SIMPOSIO ANNUALE DEL CENTRO
DI RIFERIMENTO PER LA SINDROME
DI MARFAN E PATOLOGIE CORRELATE
FOCUS SULLA SINDROME
DI EHLERS-DANLOS VASCOLARE

17 maggio 2025, ore 9:30-17:00
Aula Anfiteatro Giubileo 2000 - Policlinico Tor Vergata
Viale Oxford 81, 00133 - Roma

con il patrocinio di

IL FUTURO:
VERSO NUOVE PROSPETTIVE DI CURA

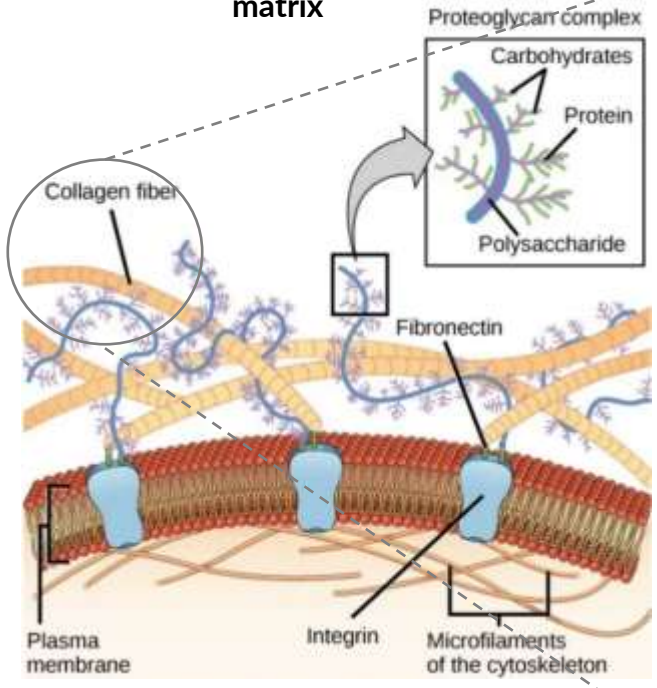
Sferoidi da fibroblasti come nuovo modello cellulare per lo studio della patogenesi e l'identificazione di nuovi target terapeutici nella sindrome di Ehlers-Danlos vascolare

Lucia Micale, MSc, PhD
UOC Genetica Medica
Fondazione IRCCS-Casa Sollievo della Sofferenza

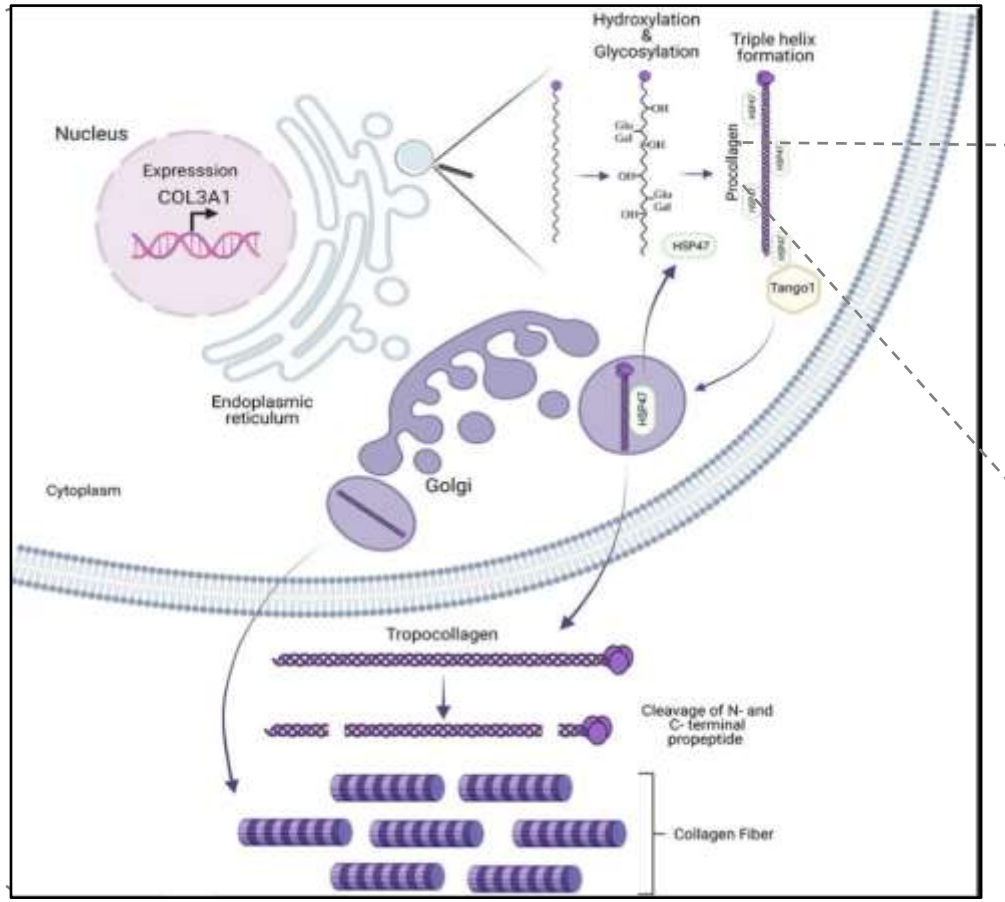
Type III Collagen: A Key Component of the Extracellular Matrix

vEDS is caused by heterozygous deleterious variants in the *COL3A1* gene that encodes the pro-alfa1 chain of type III procollagen (Collagen III, COLLIII), a major fibrillar collagen in the ECM

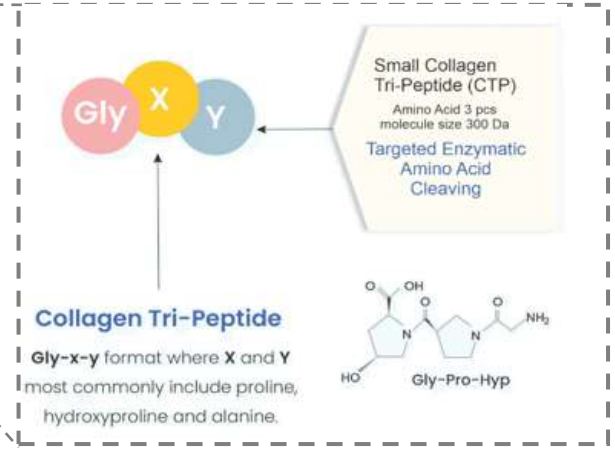
Extracellular matrix



COLLIII formation



Gly-X-Y repeats



vEDS is currently without an effective treatment

RESEARCH PROJECT (From 2022 to date)

UO:

Prof. Diego Di Bernardo
Prof. Diego Medina

Funders:

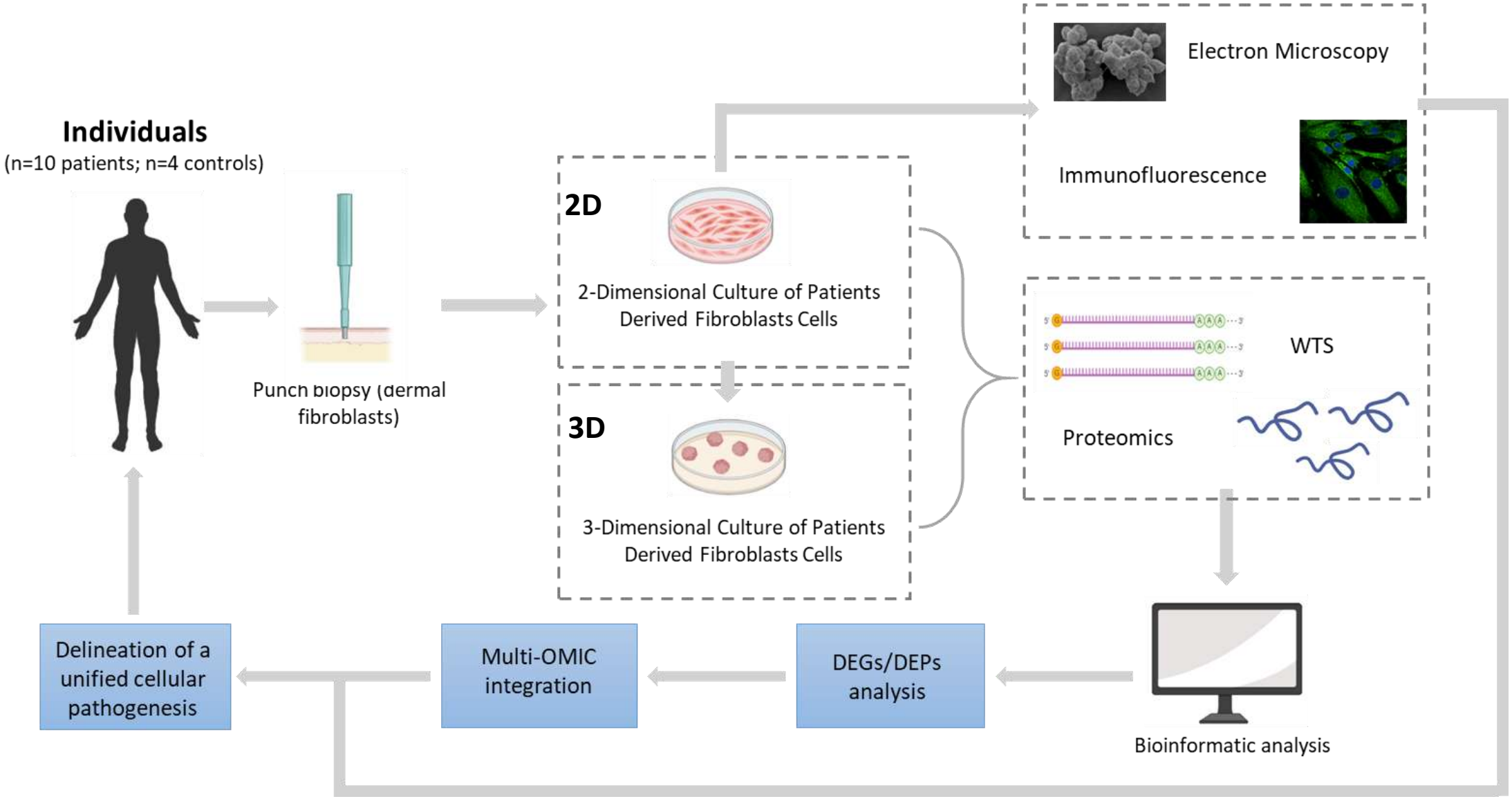
AIMs:

1. TO UNDERSTAND MOLECULAR MECHANISMS

2. TO DEFINE THE CELLULAR PHENOTYPE

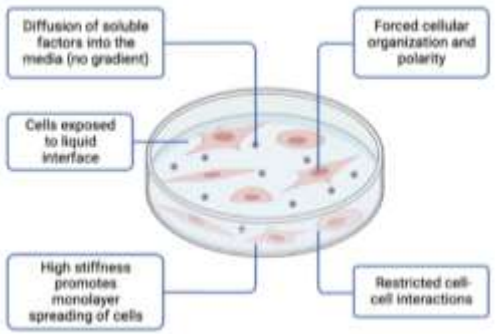
3. TO SELECT THERAPEUTIC MOLECULES BY USING A DRUG REPURPOSING APPROACH

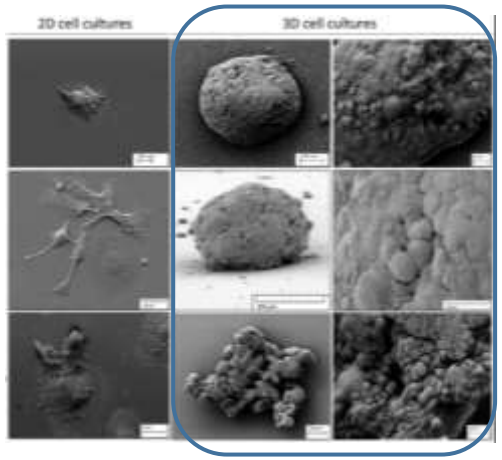
Workflow of the first study



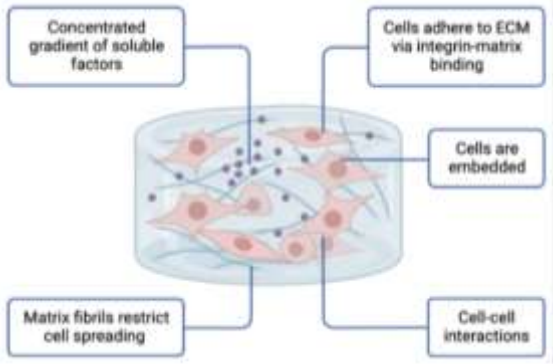
Why Use 3D Models?

2D cell culture





3D cell culture



ECM and C-C interactions

- ❑ **In vivo, cells grow in a three-dimensional environment where cell-cell and cell-ECM interactions are critical**
- ❑ **3D models can accurately replicate the microenvironment of the source tissue, both normal and pathological**
- ❑ **3D represents an excellent cellular model for studying the physiological processes of connective tissue diseases which involve altered ECM**

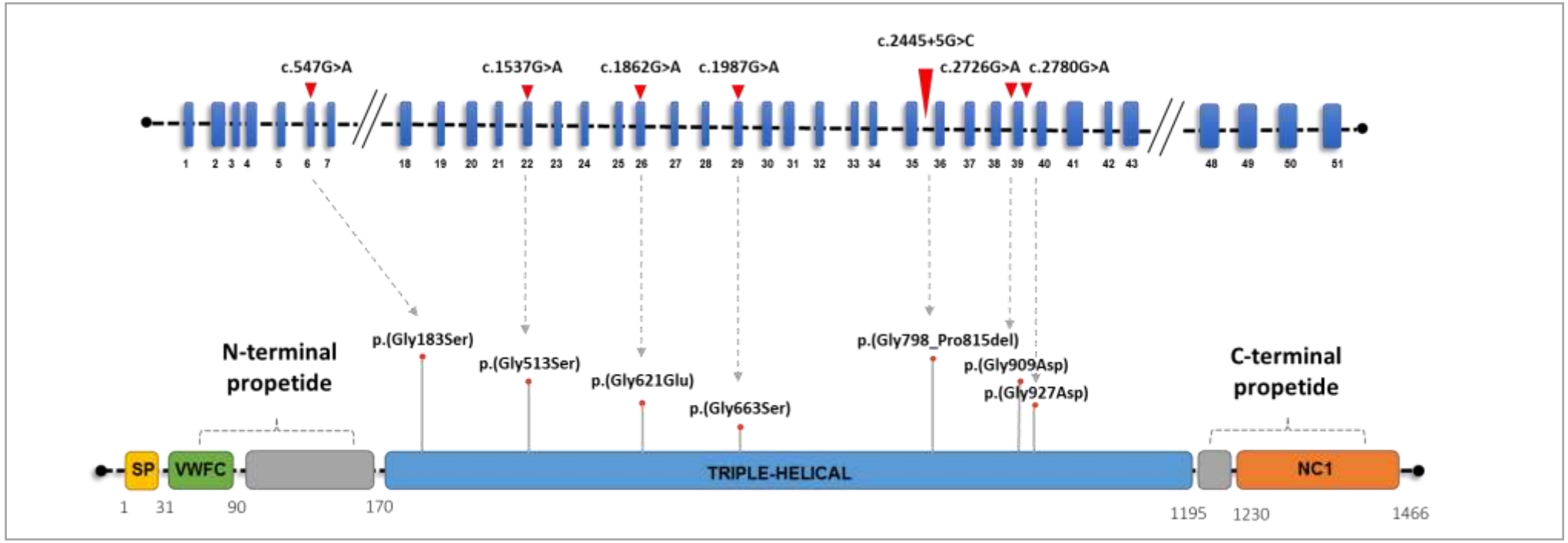
https://www.moleculardevices.com/applications/3d-cell-models/spheroids

Overview of *COL3A1* Variants identified in vEDS patients

✓ In 2022, Fondazione IRCCS-Casa Sollievo della Sofferenza has been included in the **ERN-ReCONNET** and **ERN-SKIN** European Reference Networks for the Ehlers-Danlos syndromes. To date we collected about 3.000 samples/patients with connective tissue diseases.

Patient recruitment:

- ✓ 10 patients affected by vEDS: 8 sporadic and 2 familial cases
- ✓ 9 patients harbored *COL3A1* glycine substitutions (#7 different variants) classified as P/LP, according to ACMG
- ✓ 1 intronic variant (#1) classified as VUS



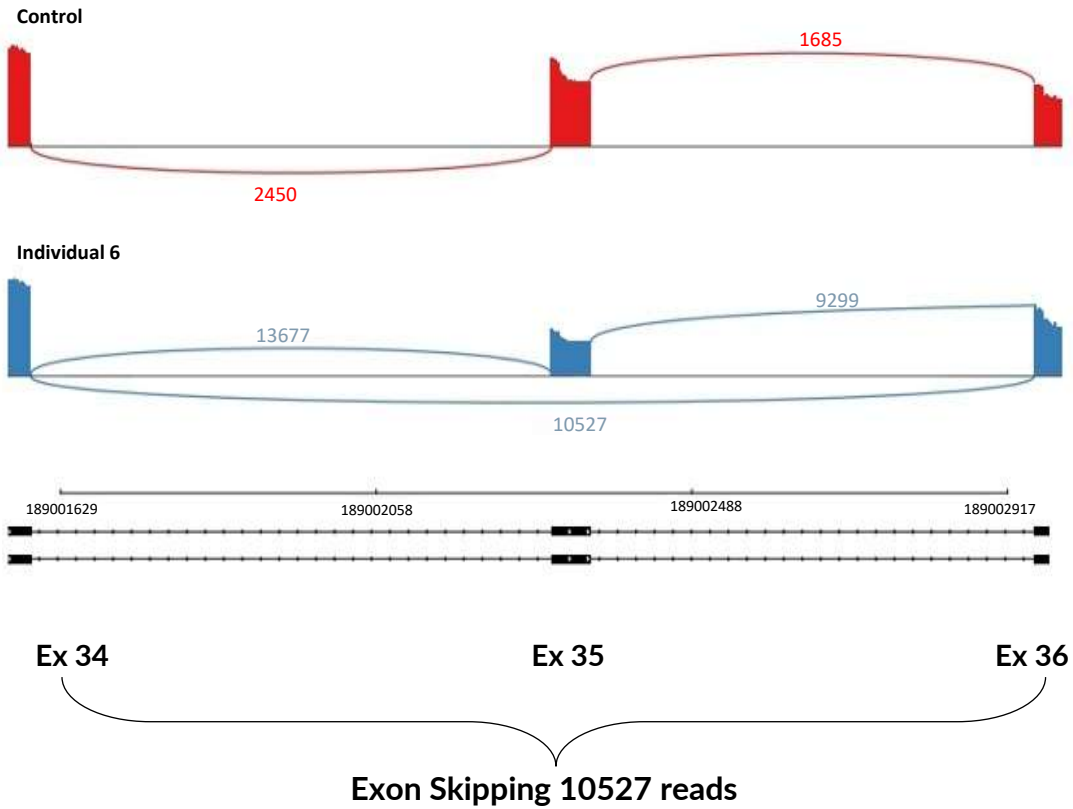
Evaluating the pathogenic effect of intronic variant:

COL3A1 **(NM_000090.4):c.2445+5G>C, p.(Gly798_Pro815del)**

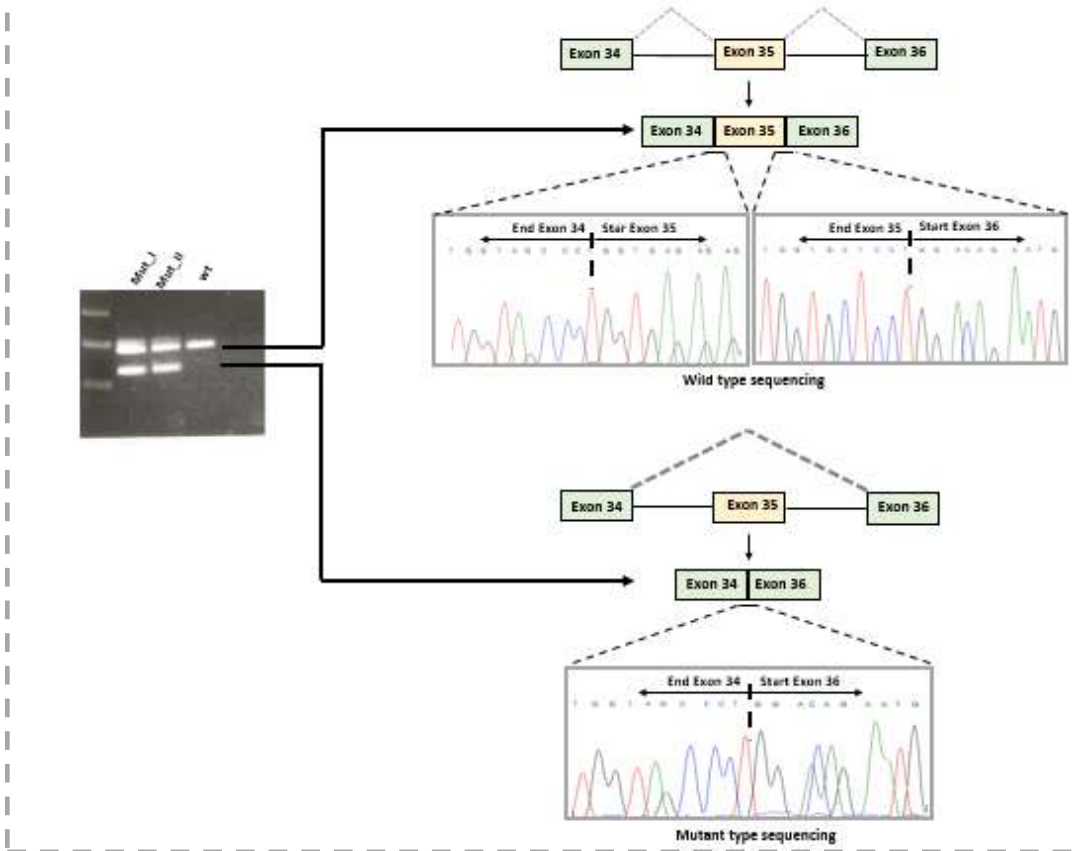
Clinical interpretation (VUS):
PM2_Moderate,
PP3_Supporting

Clinical interpretation (LP):
PS3_Strong,
PM2_Moderate,
PM6_Supporting

RNA Sequencing

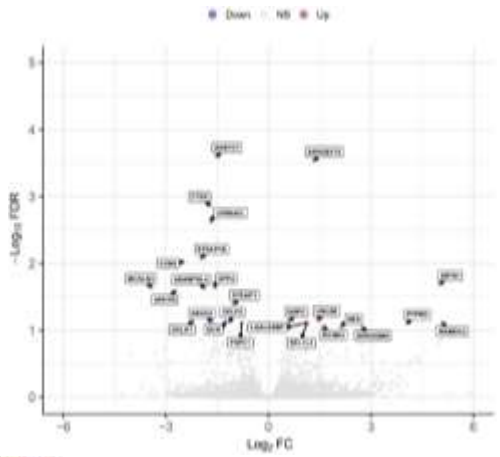


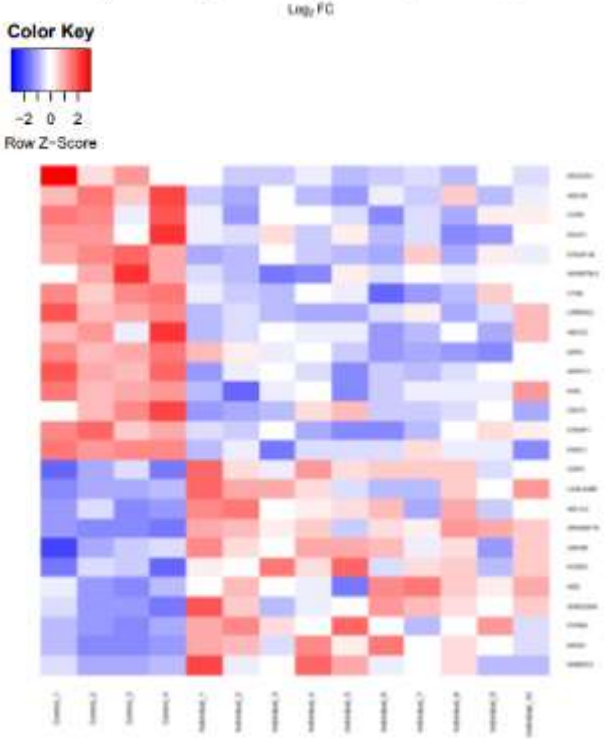
RT-PCR



Overview of 2D Transcriptomic Data

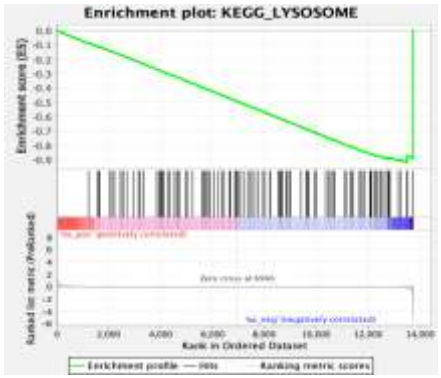
26 DEG

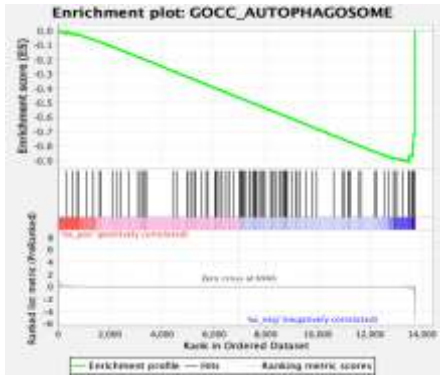


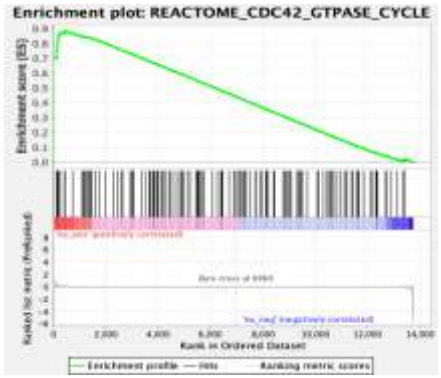


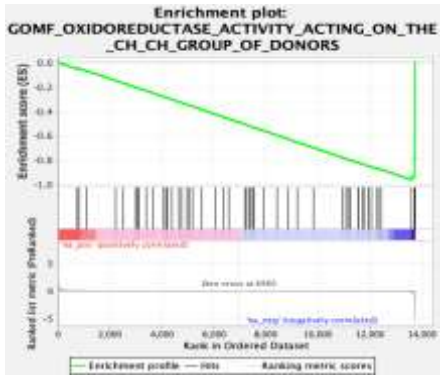
GSEA

FC = 2,5
FDR ≤0,05

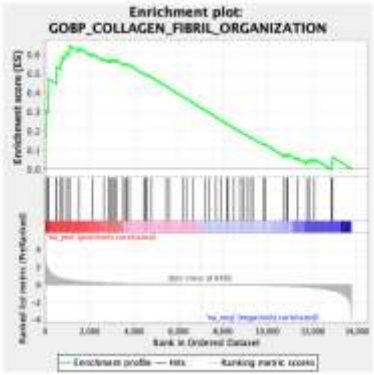


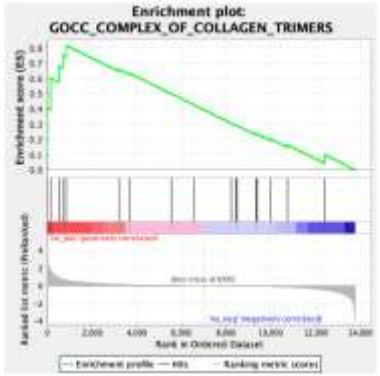


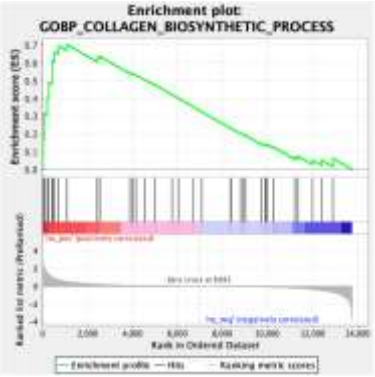


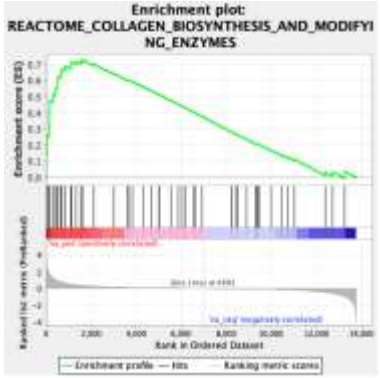


GSEA on the 2D transcriptome following Pearson Correlation test









Alteration of gene sets associated with lysosomal, autophagosomal, oxidoreductase, proteostasis, homeostasis of ER, collagen fibril organization, ATPase-coupled cation transmembrane transporter activities

PLOS ONE

RESEARCH ARTICLE

Transcriptome analysis of skin fibroblasts with dominant negative *COL3A1* mutations provides molecular insights into the etiopathology of vascular Ehlers-Danlos syndrome

Nicola Chiarelli, Giulia Carini, Nicoletta Zoppi, Marco Ritelli, Marina Colombi*

Department of Molecular and Translational Medicine, Division of Biology and Genetics, University of Brescia, Brescia, Italy

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Deciphering disease signatures and molecular targets in vascular Ehlers-Danlos syndrome through transcriptome and miRNome sequencing of dermal fibroblasts

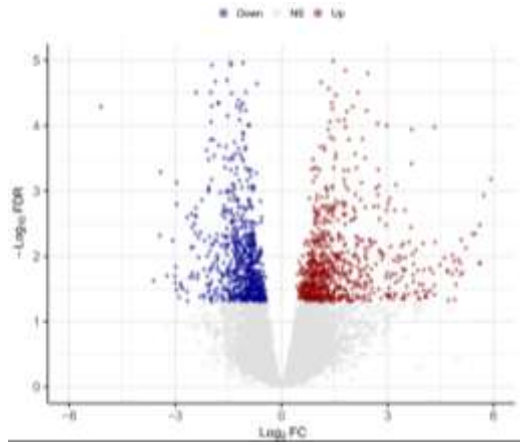
Nicola Chiarelli [a,*], Valeria Cinquina [a], Paolo Martini [a], Valeria Bertini [a], Nicoletta Zoppi [a], Marina Venturini [b], Marco Ritelli [a], Marina Colombi [a]

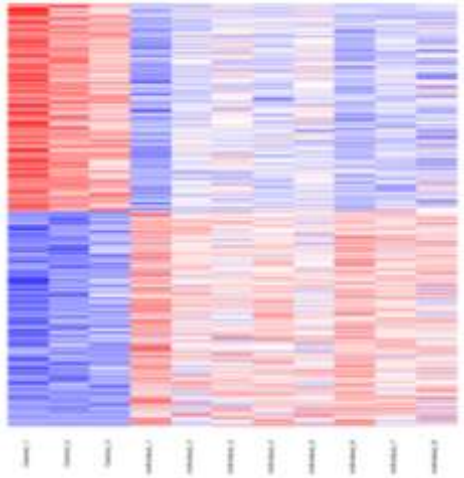
[a] *Division of Biology and Genetics, Department of Molecular and Translational Medicine, University of Brescia, 25121 Brescia, Italy*
[b] *Division of Dermatology, Department of Clinical and Experimental Sciences, Spedali Civili University Hospital Brescia, 25121 Brescia, Italy*

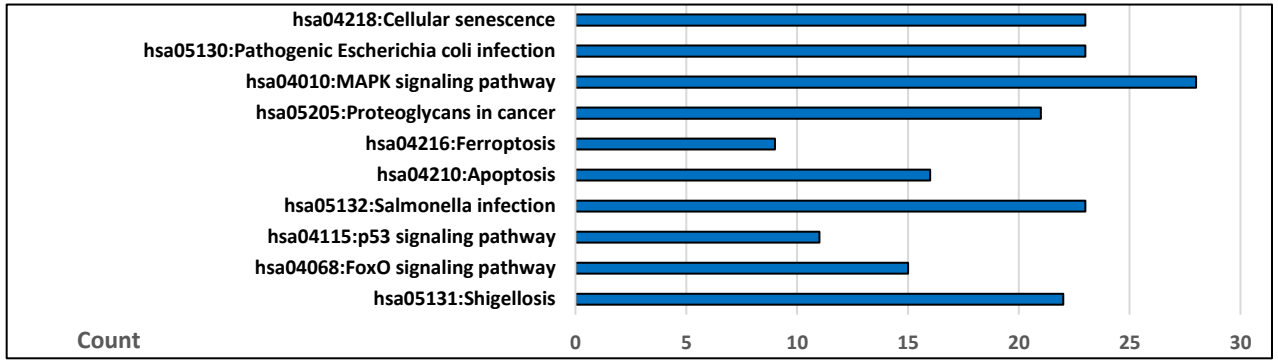
Overview of 3D Transcriptomic Data1

FC = 2,5
FDR ≤0,05

1.494 DEG

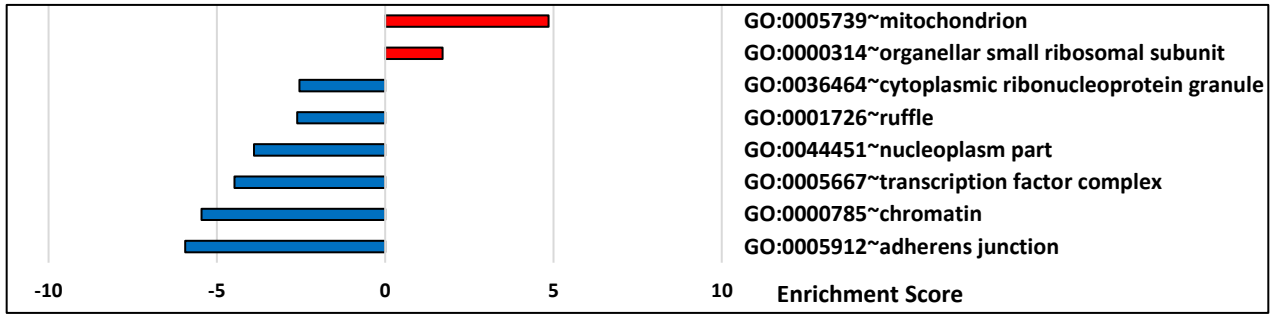




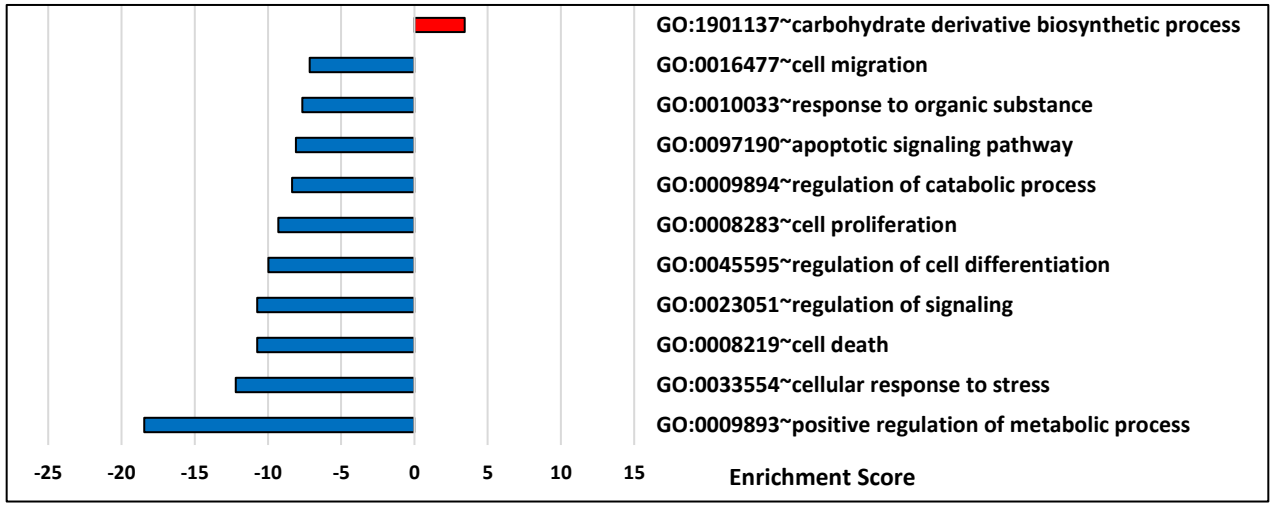


KEGG Pathways

GOEA



Cellular Component



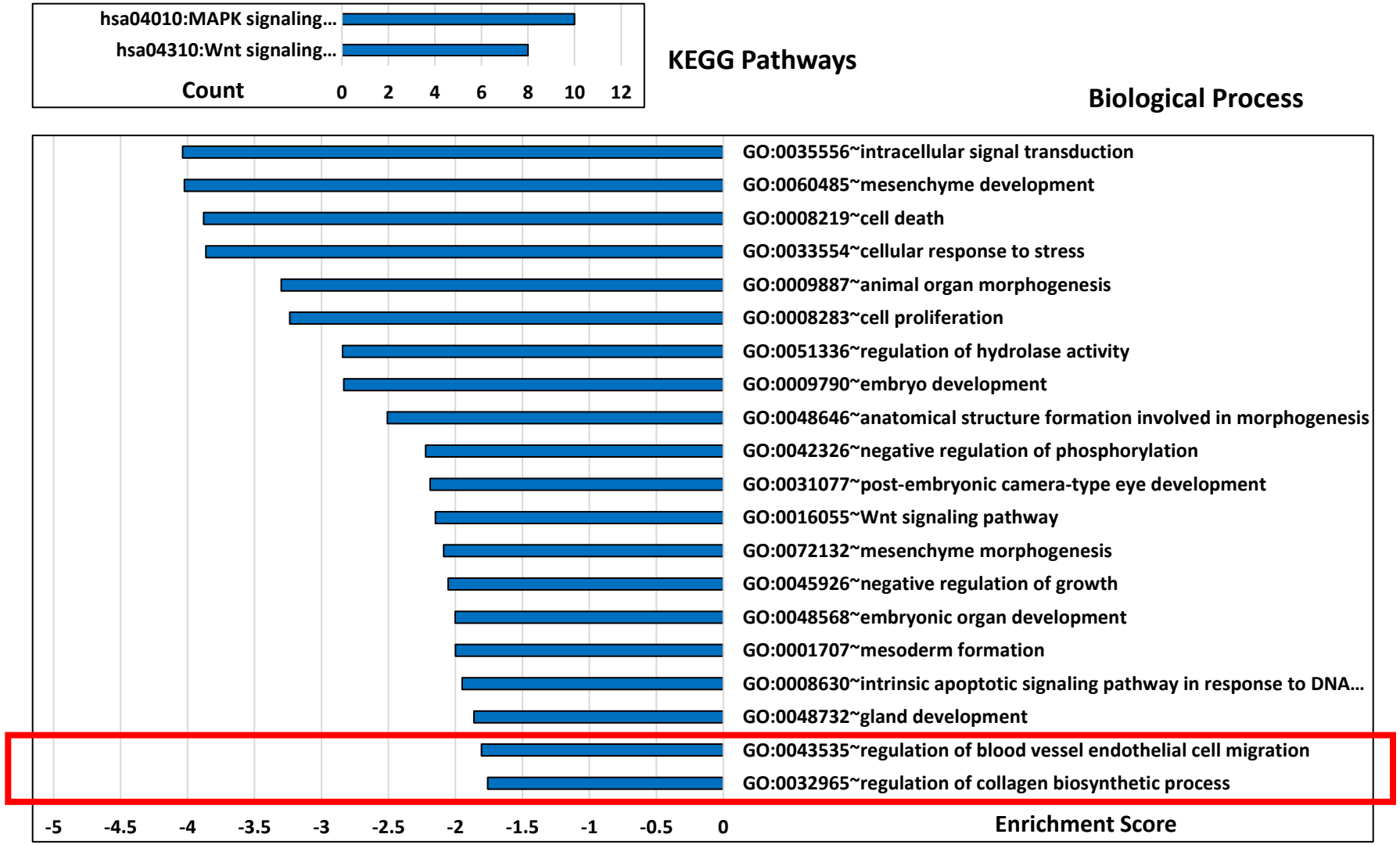
Biological Process

Overview of 3D Transcriptomic Data2

FC 1.5
FDR < 0,05

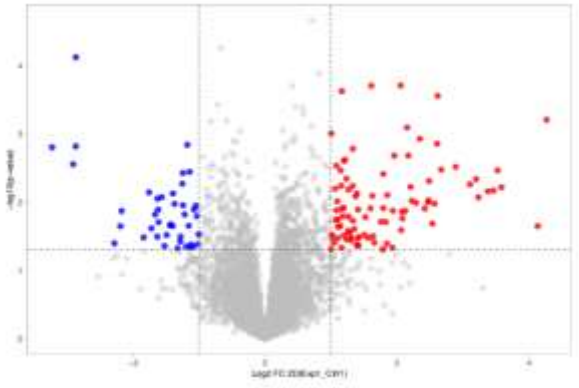
GSEA

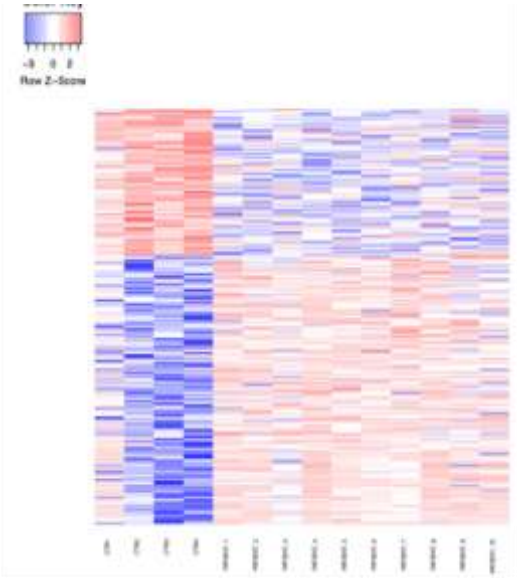
457 DEG

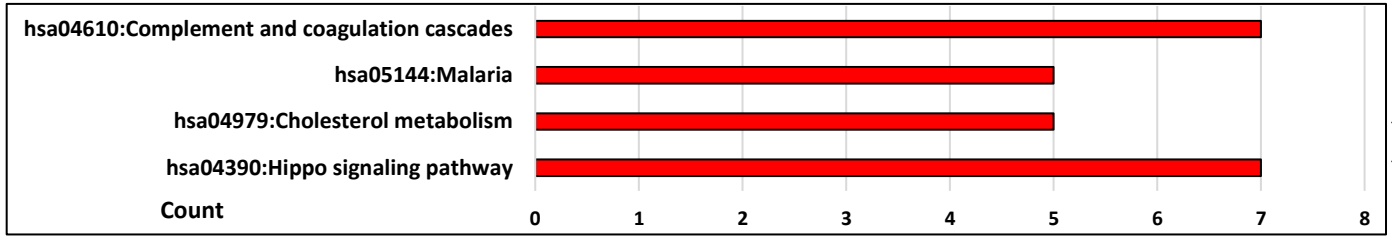


Overview of 2D Proteomic Data

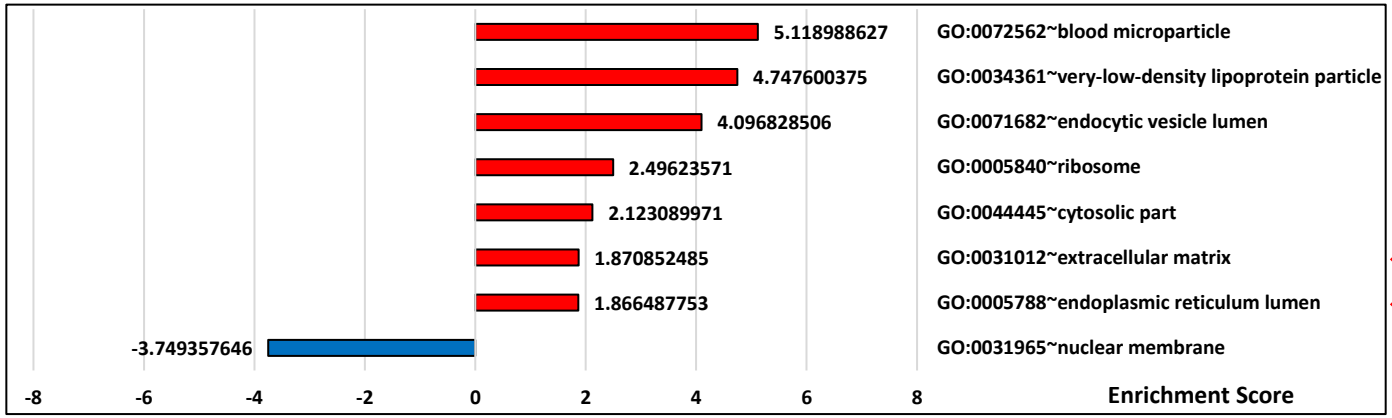
227 DEP



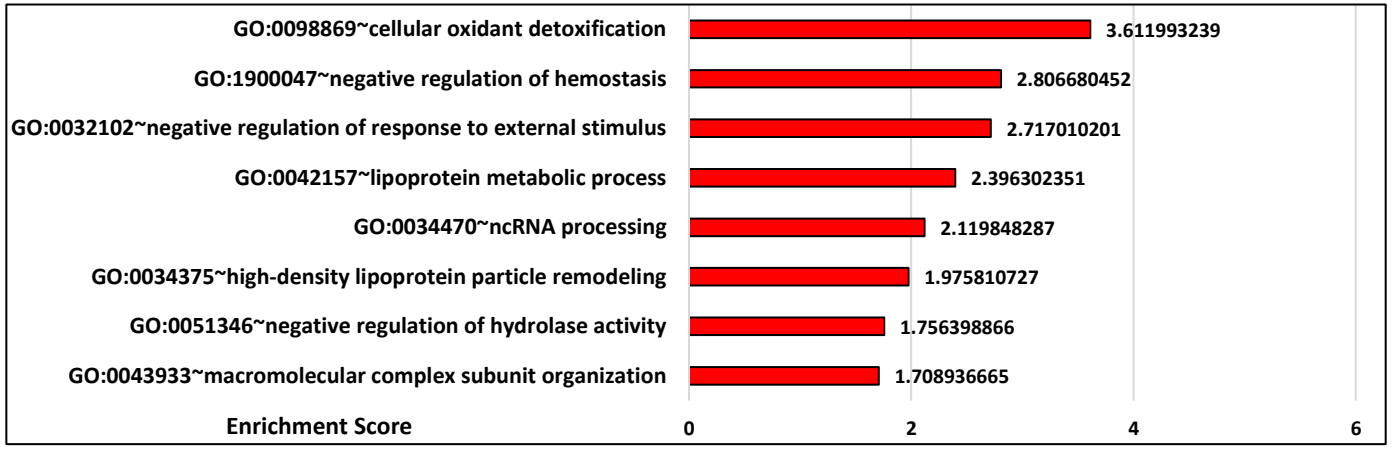




KEGG Pathways



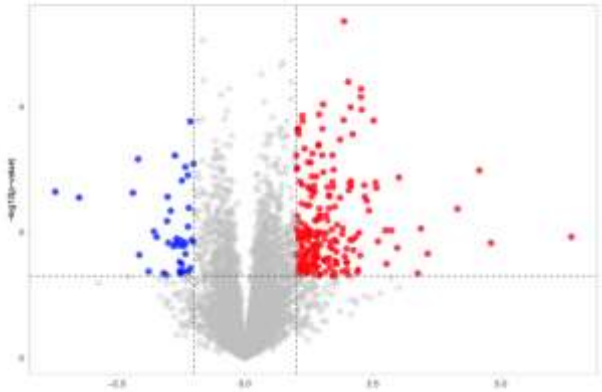
Cellular Component

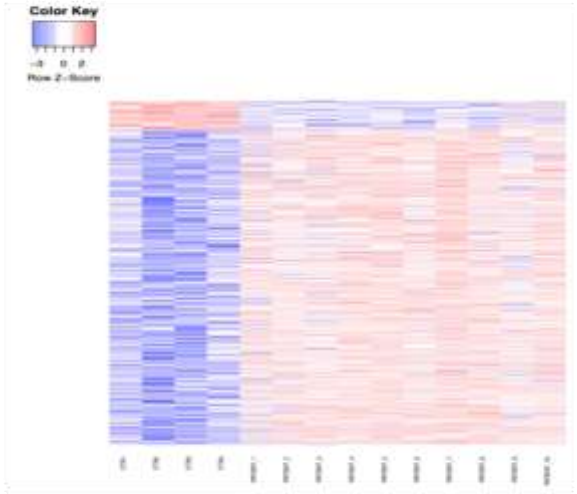


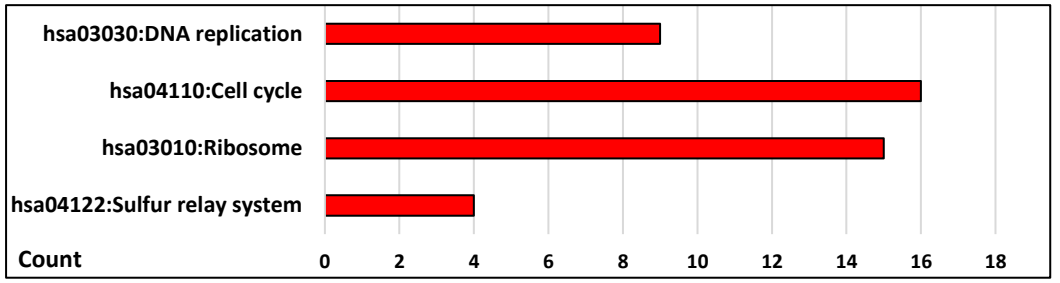
Biological Process

Overview of 3D Proteomic Data

567 DEP

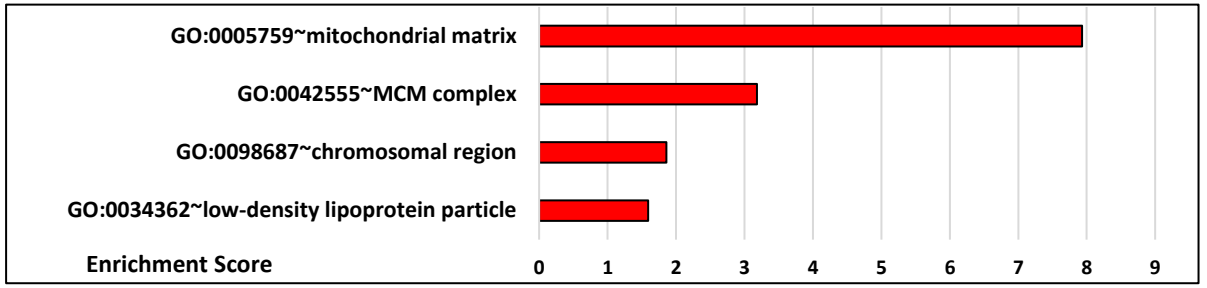






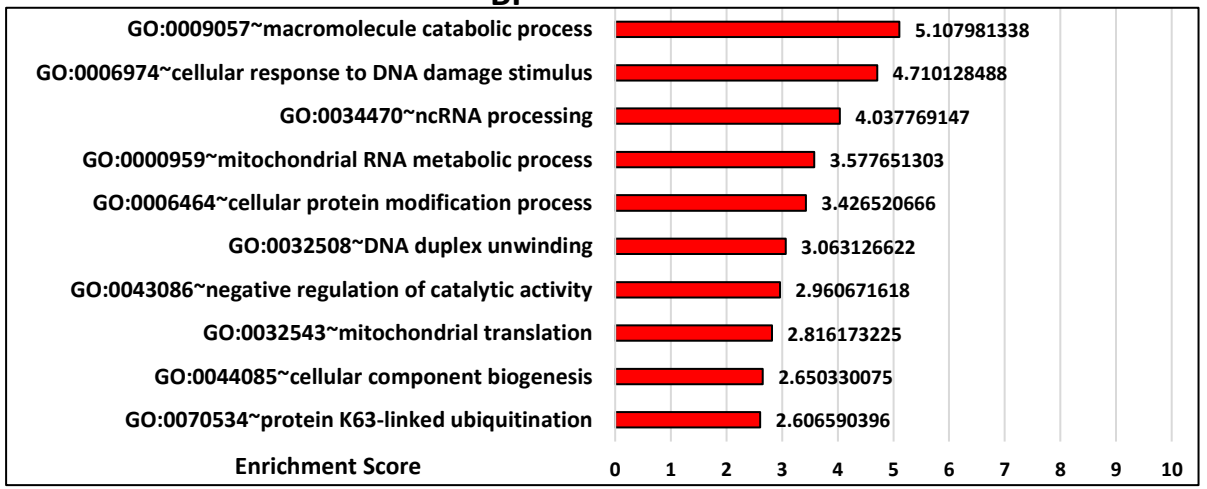
KEGG Pathways

CC



Cellular Component

BP



Biological Process

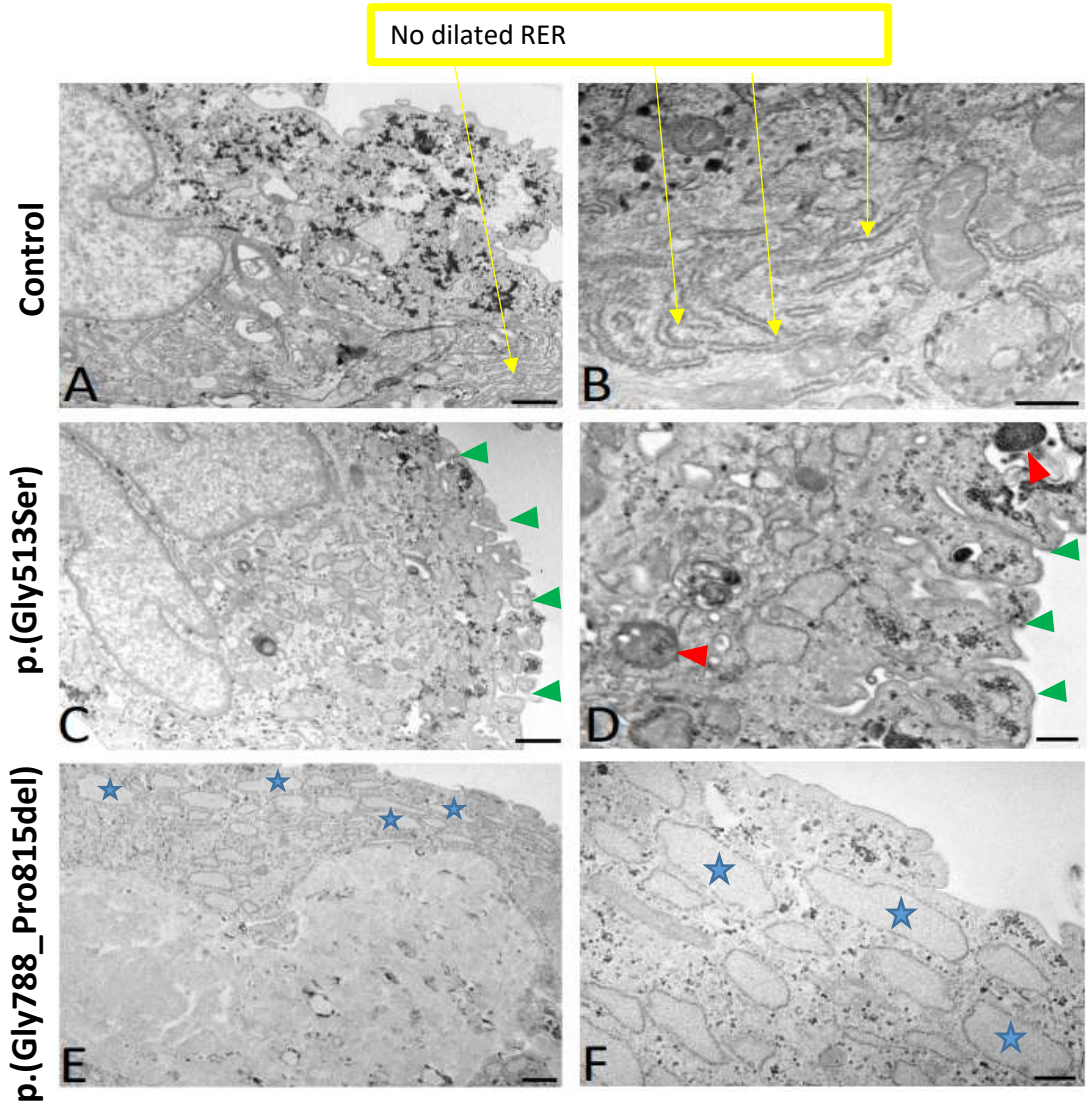
OMICs analysis revealed:

- **Upregulation** of genes related to mitochondrial function, organellar ribosomal subunits, and biosynthesis processes, indicating an **augmented adaptive metabolic response and activation of survival cell mechanisms.**

- **Downregulation** of genes linked to cell senescence, migration, differentiation, and stress response pathways, highlighting dysregulation in **cellular signaling and extracellular matrix maintenance**.

- The pronounced differences observed in the 3D cultures highlight the importance of using more physiologically relevant models to capture the complex molecular landscape of vEDS.

2D Ultrastructural Analysis images by TEM

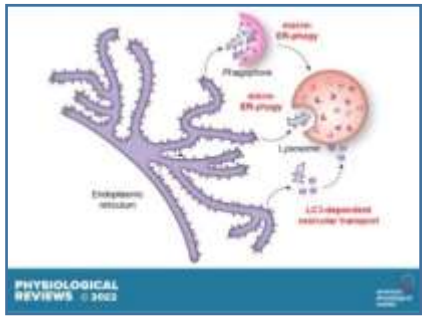


Scale bars A, C, E = 500nm;
B, D, F = 200 nm

- ❑ **Cell membrane modifications**, characterized by protrusions, suggesting altered cell-cell interactions.
- ❑ **Moderate increase in lipofuscin granules**, indicating altered functionality of mitochondria, impairment of proteasomal and autophagic-lysosomal pathways, elevations in protein oxidation.
- ❑ **Altered rough endoplasmic reticulum morphology**, which appears dilated and containing tubular-type aggregations in a matrix with low electron density.
- ❑ **Spherical to slightly oval cellular elements that aggregate into small clusters**, documenting changes in cellular behaviour and morphology.

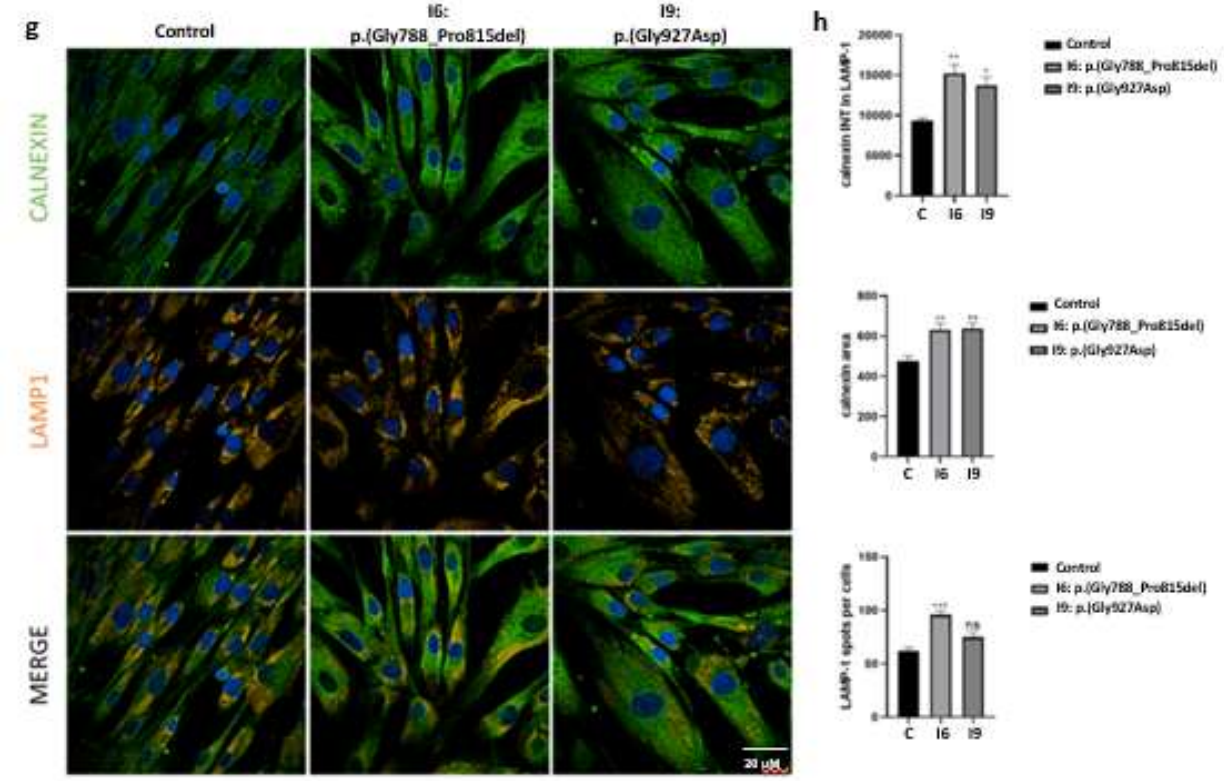
2D Immunofluorescence Analysis and High Content Imaging Analysis revealed activation of ER-phagy

ER-phagy?



ER turnover and remodeling via ER-phagy under basal and stress conditions

BafA1

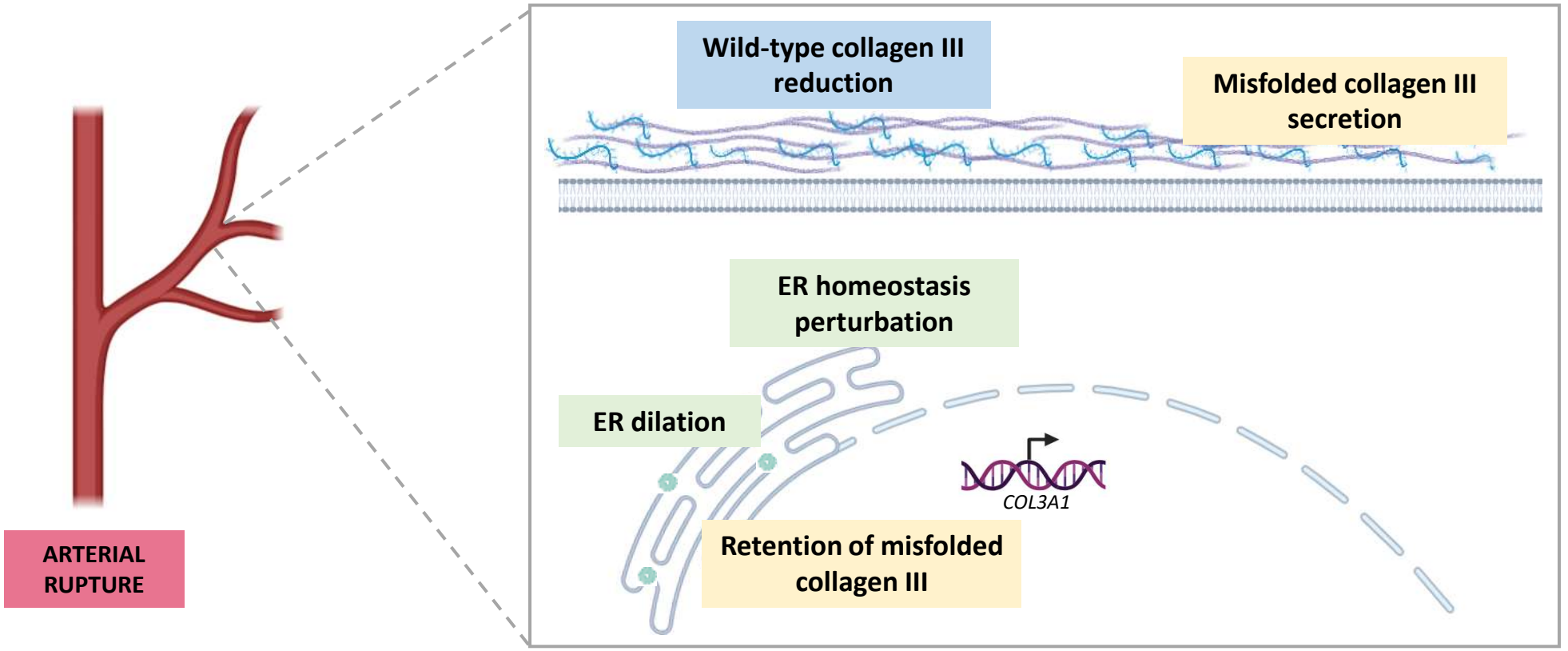


- The analysis reveals significant variations in Calnexin and LAMP-1 levels between control and patient fibroblasts
- Calnexin levels are significantly elevated in patients' cell lines suggesting **increased ER stress** and potential disruptions in protein quality control.
- LAMP-1 levels are notably higher, pointing to **enhanced selective autophagy (ER-phagy)**.

Conclusions

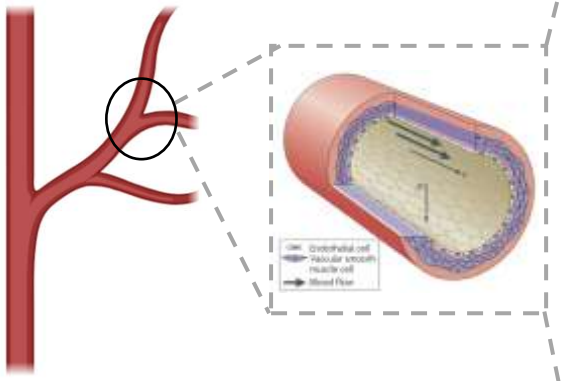
Upstream mechanisms

COL3A1 mutations act via **quantitative** and **qualitative** effects by reducing levels of extracellular collagen III coupled with secreting mutant less stable collagen III



Conclusions

Downstream mechanisms



ARTERIAL RUPTURE

ECM disorganization

Downregulated | **Altered signaling** | *Upregulated*

Rap1 · Wnt · MAPK · p53 · FoxO · cGMP-PKG

Cholesterol metabolism · DNA replication · Hippo signaling · Cell cycle

ER-phagy pathway

Altered proteostasis

Defective autophagy pathway

COL3A1

Cellular oxidant detoxification

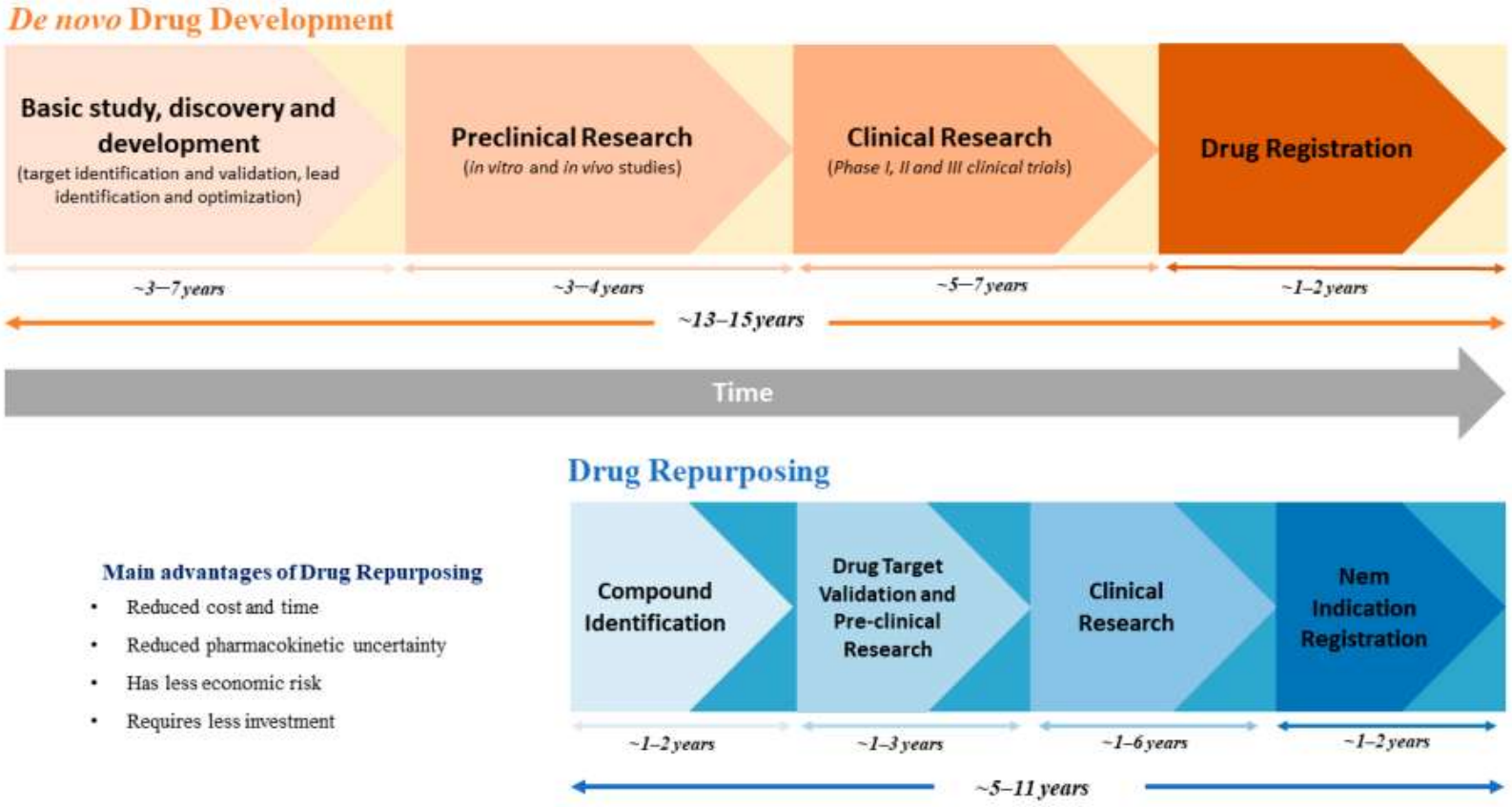
Senescence

Apoptosis

Cholesterol metabolism

Altered processes ➡ vascular fragility ➡ direct or indirect control of VSMC ➡ vessels relax and contract ➡ blood flow and pressure regulation ➡ vascular rupture predisposition

Drug repurposing approach to identify therapeutic molecules for vEDS



Pharmaceuticals 2021, 14, 280. https://doi.org/10.3390/ph14030280

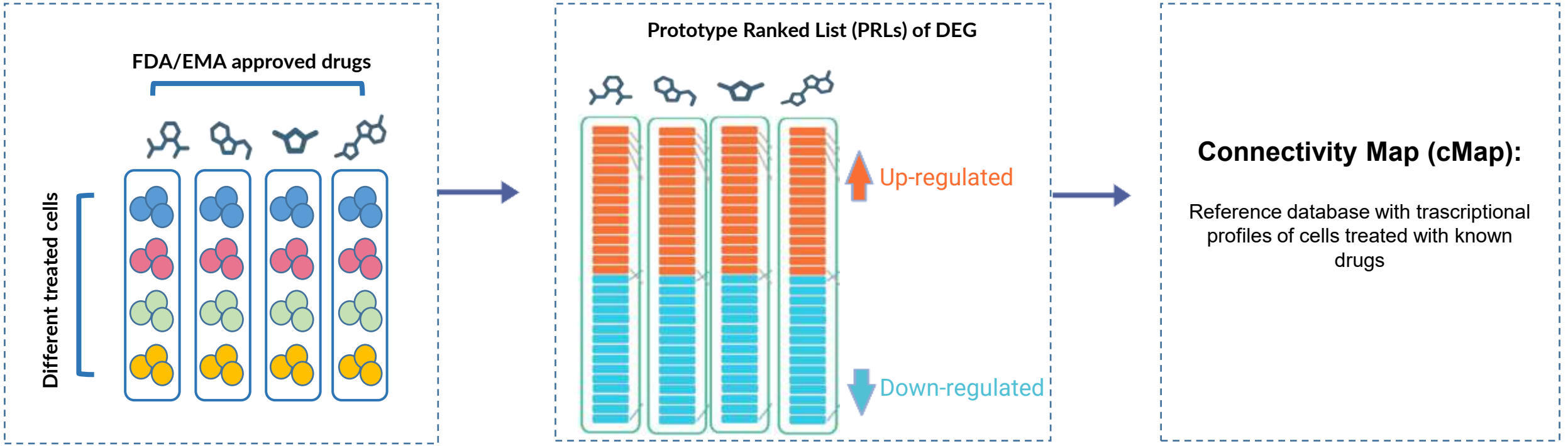
Systems and Synthetic Biology lab

Drug Repurposing

Discovery of drug mode of action and drug repositioning from transcriptional responses

Francesco Iorio[a,b], Roberta Bosotti[c], Emanuela Scacheri[c], Vincenzo Belcastro[a], Pratibha Mithbaokar[a], Rosa Ferriero[a], Loredana Murino[a], Roberto Tagliaferri[b], Nicola Brunetti-Pierri[a,d], Antonella Isacchi[c,†], and Diego di Bernardo[a,b,†]

[a]TeleThon Institute of Genetics and Medicine, Naples, Italy; [b]Department of Biotechnology, Nerviano Medical Sciences, Milan, Italy; [c]Department of Systems and Computer Science, [d]Federico II" University of Naples, Naples, Italy; [e]Department of Pediatrics, [f]Federico II" University of Naples, Naples, Italy; and [g]Department of Mathematics and Computer Science, University of Salerno, Salerno, Italy

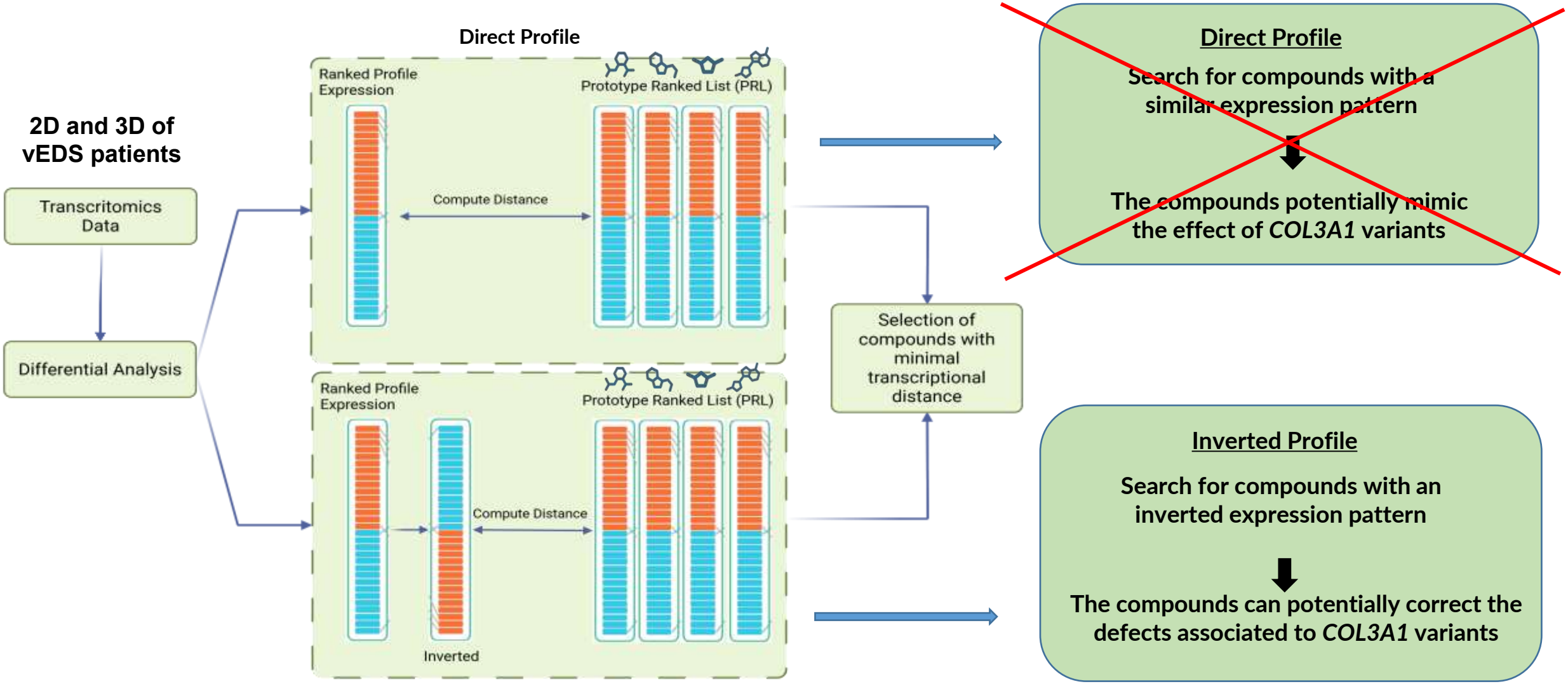


Connectivity Map (cMap):

Reference database with trascriptional profiles of cells treated with known drugs

Drug Repurposing on 2D and 3D Transcriptomics data

MANTRA (Mode of Action by NeTwoRk Analysis), a computational approach implemented by TIGEM



Kindly provided by Ettore Augello

Experimental validation of candidate drugs

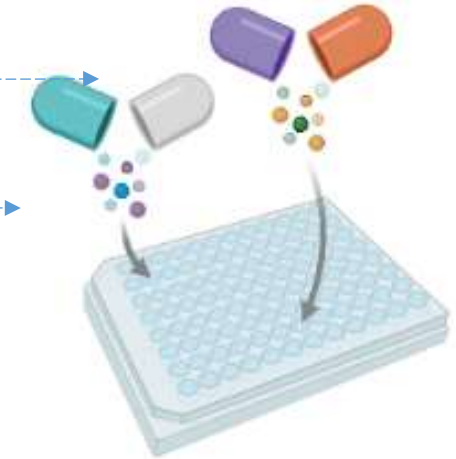
PRELIMINARY DATA

3D Inverted Profile Identification of candidate drugs

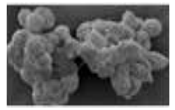
- Nocodazole
- Ouabain
- C-75
- Helveticoside
- Digoxin
- Beta-escin
- Niclosamide
- Piperlongumine

IN PROGRESS

Experimental validation on patient's cells

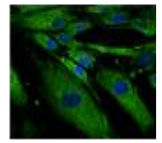


Functional analysis to verify a reverted transcriptome signature



Electron Microscopy

Immunofluorescence



WTS

Bioinformatic analysis

DEGs/DEPs analysis

Multi-OMIC integration

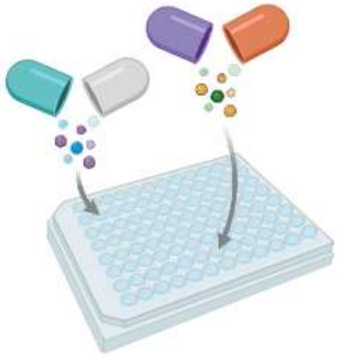
Reverted phenotype?

Human experimentation

Work in progress……

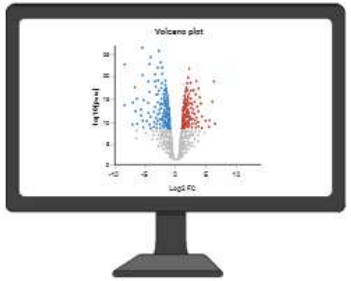
Therapeutics validation

Experimental drugs validation



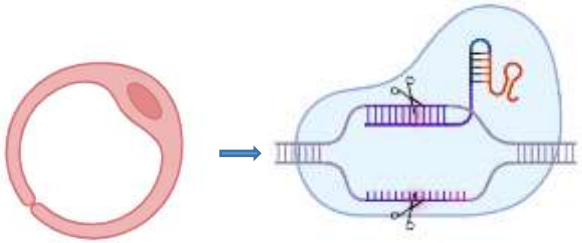
vEDS Pathogenesis

Secretome analysis



Cellular model for vEDS

HUVEC-CRISPR/Cas9



Human iPSC-derived VSMC

……Looking for funding

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Beta-escin:.
è una saponina, si trova principalmente nei semi dell'ippocastano. È nota per le sue proprietà antiossidanti, vasoprotettive, vasocostrittrice, drenanti, disintossicanti, antiedemigene e stimolanti del microcircolo. Viene utilizzata per ridurre gonfiori e migliorare la circolazione, e in farmaci per il trattamento di fragilità capillare, emorroidi e vene varicose **Beta-escin:** la forma β dell'escina è stata descritta per i suoi effetti antinfiammatori attraverso la soppressione dei prodotti genici di proliferazione, apoptotici e metastatici regolati dall'NF-κB. Nelle cellule HUVEC o ECV304, la β-escina sodica (10, 20 o 40 µg/mL) ha inibito in modo dose-dipendente la proliferazione delle cellule endoteliali e a 40 µg/mL ha anche indotto l'apoptosi delle cellule endoteliali.
DOI https://doi.org/10.1038/s41577-022-00792-3 (Death by TNF: a road to inflammation)

https://doi.org/10.1016/j.vph.2008.07.005 **(Effect of β-escin sodium on endothelial cells proliferation, migration and apoptosis)**
The results indicate **that β-escin sodium reduced proliferation of ECs.** The dose-dependent inhibition of β-escin sodium on HUVECs proliferation was same as that on ECV304 cells. Our data also suggested that β-escin sodium induced apoptosis both in HUVECs and ECV304 cells. (inhibition of proliferation and induction of apoptosis; suppression of adhesion molecule expression). Our results suggest that β-escin sodium inhibits angiogenesis by depressing ECs proliferation and migration, and by inducing ECs apoptosis. Western blot also implicate that TSP-1 and the signal proteins: PKC-α, ERK, p38 may be involved in the effects of β-escin sodium on ECs. The comprehensive effects of β-escin sodium on angiogenesis need further research. Our findings give the first evidence of the direct effect of β-escin sodium on ECs proliferation, migration and apoptosis.
doi: 10.3892/or.2021.8006 **(Escin inhibits angiogenesis by suppressing interleukin-8 and vascular endothelial growth factor production by blocking nuclear factor-κB activation in pancreatic cancer cell lines).** These results indicated that escin inhibited angiogenesis by reducing the secretion of IL-8 and VEGF by blocking NF-κB activity in PaCa. In conclusion, escin could be used as a novel molecular therapy for PaCa.

C-75: inibitore della sintesi degli acidi grassi (FAS), riduce la sintesi dei lipidi.

C-75 and endothelial cells

https://doi.org/10.1161/01.RES.84.6.688 **(Identification of Endothelial Cell Binding Sites on the Laminin γ1 Chain)**

Peptides C25, C38, C75, and C102 have not been previously reported to possess any type of biological activity when tested in full peptide screens for adhesion or differentiation of various nonendothelial cell lines.10 These peptides, however, disrupted tube formation, promoted endothelial cell adhesion, and induced angiogenesis from vascular explants. Such cell-type specificity for endothelial cells is not observed with peptides from the α1 and β1 chains.22 More than 679 peptides from all 3 laminin-1 chains have been screened now in our laboratory with endothelial cells as well as with other cell types, and only these 4 γ1 chain peptides are active with endothelial cells and not with either HSG, B16F10, or PC12 cells.

Helveticoside: simile a Ouabain e Digoxin. Modula la segnalazione del calcio.

Piperlongumine: Questo composto è selettivamente citotossico contro le cellule tumorali attraverso l'induzione dello stress ossidativo, induce genotossicità, come strategia alternativa per uccidere le cellule tumorali, ha un'eccellente biodisponibilità orale nei topi, inibisce la crescita tumorale nei topi e presenta solo una debole tossicità sistemica.

Piperlongumine and vascular smooth cells

https://doi.org/10.1016/j.bbrc.2012.09.061 **(Piperlongumine inhibits atherosclerotic plaque formation and vascular smooth muscle cell proliferation by suppressing PDGF receptor signaling)**

We therefore further investigated the inhibitory mechanism of the anti-proliferative effect exerted by PL on PDGF-BB-induced VSMC proliferation via PDGF-R signaling pathway. Activation of downstream signals of PDGF-Rβ, such as ERK1/2, Akt and PLCγ1, appears to be essential for the VSMC proliferation [10], [11], [43]. PL potently inhibited PDGF-Rβ phosphorylation (Fig. 3A), and also inhibited the PLCγ1 (Fig. 3B), ERK1/2 (Fig. 3C) and Akt (Fig. 3D) phosphorylation. These results suggest that PL may directly target PDGF-Rβ phosphorylation leading to the inhibition of VSMC proliferation.

Digoxin

Viene utilizzato per aumentare la forza di contrazione delle fibre miocardiche sia atriali che ventricolari, determinando così un effetto inotropo positivo.

DOI: 10.1111/j.1600-0897.2011.01055.x (Digoxin immune fab protects endothelial cells from ouabain-induced barrier injury)

DIF protects ECs from ouabain-induced barrier injury, providing evidence of beneficial effects of DIF on EC function and supporting that Na+ /K+ATPase might be a therapeutic target to ameliorate endothelial dysfunction.

Niclosamide

La niclosamide, viene utilizzato per trattare le infezioni da tenia.

DOI: 10.1111/bph.14182 (Niclosamide inhibits vascular smooth muscle cell proliferation and migration and attenuates neointimal hyperplasia in injured rat carotid arteries)

Niclosamide inhibited vascular smooth muscle cell proliferation and migration and attenuated neointimal hyperplasia in balloon-injured rat carotid arteries through a mechanism involving inhibition of STAT3.
https://doi.org/10.1085/jgp.202313460 **(Niclosamide potentiates TMEM16A and induces vasoconstriction)**
Niclosamide potentiates TMEM16A from the extracellular side. In VSMCs expressing endogenous TMEM16A, niclosamide stimulates the TMEM16A, which increases cytosolic Ca2+ and produces vasoconstriction.

Ouabain

A cardiac glycoside similar to digitoxin, **is used to treat congestive heart failure and supraventricular arrhythmias**

Nocodazole: agente antineoplastico che esercita il suo effetto depolimerizzando i microtubuli. Il nocodazolo si lega alla tubulina, la subunità proteica dei microtubuli, impedendone la polimerizzazione. Questa interruzione interessa il fuso mitotico, cruciale per la segregazione dei cromosomi durante la divisione cellulare. L'interferenza con la dinamica dei microtubuli provoca l'arresto delle cellule nella fase G2/M del ciclo cellulare. Questo arresto avviene perché il fuso mitotico non può formarsi correttamente, arrestando la mitosi. L'impossibilità di procedere attraverso la mitosi innesca una risposta di checkpoint, mettendo in pausa il ciclo cellulare per prevenire errori nella segregazione dei cromosomi, che potrebbero portare ad aneuploidie e a trasformazioni potenzialmente oncogene.

doi: 10.1134/S1990747808020049 **(Dose-Dependent Effect of Nocodazole on Endothelial Cell Cytoskeleton)**

The results obtained can be summarized as follows. At 100 nM, nocodazole causes partial disruption of the microtubule network at the cell periphery without any notable effect on the population of stress fibers and insignificant transient increase in vascular endothelium permeability. At 200 nM, nocodazole initiates significant depolymerization of dynamic microtubules and formation of additional stress fibers in the internal cytoplasm; these changes are consistent with stable dysfunction of endothelial cells. This suggests that microtubules are indeed the first effector link in the development of vascular endothelium dysfunction.

doi: 10.1126/scisignal.aan2694 **(Microtubule structures underlying the sarcoplasmic reticulum support peripheral coupling sites to regulate smooth muscle contractility)**

In contrast, our findings showed that nocodazole treatment did not alter vasoconstriction induced by increased extracellular K+ or a Gαq protein receptor agonist, suggesting that this treatment did not affect Ca2+ sensitization. These apparent discrepancies could be attributable to heterogeneity in the influence of Ca2+-activated ion channel activity among vascular beds, such that BK channels have a higher open probability and a larger effect on contractile responses in cerebral arteries than in skeletal muscle arteries

doi.org/10.1074/jbc.M310721200 **(Calphostin-C Induction of Vascular Smooth Muscle Cell Apoptosis Proceeds through Phospholipase D and Microtubule Inhibition)**

This cell response is similar to what we observed in response to microtubule-disruptive agents such as nocodazole, which also induces VSMC apoptosis.

Ouabain: Glicoside cardioattivo composto da ramnosio e ouabagenina, ottenuto dai semi di Strophanthus gratus e di altre piante delle Apocynaceae; usato come la digitale. È comunemente usato negli studi di biologia cellulare come inibitore dell'ATPasi di scambio NA(+)-K(+). La proliferazione cellulare dipendente dall'ouabain è stata riscontrata anche in altri tipi di cellule, tra cui le cellule endoteliali della vena ombelicale umana (HUVEC), le cellule muscolari lisce vascolari di bovino, canino e ratto. Nelle piccole arterie mesenteriche di ratto, mantenute in vitro, l'ouabain regola la comunicazione intercellulare, riducendo la vasomozione indotta dalla noradrenalina e desincronizzando i transienti di Ca2+ nelle cellule. Queste azioni vascolari dell'ouabaina rappresentano uno dei fattori che contribuiscono alle azioni ipertensive di questo cardenolide. (Cancers 2020, 12(12), 3840; https://doi.org/10.3390/cancers12123840

Ouabain and vascular smooth cells

https://doi.org/10.1074/jbc.M106178200 **(Ouabain-induced Signaling and Vascular Smooth Muscle Cell Proliferation)**

We observed at 10−10 and 10−9mthat ouabain induced MAPK42/44 activation, DNA synthesis, and proliferation in VSMCs. The increase in DNA synthesis and proliferation effects disappeared with increased ouabain concentrations, but a small increment in MAPK activation remained. Considering the limited number of pump sites and expression of only one isoform of the sodium pump in these cells (15), this finding suggests that at higher concentrations the pump-inhibitory effect of ouabain might interfere with its proliferative effect.

DOI: 10.1590/s0100-879x1997000400016 **(Effects of ouabain on vascular reactivity)**

The results showed that ouabain pretreatment increased the vasopressor responses to PE in vitro and in vivo. This sensitization after ouabain treatment was also observed in hypertensive animals which presented an enhanced vasopressor response to PE in comparison to normotensive animals. It is suggested that ouabain at nanomolar concentrations can sensitize vascular smooth muscle to vasopressor stimuli possibly contributing to increased tone in hypertension.