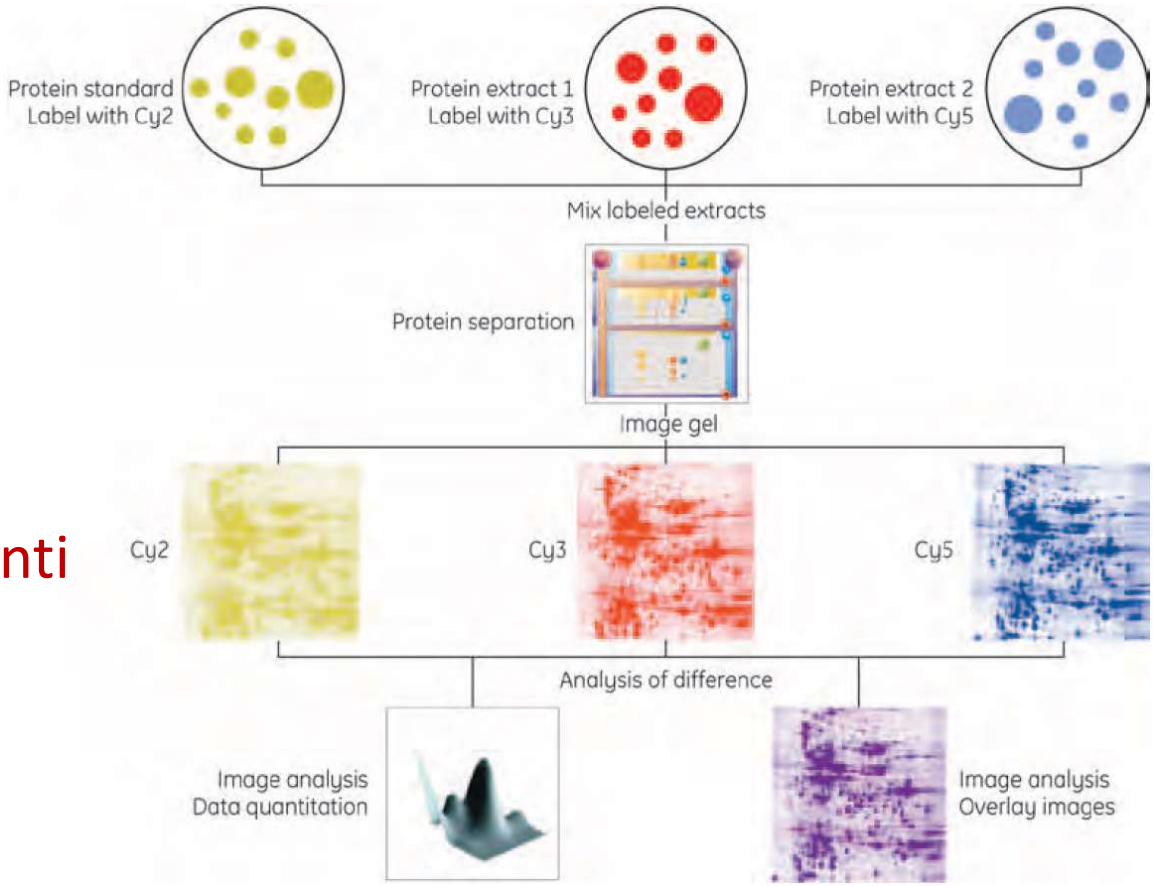

2-D Fluorescence Difference Gel
Electrophoresis

2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE)

Possibilità di analisi in **multiplexing**

- Marcatura delle proteine prima dell'elettroforesi 2-D.
- Analisi fino a 3 campioni
(es. 2 campioni + riferimento/controllo)



Fluorocromi:

- Spettri (e quindi segnali) ben distinti
- Insensibili alle variazioni di pH
- Fotostabili

Proteine identiche migreranno nella stessa posizione all'interno del gel 2-D

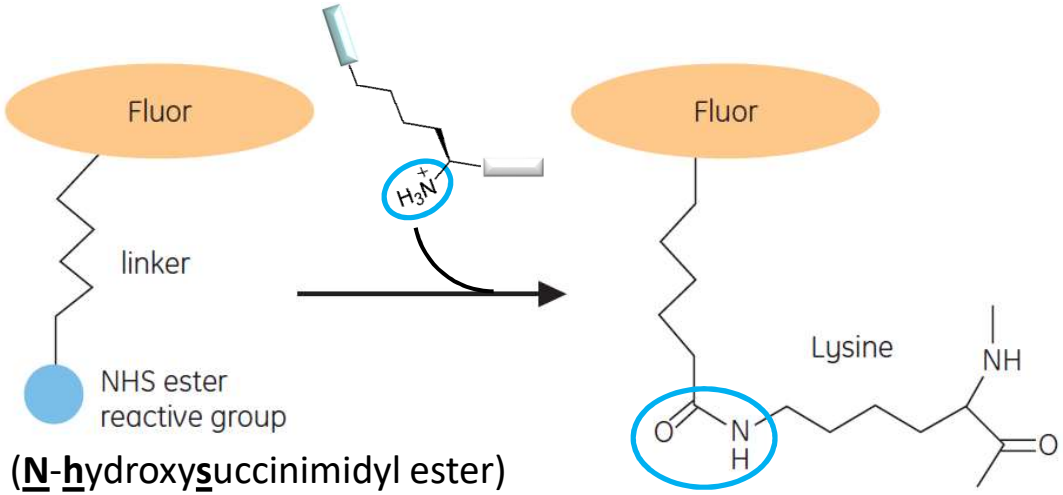
Labeling DIGE

- Protein Desalting Spin Columns
- Acetone precipitation step needed to obtain the sample volume for an efficient DIGE labeling
- Samples were reduced with 1 nmol Tris 2-carboxyethyl phosphine at 37 °C for 1 h in the dark
- **Samples were labeled with 2 nmol of Cy5 dye and the pool with an equivalent amount of Cy3, at 37 °C for 30 min in the dark**

Half of each sample of the study was added to a pool to be used as internal standard in all gels for the two-dimensional DIGE analysis.

2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE)

Marcatura dei campioni



(**N**-**h**ydroxy**s**uccinimidyl ester)

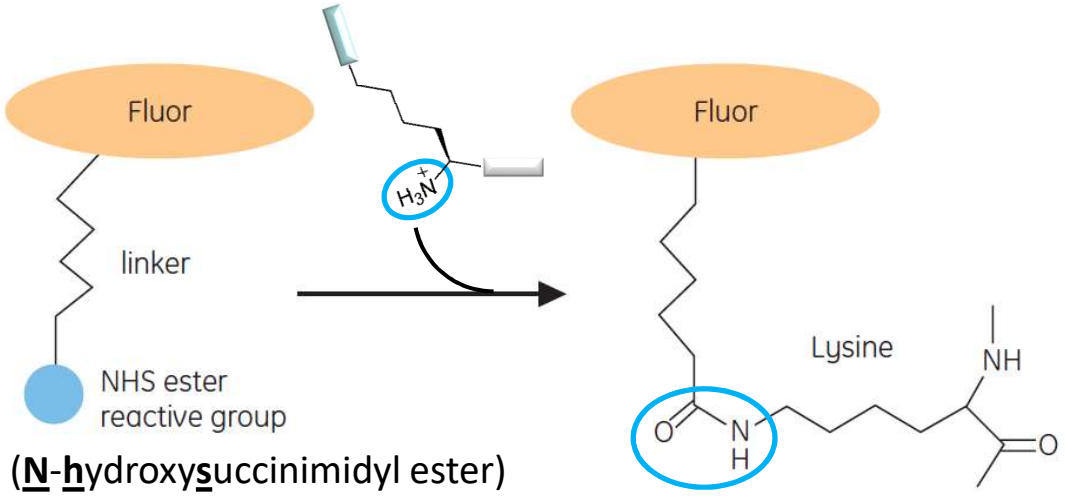
Gruppi reattivi che formano **legami covalenti** (legami amidici) con gli ammino gruppi ε di **residui di lisina**

Il fluorocromo ha carica + che "bilancia" quella persa dalla lisina

⟶ il pI non viene modificato

2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE)

Marcatura dei campioni



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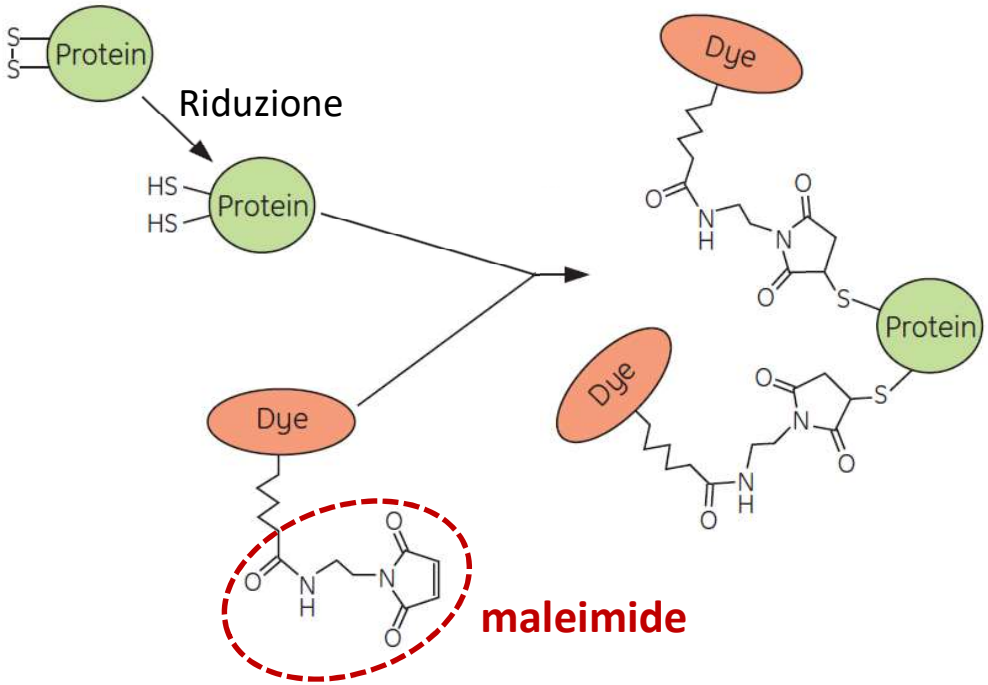
Fluorocromi coniugati con **maleimide** formano **legami covalenti** reagendo con i **tioli** liberi (**-SH**) di **residui di cisteina** a formare complessi proteina-fluorocromo



INTIMA PROTEOMICS
Two-dimensional Gel Electrophoresis

Each Cy5-labeled sample was mixed with an equal amount of Cy3-labeled pool

loaded on 18 cm, pH 4–7, IPG strips, by anodic cup-loading.

Second dimension was performed on 12.5% polyacrylamide gels

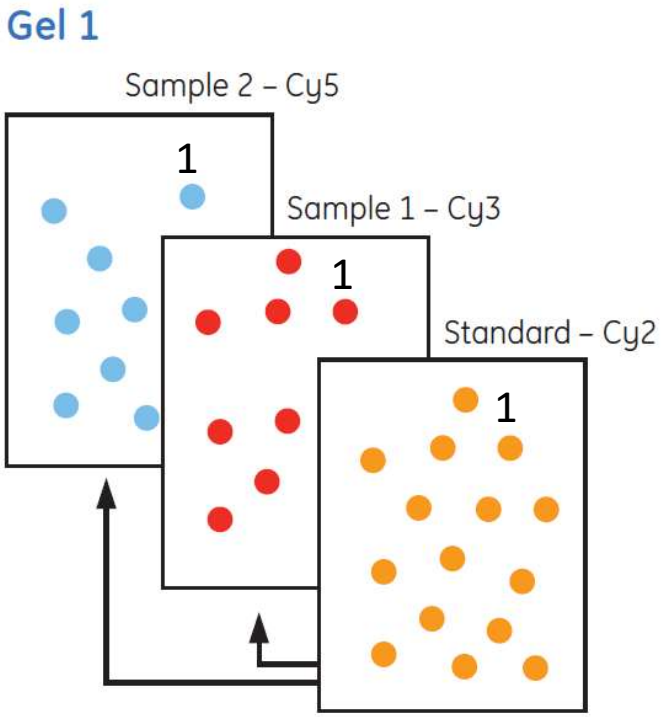
2-DE gels were scanned in a Typhoon 9400 scanner.

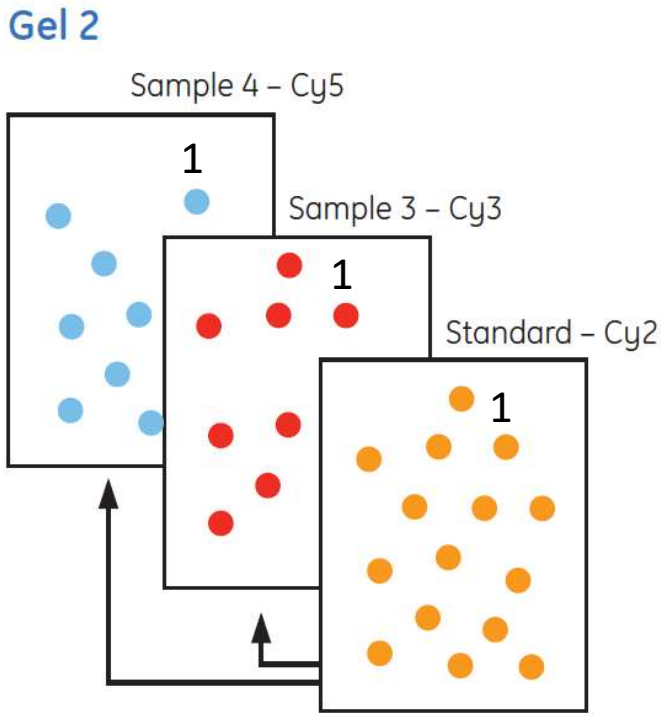
spot detection and matching performed with the internal standard loaded on each gel.

2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE)

Analisi campione-controllo

Analisi relative tra campioni in uno stesso gel e il controllo interno (standard)





Proteina 1 (campione 1) : Proteina 1 (standard)
Proteina 1 (campione 2) : Proteina 1 (standard)

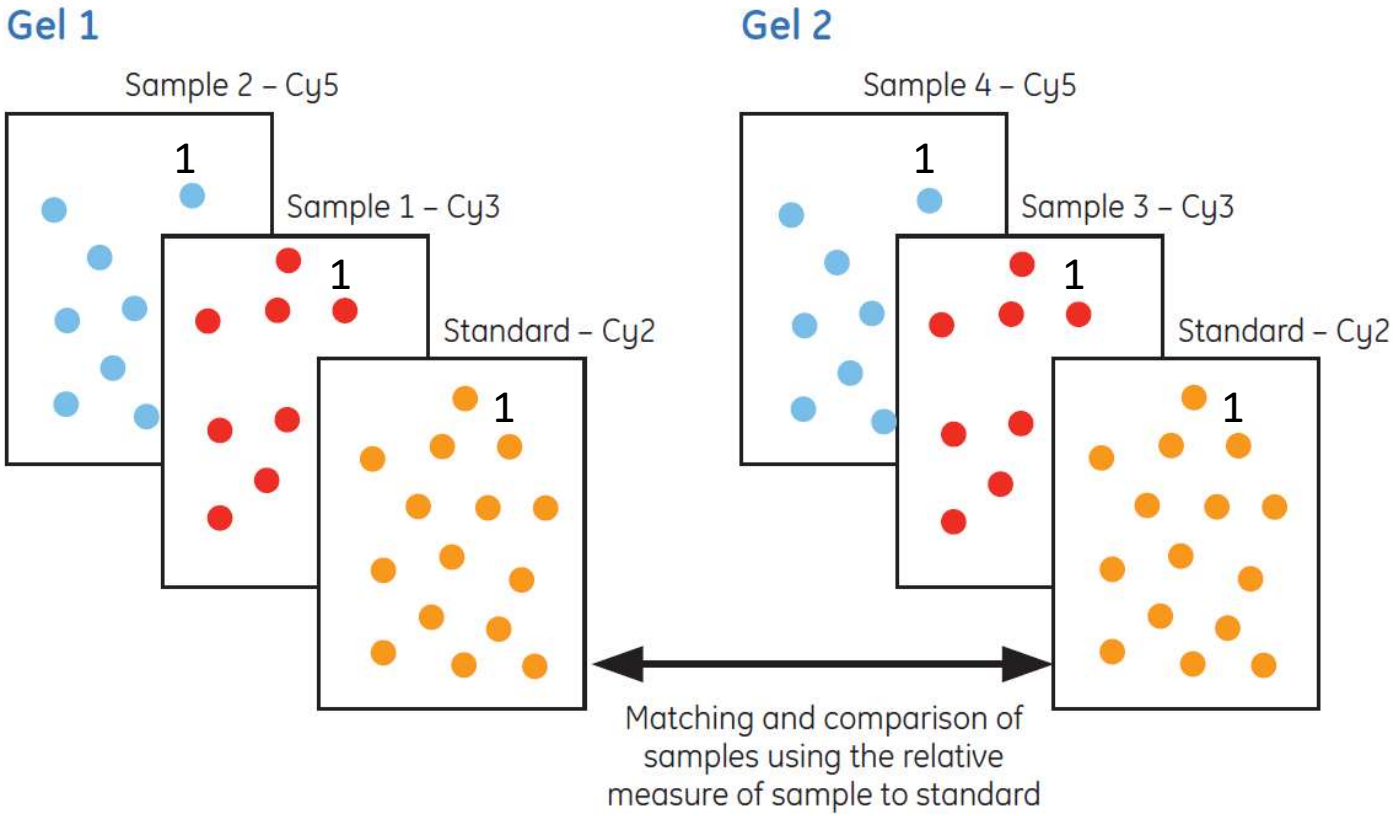
Proteina 1 (campione 3) : Proteina 1 (standard)
Proteina 1 (campione 4) : Proteina 1 (standard)

I livelli di ogni singola proteina vengono espressi come rapporto relativo tra gli spot del campione e gli spot dello standard interno

2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE)

Analisi tra più campioni

Analisi relative tra campioni su gel diversi



Il rapporto tra ogni campione e lo standard interno viene utilizzato per comparare i livelli delle proteine tra campioni su gel diversi.

INTIMA PROTEOMICS
Two-dimensional Gel Electrophoresis

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Second dimension was performed on 12.5% polyacrylamide gels

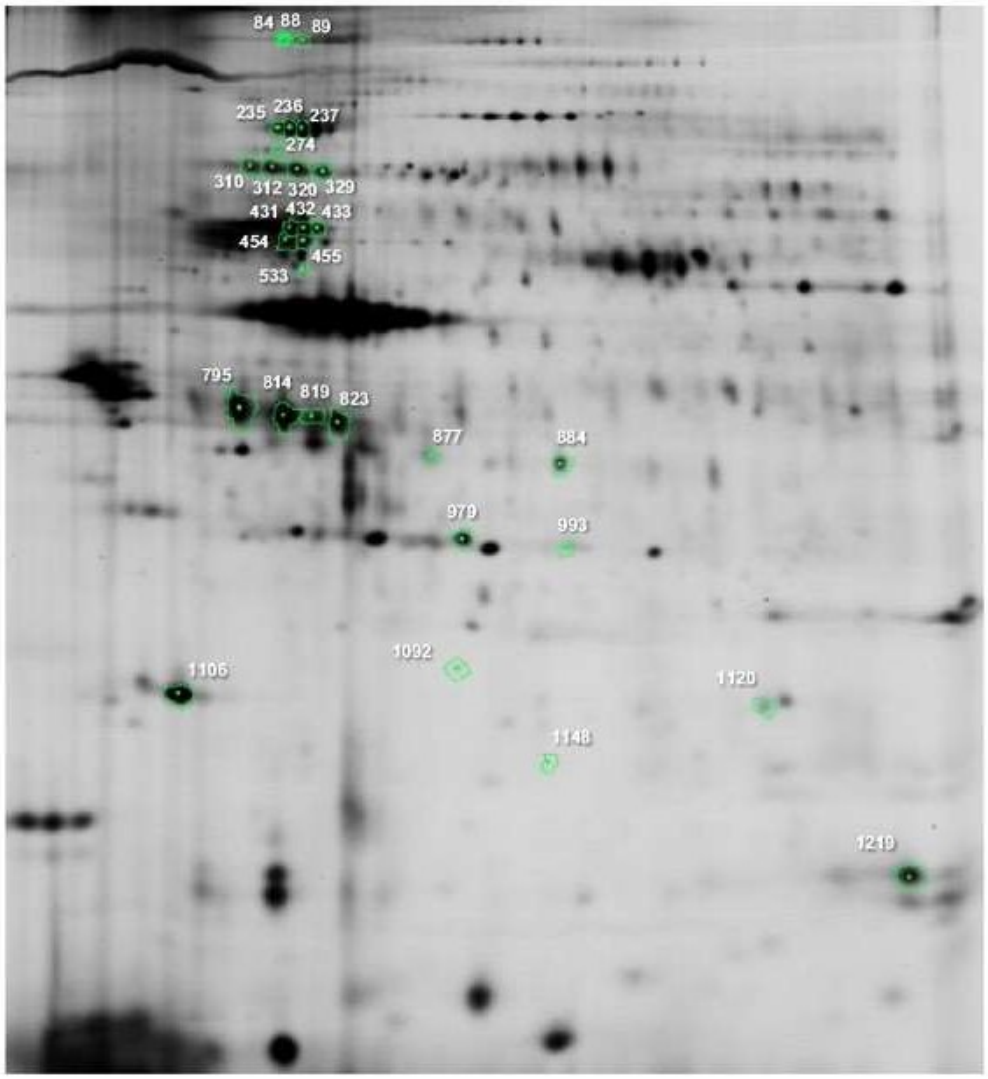
2-DE gels were scanned in a Typhoon 9400 scanner.

spot detection and matching performed with the internal standard loaded on each gel.

Differential abundance analysis of matched spots was performed by Student′s t-Test

Results from the differential protein abundance analysis of atherosclerotic coronary intima.

A



Spots found significantly varied (p value ≤ 0.05) and

with a fold change greater than 2.0 or lower than −2.0

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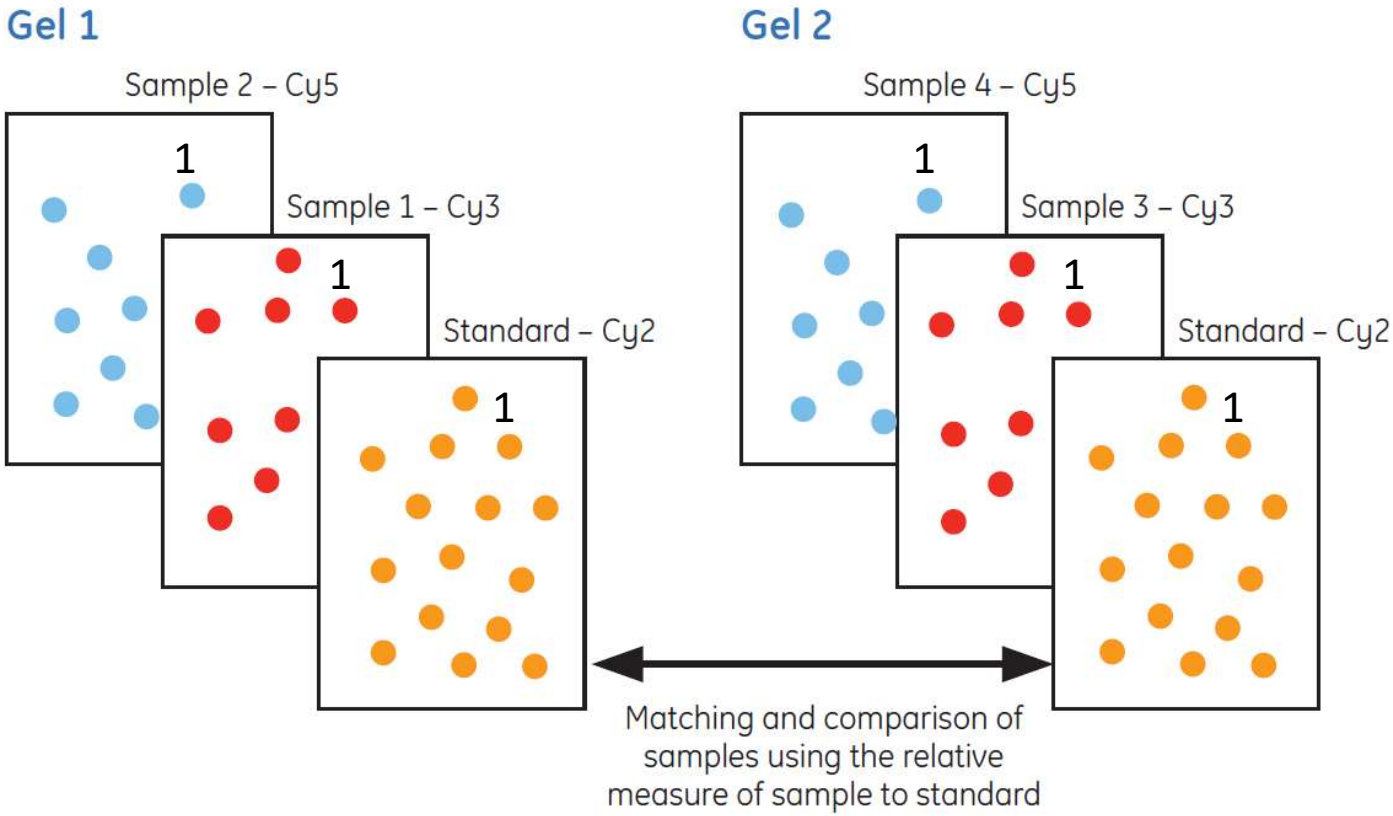

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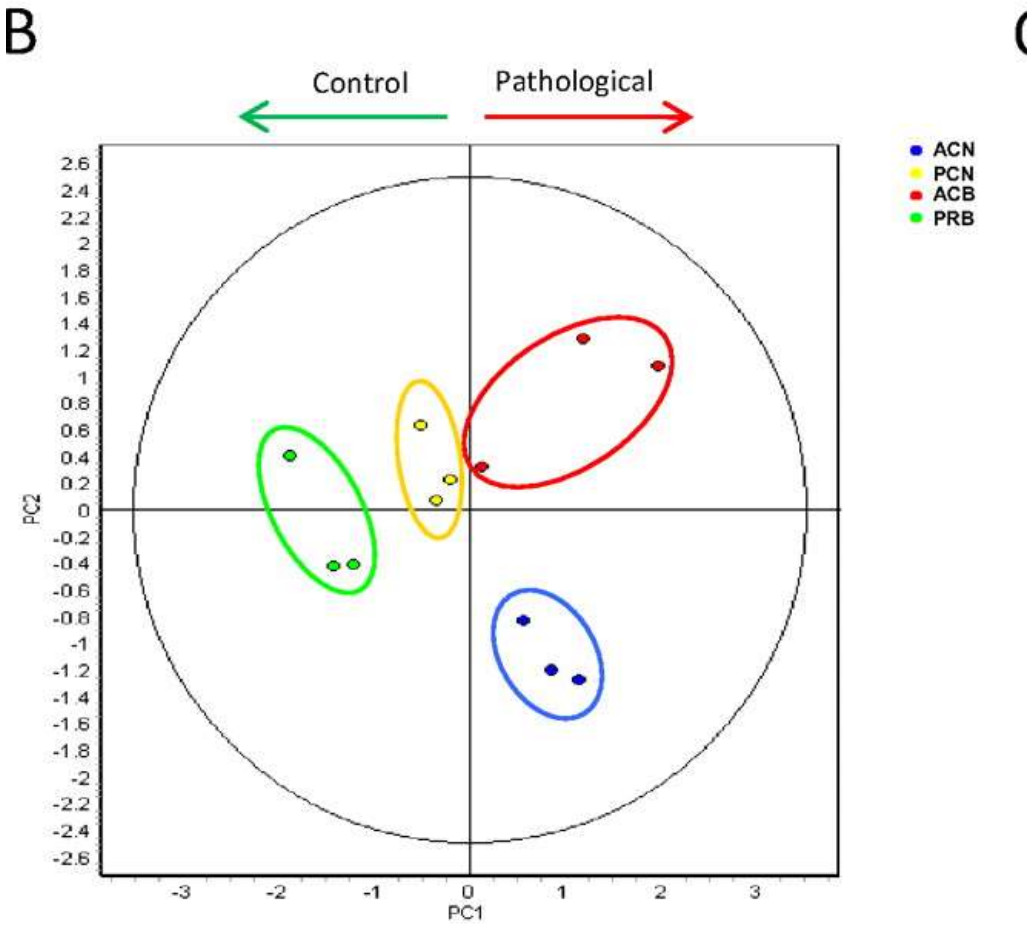

principal components analysis (PCA) was performed with the spots found significantly varied in an Anova Test performed with the groups of samples under study

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Preparative Two-dimensional DIGE Gels (1)

Microdissected material collected was insufficient to run preparative gels to perform MS protein identifications

whole artery tissue extracts from two of the arteries used in the DIGE experiment were obtained

Proteins were extracted in lysis buffer (7 m urea, 2 m thiourea, 30 mm Tris, and 4% CHAPS) after having crushed the frozen tissue with a mortar.

Extracts were subsequently sonicated for 15 min and tissue debris were removed by centrifugation at 12000 × g.

Supernatant was stored at −80 °C until use.

Preparative Two-dimensional DIGE Gels (2)

Supernatant was stored at −80 °C until use. An amount of 300 μg of total protein was reduced with 60 nmol Tris 2-carboxyethyl phosphine and labeled with 120 nmol preparative Cy3 dye.

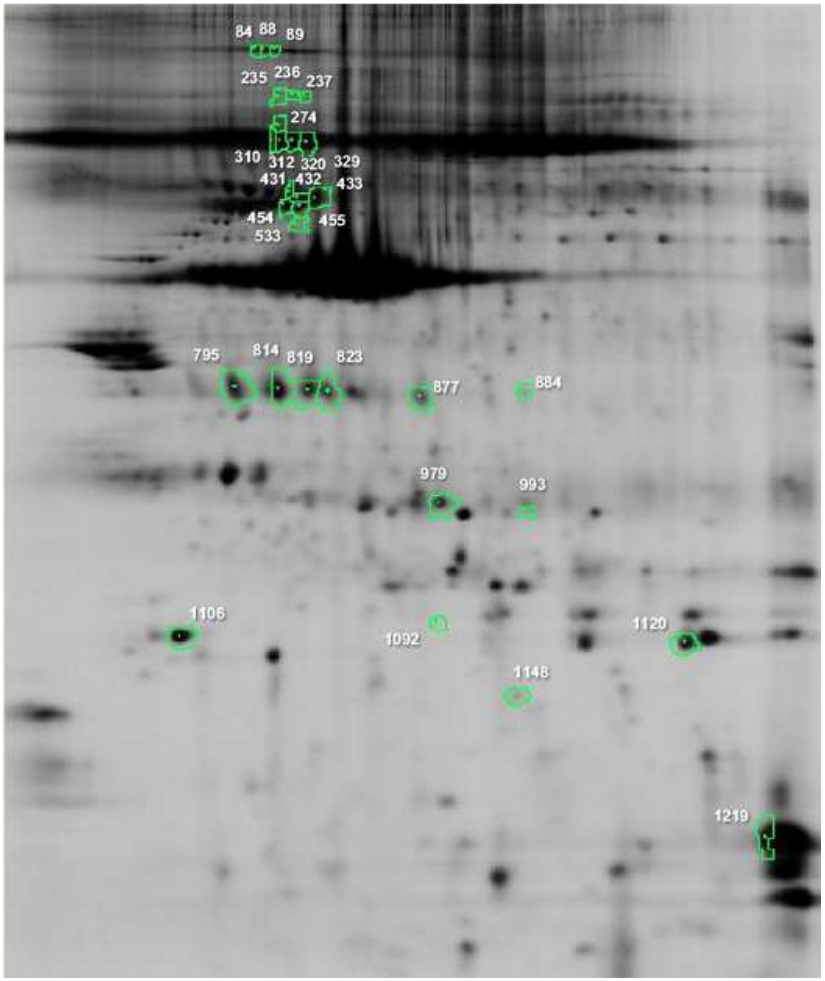
Following labeling, tissue extracts were loaded on 18-cm, pH 4–7, IPG strips. Following an active rehydration 12 h step, the same IEF program applied for saturation labeling DIGE samples was performed.

Second dimension was performed in the same conditions as two-dimensional DIGE experiment in 12.5% polyacrylamide gels.

Fluorescence images of the gels were obtained using a Typhoon 9400 scanner at a resolution of 100ppm.

Gels were subsequently poststained by a conventional silver staining and scanned using a GS-800 Calibrated Densitometer (BioRad).

Results from the differential protein abundance analysis of atherosclerotic coronary intima.



The altered spots were identified on an extract of whole atherosclerotic coronary artery tissue, where correspondent proteins are noted

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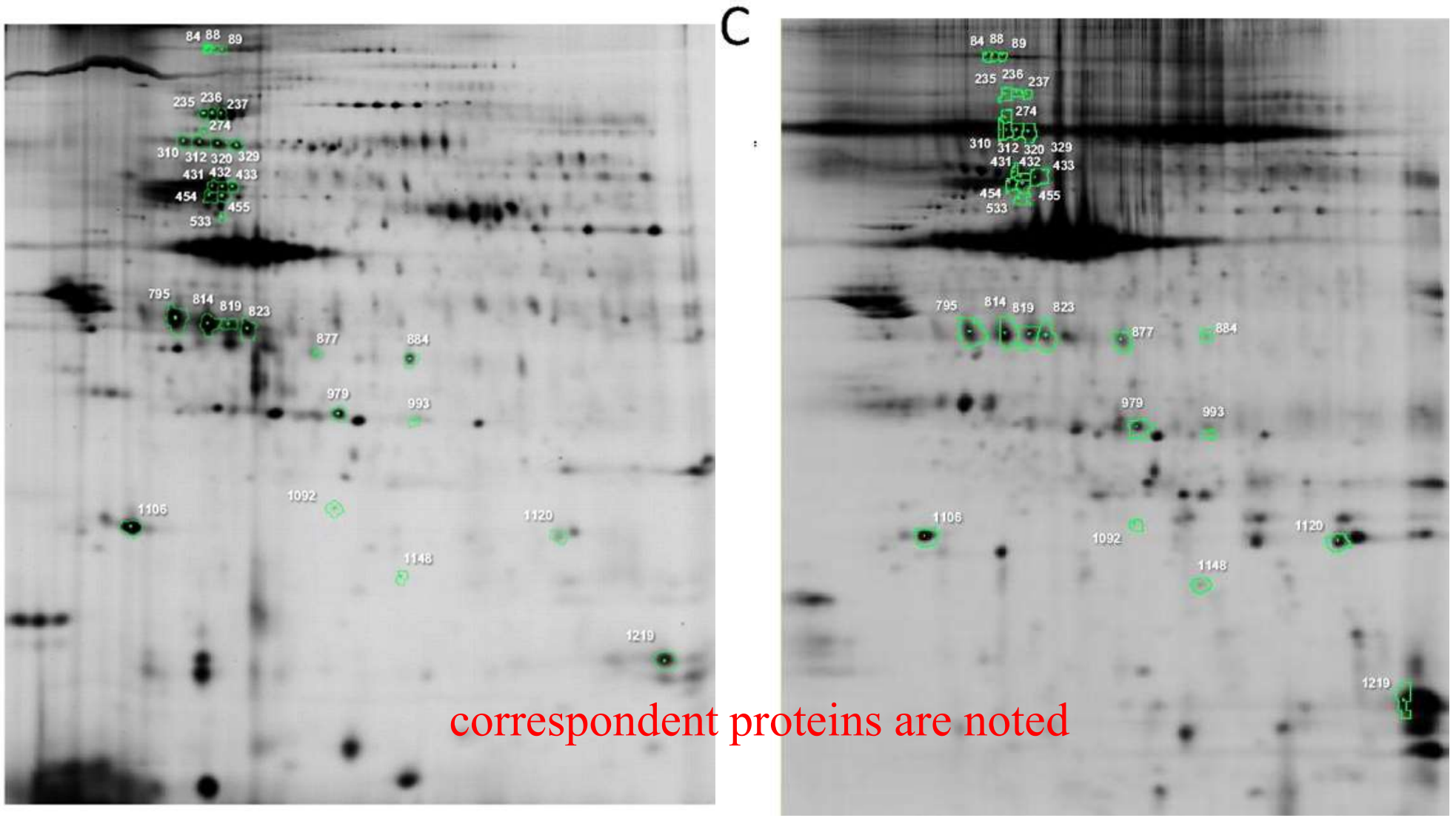

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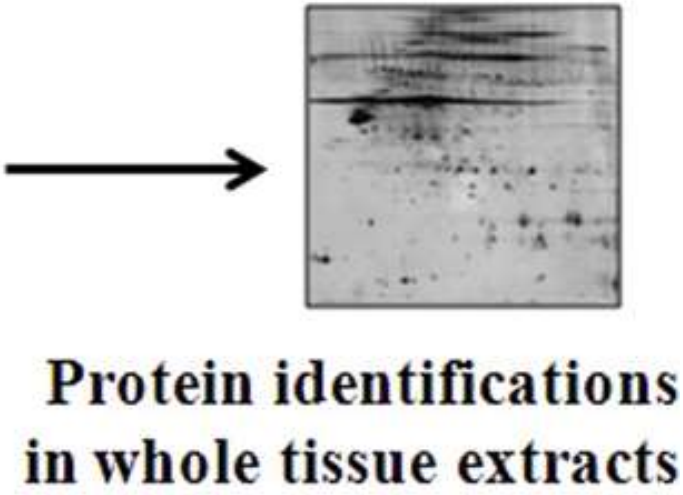

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Mass Spectormetry Identification of Spots

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Preparative gels were matched with the master gel from the DIGE experiment and the differential **spots were picked manually** from the gel.

Protein spots were automatically **digested** in-gel with porcine **trypsin** at a final concentration of 20 ng/µl in 20 mM ammonium bicarbonate and the digestion proceeded at 37 °C overnight.

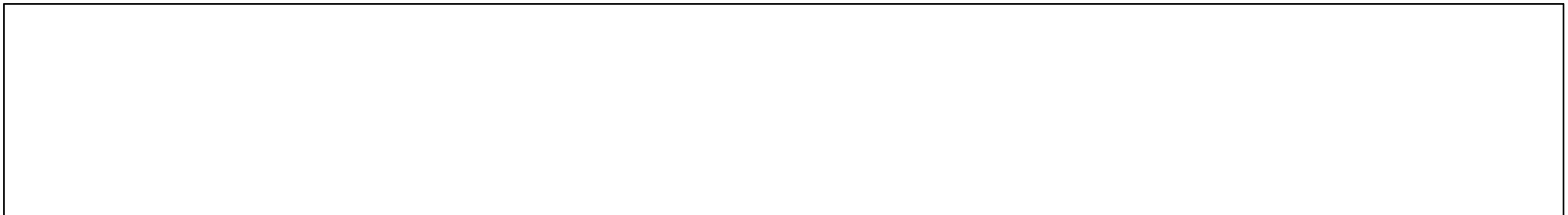
60% acetonitrile and 0.1% formic acid was added for **peptide extraction.**

An aliquot of the above digestion solution was deposited onto a 384 Opti-TOF 123 × 81 mm **matrix-assisted laser disorption ionization (MALDI)** plate

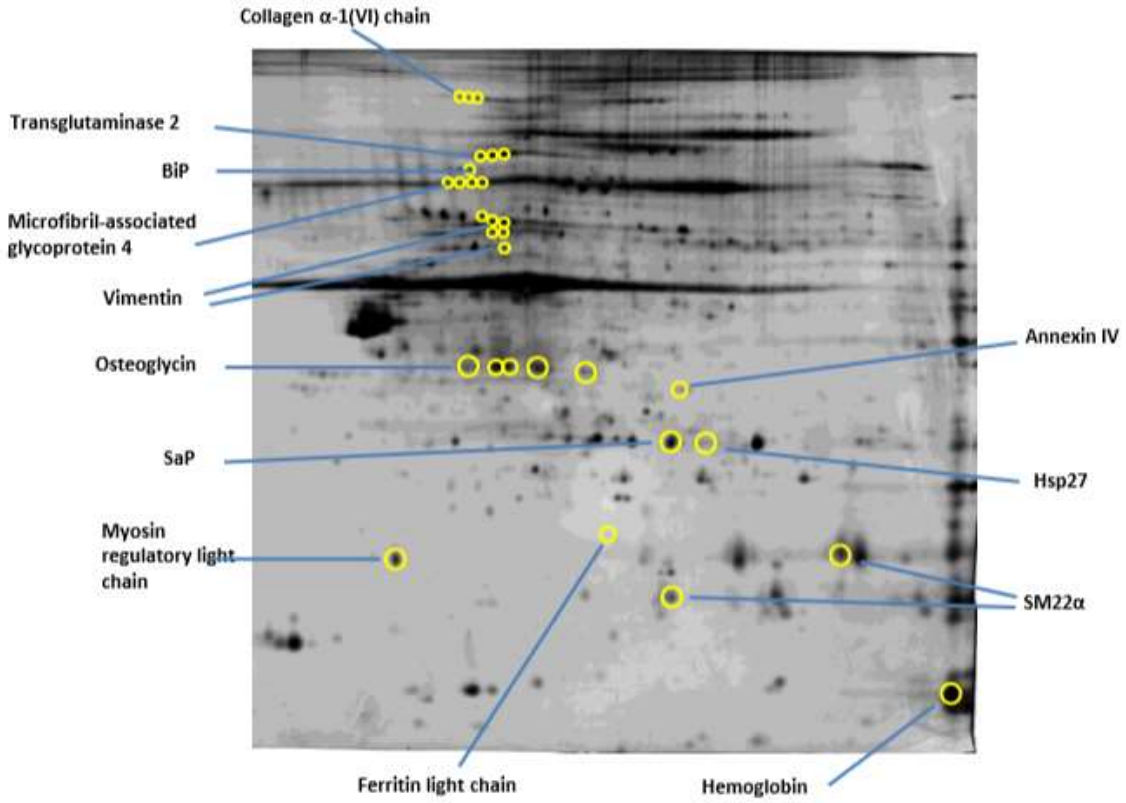
Differential spots were identified by MS/MS

Protein identifications of spots from DIGE analysis.

SPOT Num	Comparison	t-test p value	Av. Ratio	Protein identification	Accesion number (NCBInr or SwissProt)	Unique peptides detected	Sequence coverage (%)
84	ACB/PRB	0.022	3.49	————————	–	–	–
88	ACB/PRB	0.017	3.31	Collagen α-1(VI) chain	P12109	11	11
	ACN/PRB	0.0025	2.07				
89	ACB/PRB	0.049	2.47	Collagen α-1(VI) chain	P12109	5	6
235	ACN/PCN	0.0048	−3.68	Transglutaminase 2	gi 39777597	9	18
	ACN/PRB	0.044	−4.28				
236	ACN/PCN	0.048	−2.42	Transglutaminase 2	gi 39777597	9	18
237	ACN/PCN	0.0083	−2.79	Transglutaminase 2	gi 39777597	10	20
274	ACB/PRB	0.024	2.63	GRP78	gi 73968066	8	16
310	ACN/PRB	0.024	−2.59	Microfibril-associated glycoprotein 4	P55083	6	19



CONFERMA!!!



Supplementary Figure 3. Silver stained preparative gel. Non-labeled whole artery extracts were run in addition to the Cy3-labeled gels and differential spots excised for further MS/MS identifications, which corroborated the 29 identifications obtained.

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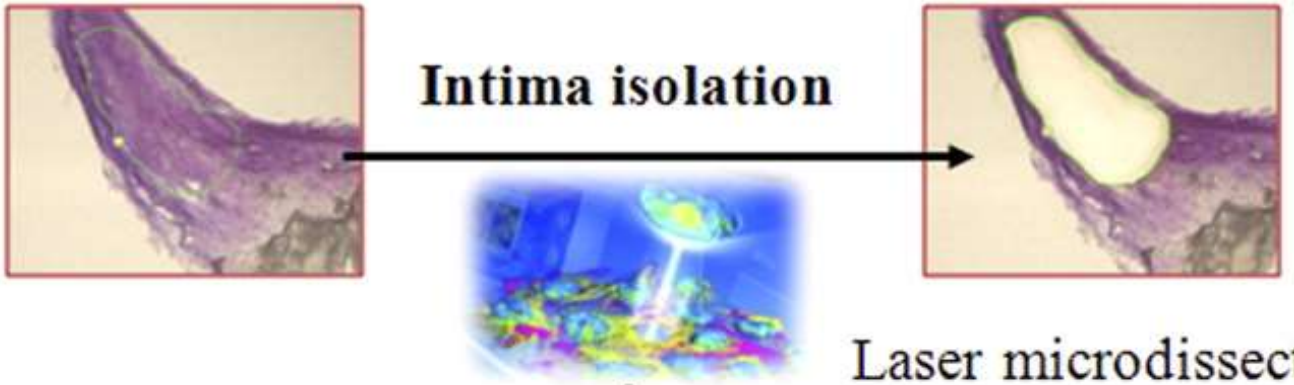
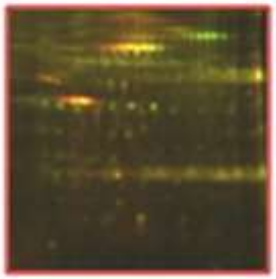
STRATEGIA DI CONFERMA e
STUDIO DEL SIGNIFICATO DEGLI SPOT
NEI TESSUTI (CORONARIE)

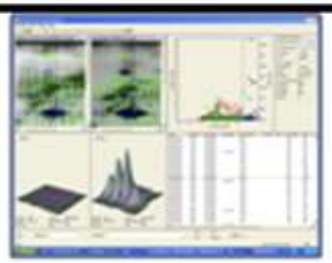


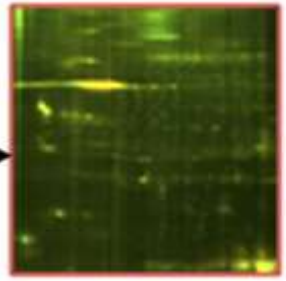
Laser microdissection

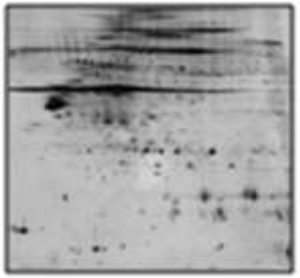


Saturation labeling DIGE

Differential protein abundance analysis

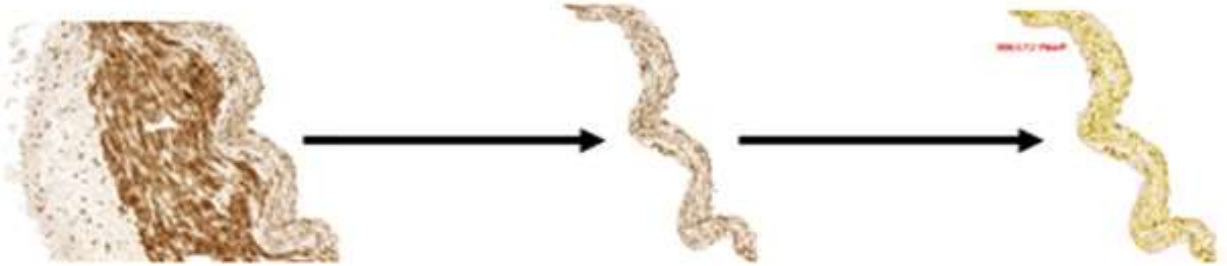






Protein identification in whole tissue extract

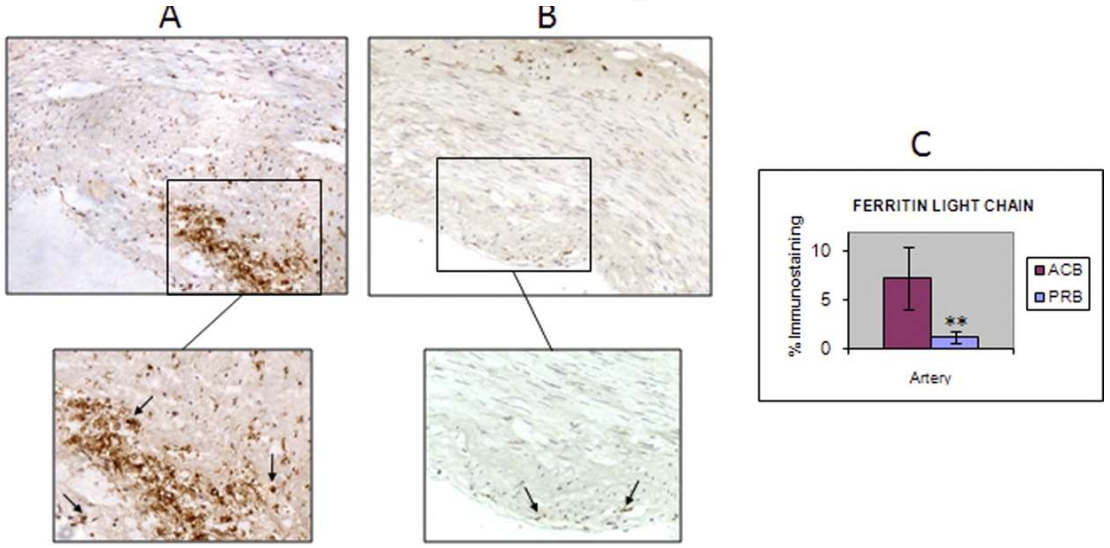
Validations by IHC



ferritin light chain (Spot 1092)

atherosclerotic coronary

preatherosclerotic



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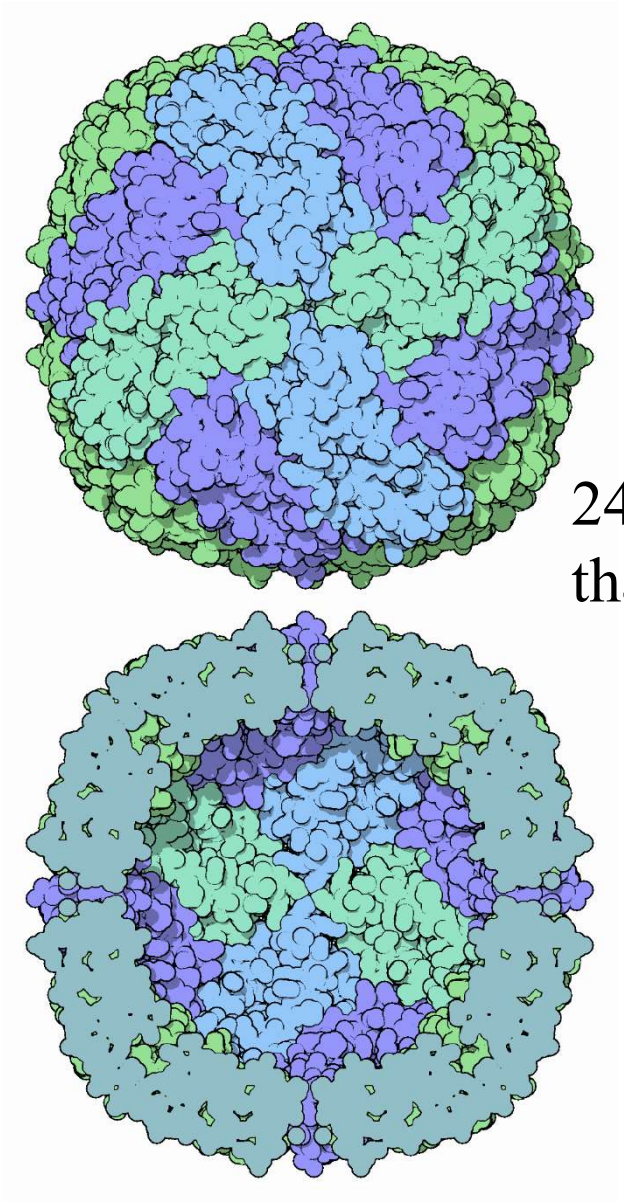
1092	ACN/PRB	0.025	16.04	Ferririn light chain	P02792	2	14

Fernando de la Cuesta et al. Mol Cell Proteomics 2011;10:M110.003517

Ferritin exterior and interior cross section



24 identical protein subunits that form a hollow shell

4500 iron «Ferric» ions inside!!

Iron transport:

- **Transferrin** (Fe^{3+})

$$\text{Transferrin} + Fe^{3+} + CO_3^{2-} \rightarrow \text{Transferrin} \bullet 2(Fe^{3+}CO_3^{2-})$$

  - only one third saturated with iron
  - unsaturated transferrin protects against infections (iron overload and infection)
- **Lactoferrin**
  - binds iron in milk
  - antimicrobial effect (protects newborns from gastrointestinal infections)
- **Haptoglobin** - binds hemoglobin in the plasma

Iron storage:

- **Ferritin** (Fe^{3+})
  - storage of iron (hepatocytes, muscles)
  - in the blood → sensitive indicator of the amount of iron in the body
- **Hemosiderin** - when iron is in excess (amorphous iron deposition)

Intracellular Iron Chelates

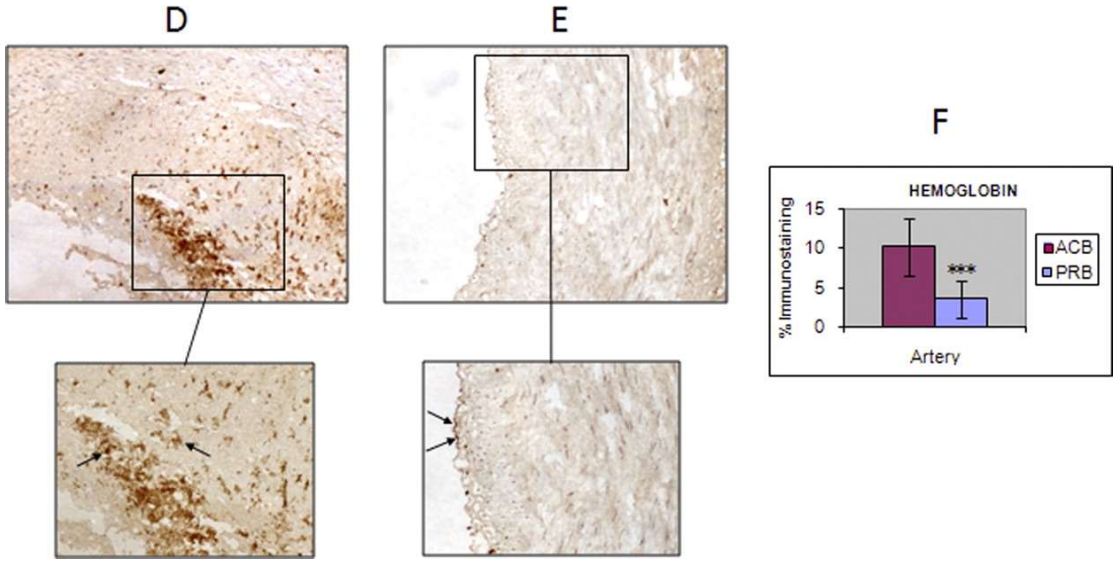
- **Ferritin**
  - **Long term storage**
  - **4500 atoms Fe/molecule**
  - **Fe^{3+}**
- **Labile Iron Pool**
  - **Poorly characterized**
  - **Transient storage**
  - **Exchanges with ferritin**

Hemoglobin (spot 1219)

0.028	4.69	Hemoglobin	gi 999565	7	63

atherosclerotic coronary

preatherosclerotic

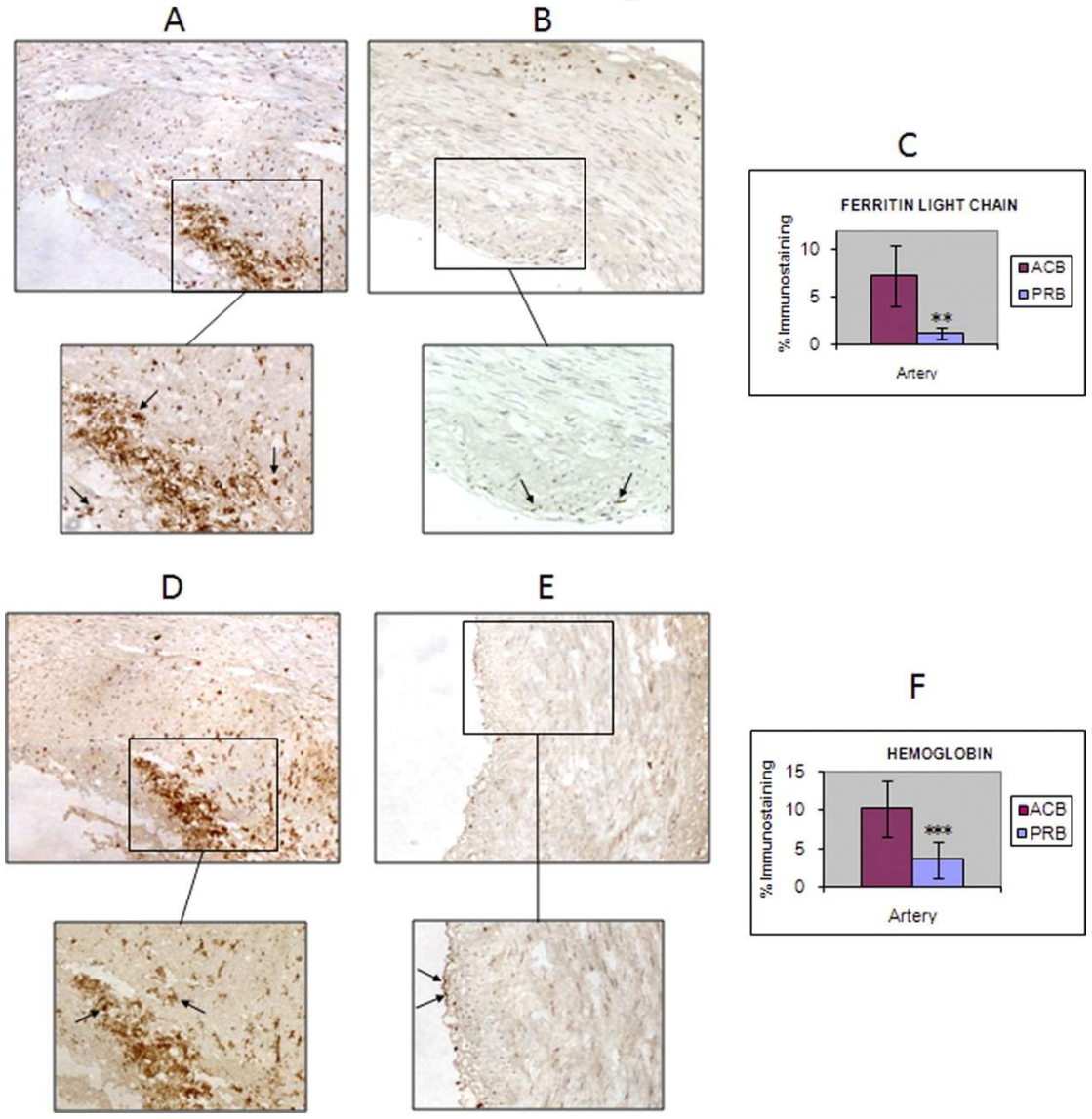


Fernando de la Cuesta et al. Mol Cell Proteomics 2011;10:M110.003517

IHC results from ferritin light chain and hemoglobin point out colocalization

atherosclerotic coronary

preatherosclerotic



intraplaque hemorrhage deposition?

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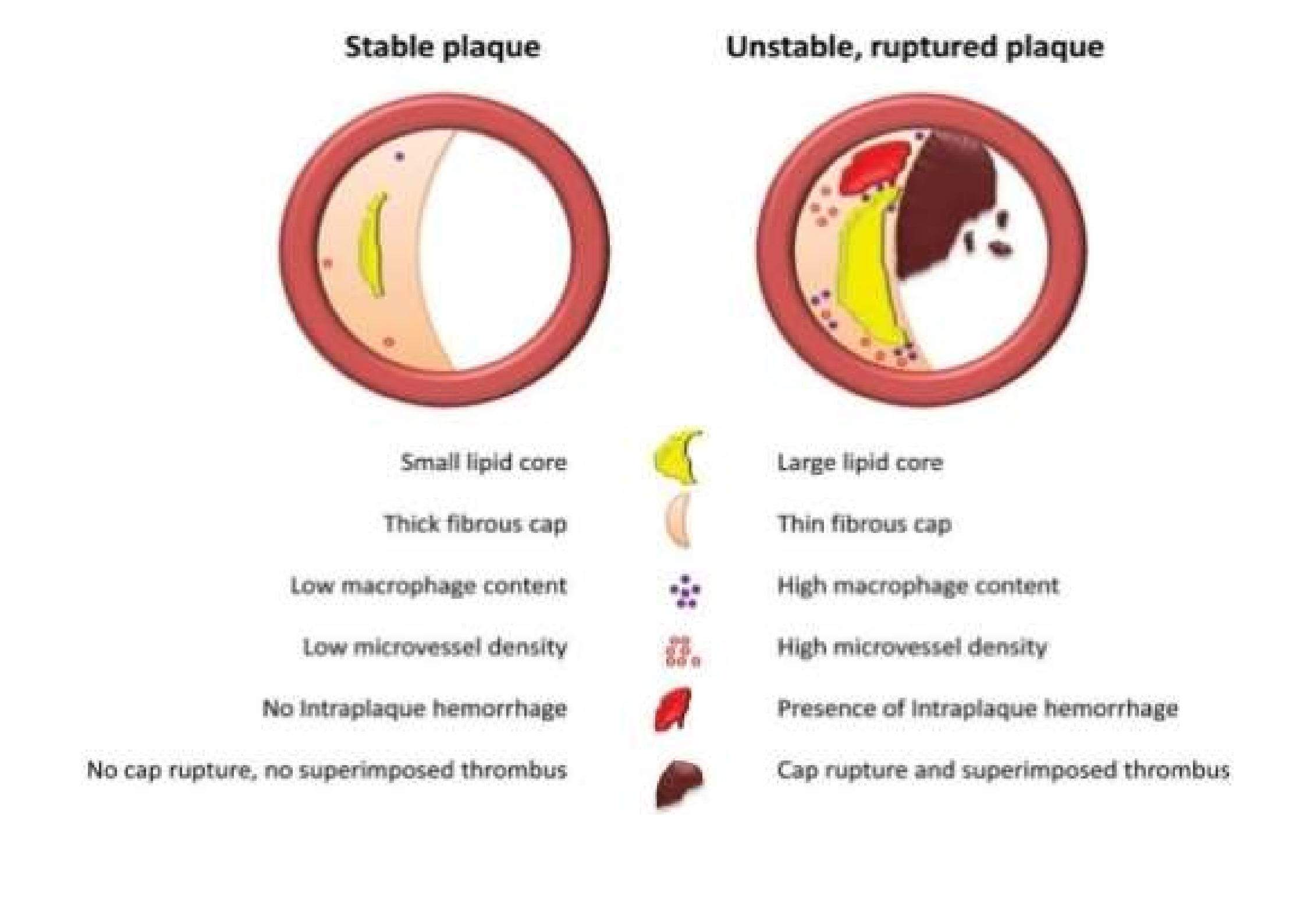
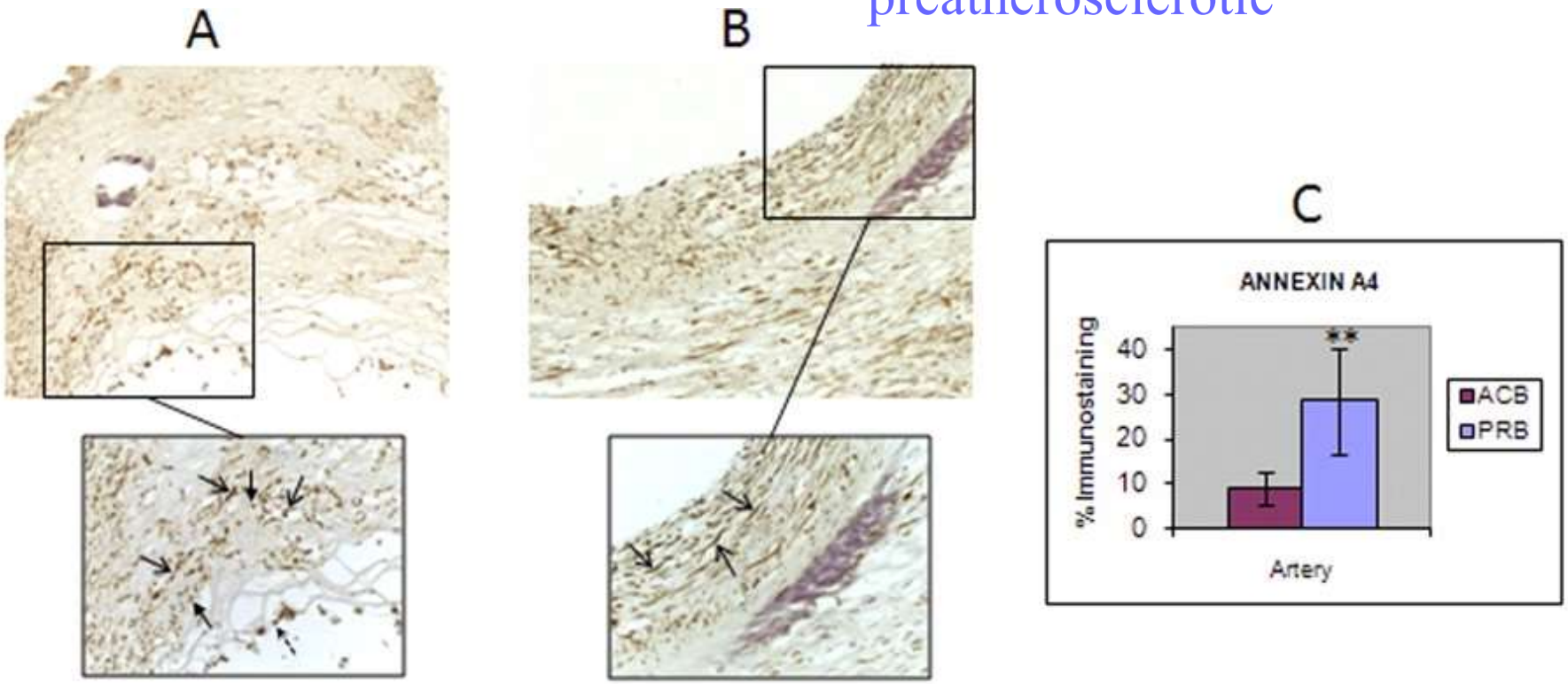

Stable plaque
Unstable, ruptured plaque
Small lipid core
Large lipid core
Thick fibrous cap
Thin fibrous cap
Low macrophage content
High macrophage content
Low microvessel density
High microvessel density
No Intraplaque hemorrhage
Presence of Intraplaque hemorrhage
No cap rupture, no superimposed thrombus
Cap rupture and superimposed thrombus

IHC results from annexin A4 (Spot 884)

atherosclerotic coronary

preatherosclerotic



884 ACB/PRB 0.024 —4.2 Annexin IV P09525 24 64

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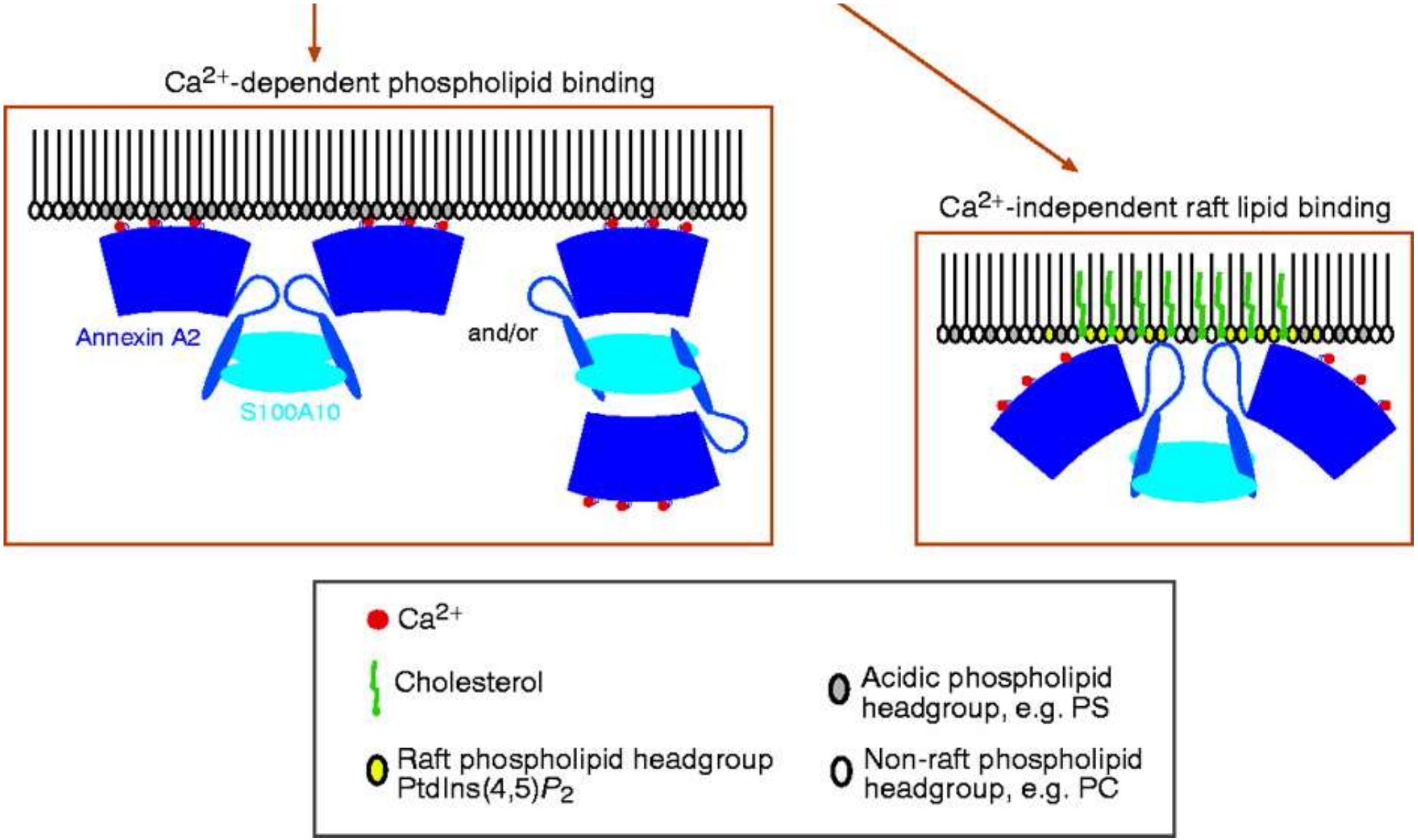
annexins

the annexin family - 97% homology between A4 and A5.

Annexins have a crucial anticoagulant role

Fernando de la Cuesta et al. Mol Cell Proteomics 2011;10:M110.003517

Model of membrane domain binding mediated by annexin



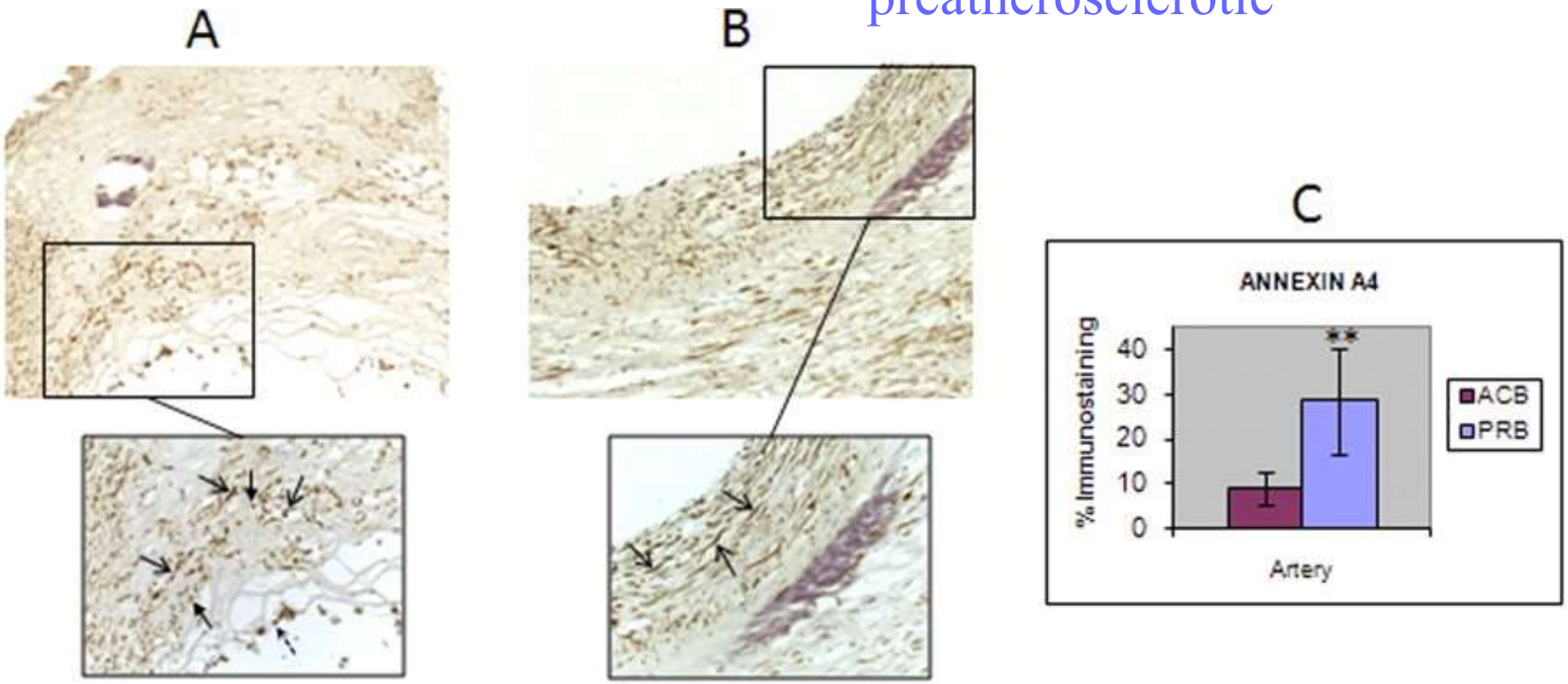
Ursula Rescher, and Volker Gerke J Cell Sci 2004;117:2631-2639

Journal of Cell Science

IHC results from annexin A4

atherosclerotic coronary

preatherosclerotic



crucial anticoagulant role

the decrease in ANXA4 found in the atherosclerotic arterial intima may contribute to an impairment of the anticoagulation

increasing the risk of thrombosis?

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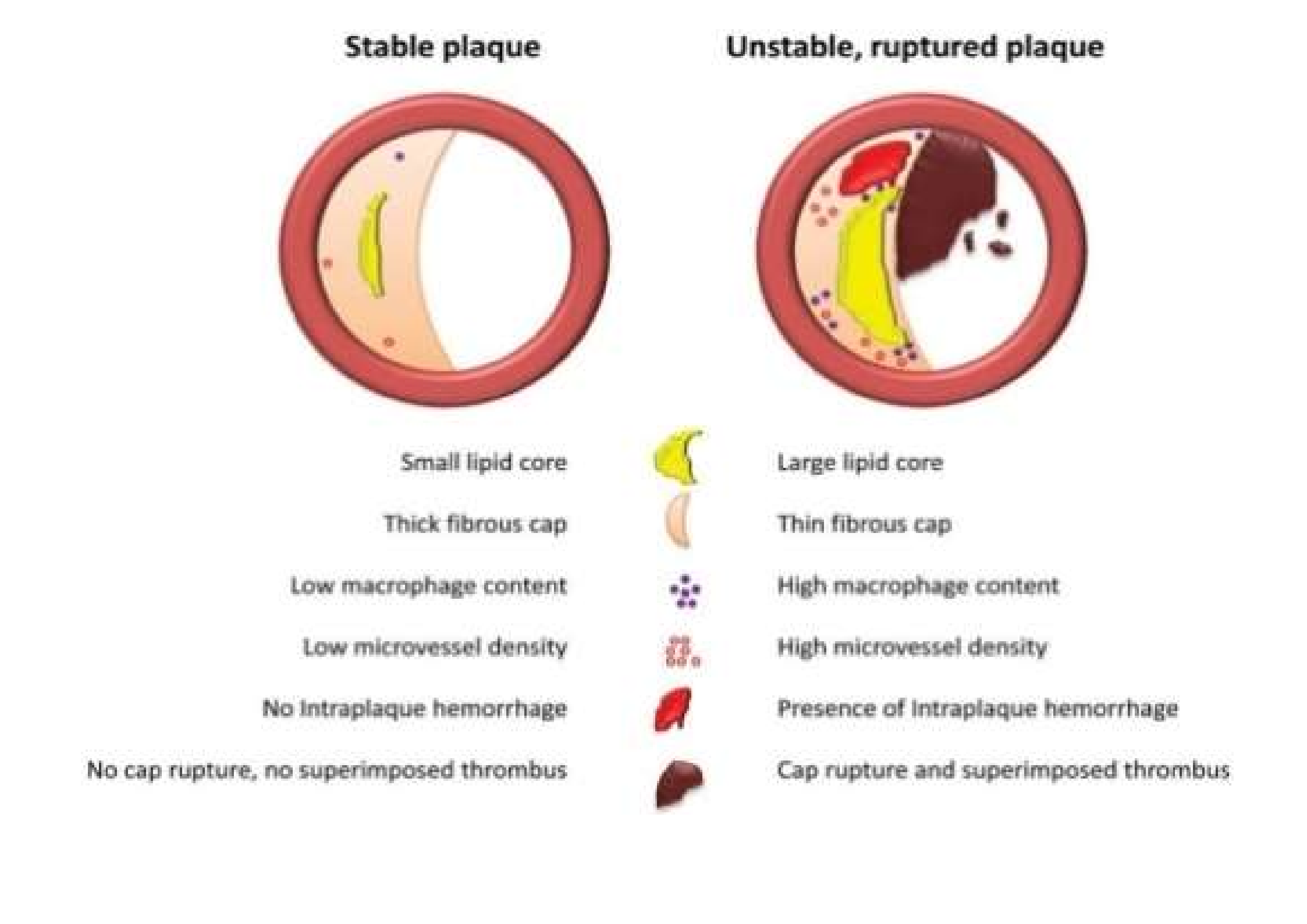
Stable plaque
Unstable, ruptured plaque
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Low macrophage content
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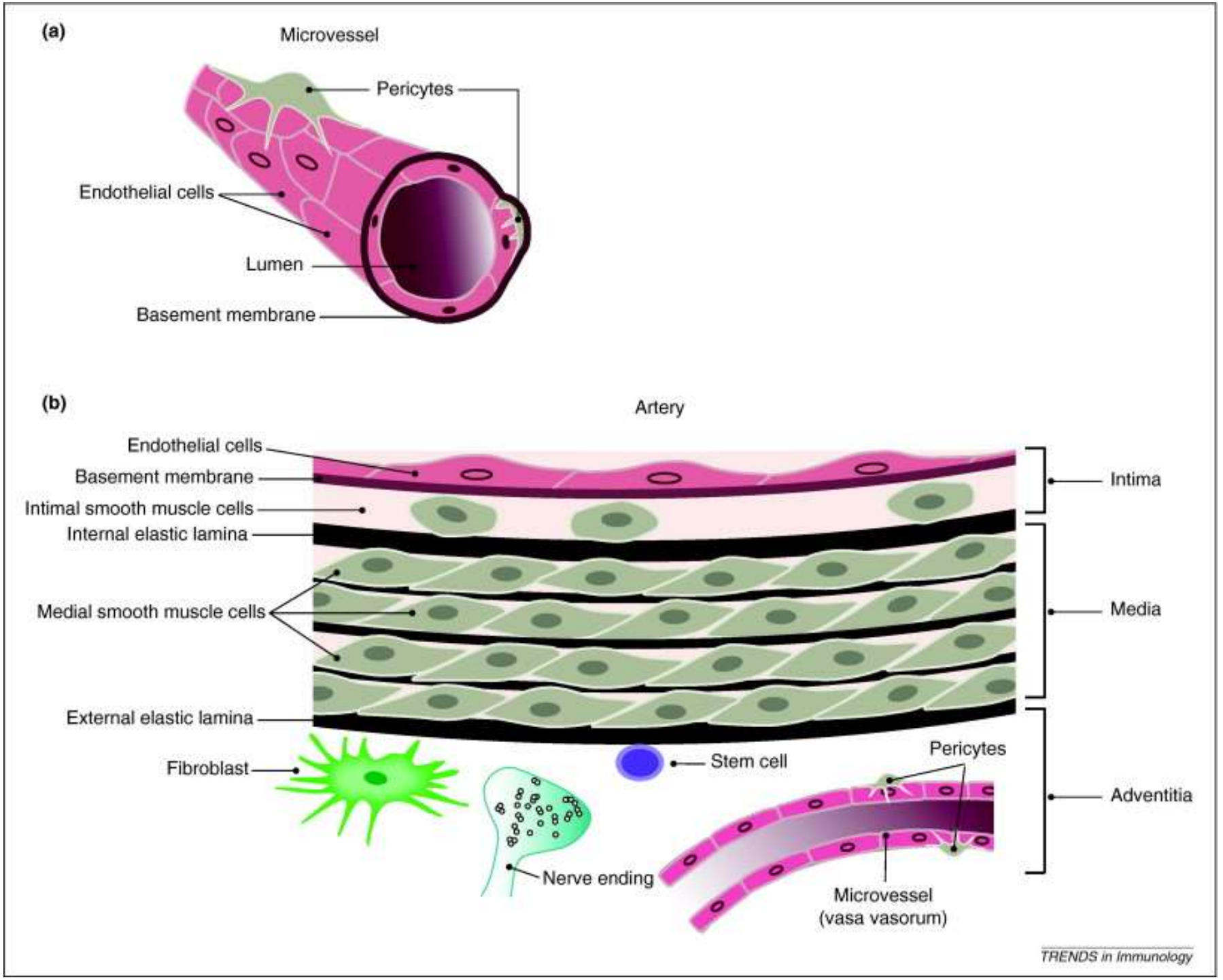
No Intraplaque hemorrhage
Presence of Intraplaque hemorrhage
No cap rupture, no superimposed thrombus
Cap rupture and superimposed thrombus

Myosin regulatory light chain MRLC Spot 1106

a subunit of the myosin complex
which regulates the contractility of vascular cells
and their migration ability

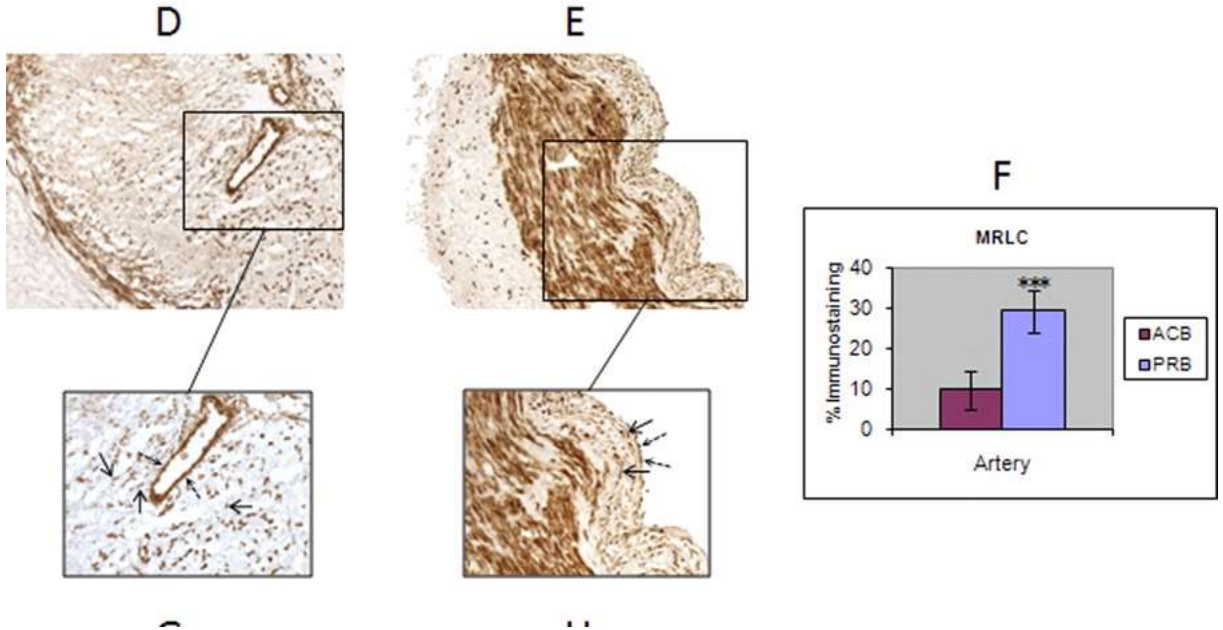
1106	ACB/PCN	0.049	—4.9	Myosin regulatory light 2, smooth muscle isoform	gi 119936529	11	66

Actin fibers, which are known as sensors of mechanical stress, exert traction force by interacting with myosin II
The tensional force is regulated by phosphorylation of myosin regulatory light chain (MRLC) at Thr18 and Ser19


(a)
Microvessel
Pericytes
Endothelial cells
Lumen
Basement membrane
(b)
Artery
Endothelial cells
Basement membrane
Intimal smooth muscle cells
Internal elastic lamina
Medial smooth muscle cells
External elastic lamina
Fibroblast
Stem cell
Pericytes
Nerve ending
Microvessel
(vasa vasorum)
Intima
Media
Adventitia
TRENDS in Immunology

IHC results from MRLC

Myosin regulatory light chain MRLC is a subunit of the myosin complex which regulates the contractility of vascular cells and thereby their migration ability.

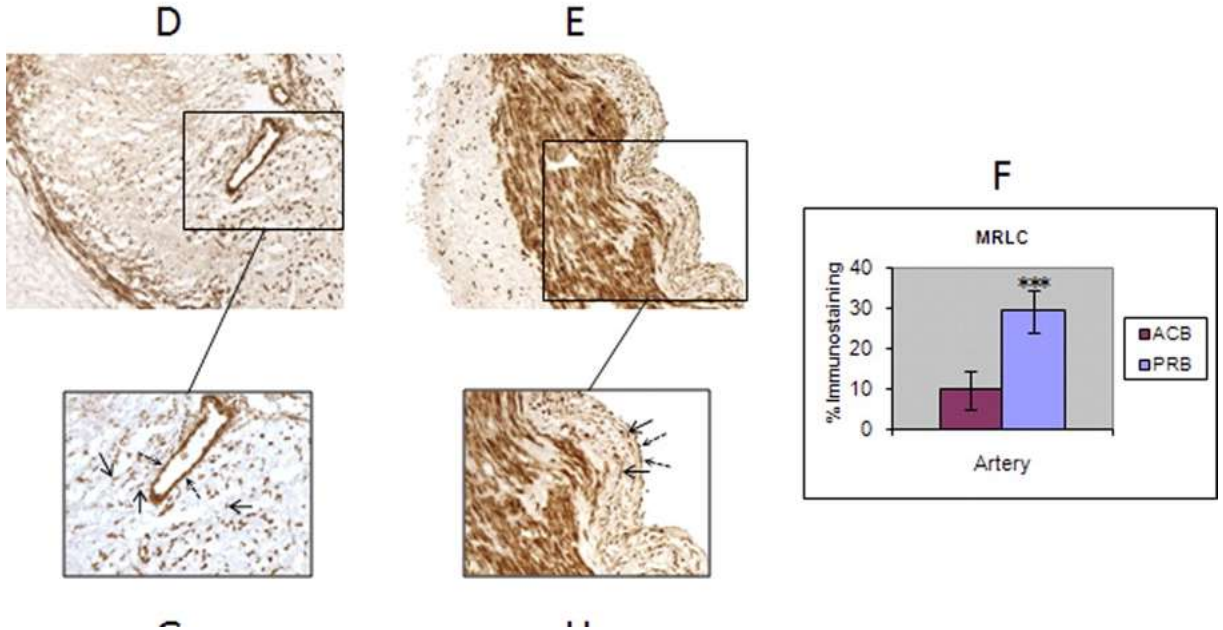


atherosclerotic coronary preatherosclerotic

Fernando de la Cuesta et al. Mol Cell Proteomics 2011;10:M110.003517

IHC results from MRLC

Myosin regulatory light chain MRLC is a subunit of the myosin complex which regulates the contractility of vascular cells and thereby their migration ability.



The reduction in positive Vascular Smooth Muscle Cells within the atherosclerotic intima points out a change in this cell s phenotype, lacking of migrative properties

Fernando de la Cuesta et al. Mol Cell Proteomics 2011;10:M110.003517

Premessa Initiation of atherosclerosis occurs within the intima, where major molecular changes are produced during pathogenesis.

Risultati
13 proteins altered (seven up-regulated and six down-regulated)

implicated in
Migrative capacity of vascular smooth muscle cells
Coagulation
Intraplaque hemorrhage deposition

Altre …extracellular matrix composition
apoptosis,
heat shock response

Novità annexin 4, myosin regulatory light 2s-mooth muscle isoform and ferritin light chain not previously identified in the atherosclerotic coronary intima