SISET

Workshop

SISET

Società Italiana
per lo Studio dell'Emostasi
e della Trombosi

POST-ISTH:
Novità dal meeting
di Toronto 2015

29-30 Gennaio 2016
Ospedale Papa Giovanni XXIII
Bergamo

Biologia vascolare

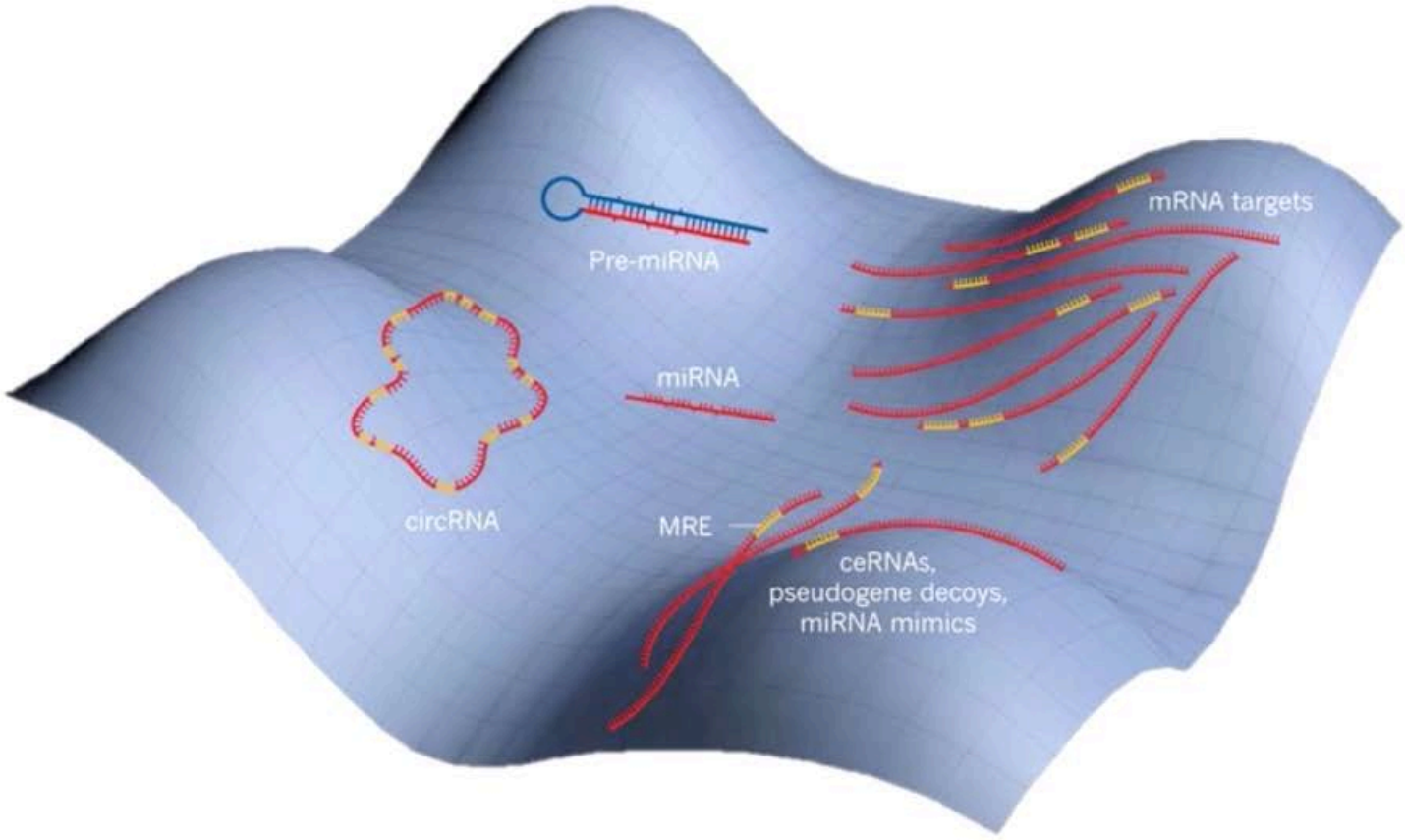
Marina Camera

Università degli Studi di Milano
Centro Cardiologico Monzino IRCCS, Milano

Conflitto d’interessi

Nessuno

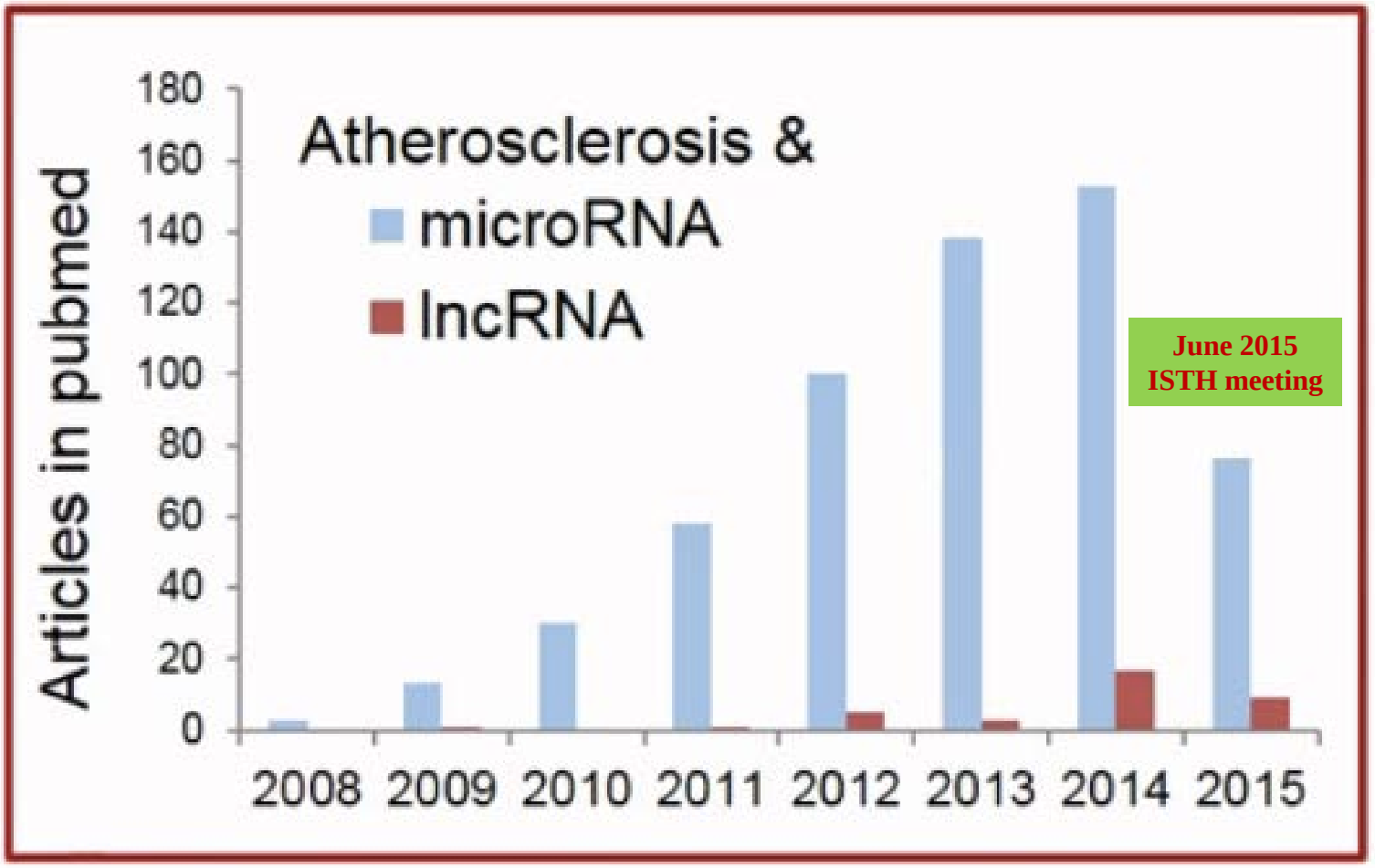
The RNA world



(taken from Kosik Nature 495:322-324, 2013)

Aim: understand the biology of RNAs and develop novel RNA therapeutics for the treatment of cardiovascular disease

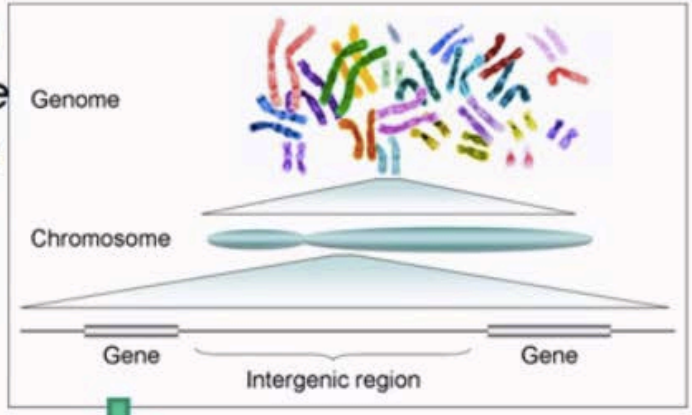
Non coding RNAs and Atherosclerosis



Non-coding DNA & RNA and microRNAs

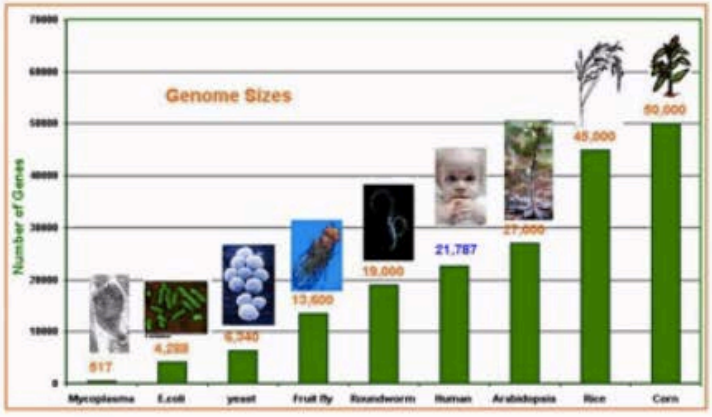
Human Genome Project: Craig Venter, 2003

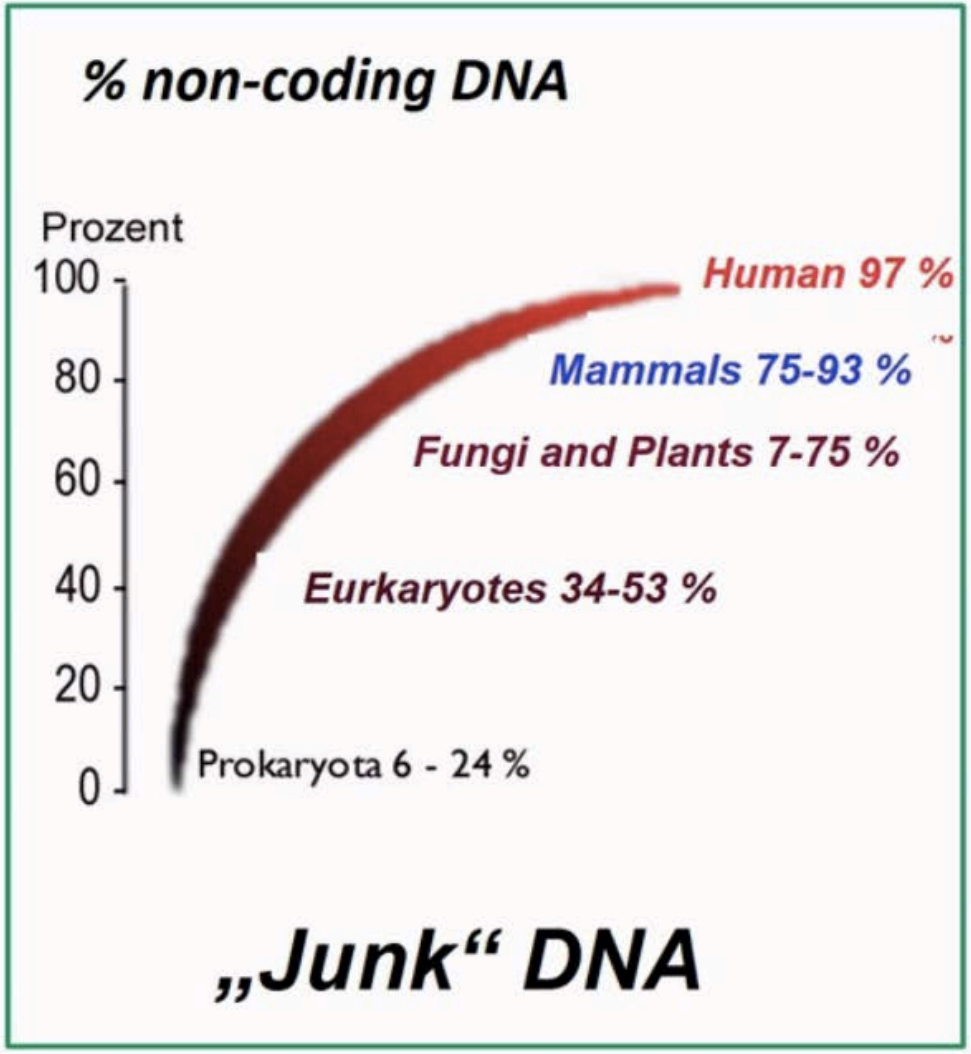
$3x10^9$ bp



Proteins

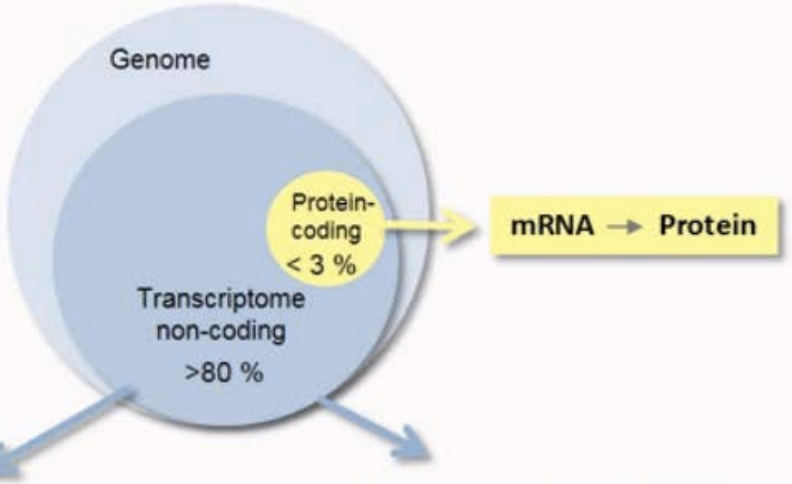
Human Genome: ca. 25.000 Genes
(only 2 x more than worms or flies)





Source: Wikipedia

Non-coding RNAs



Small non-coding RNAs
"microRNAs"

- <200nt
- transcribed by RNA Polymerase II
- endogenously processed by endonucleases
- well conserved

> 2,000 microRNAs

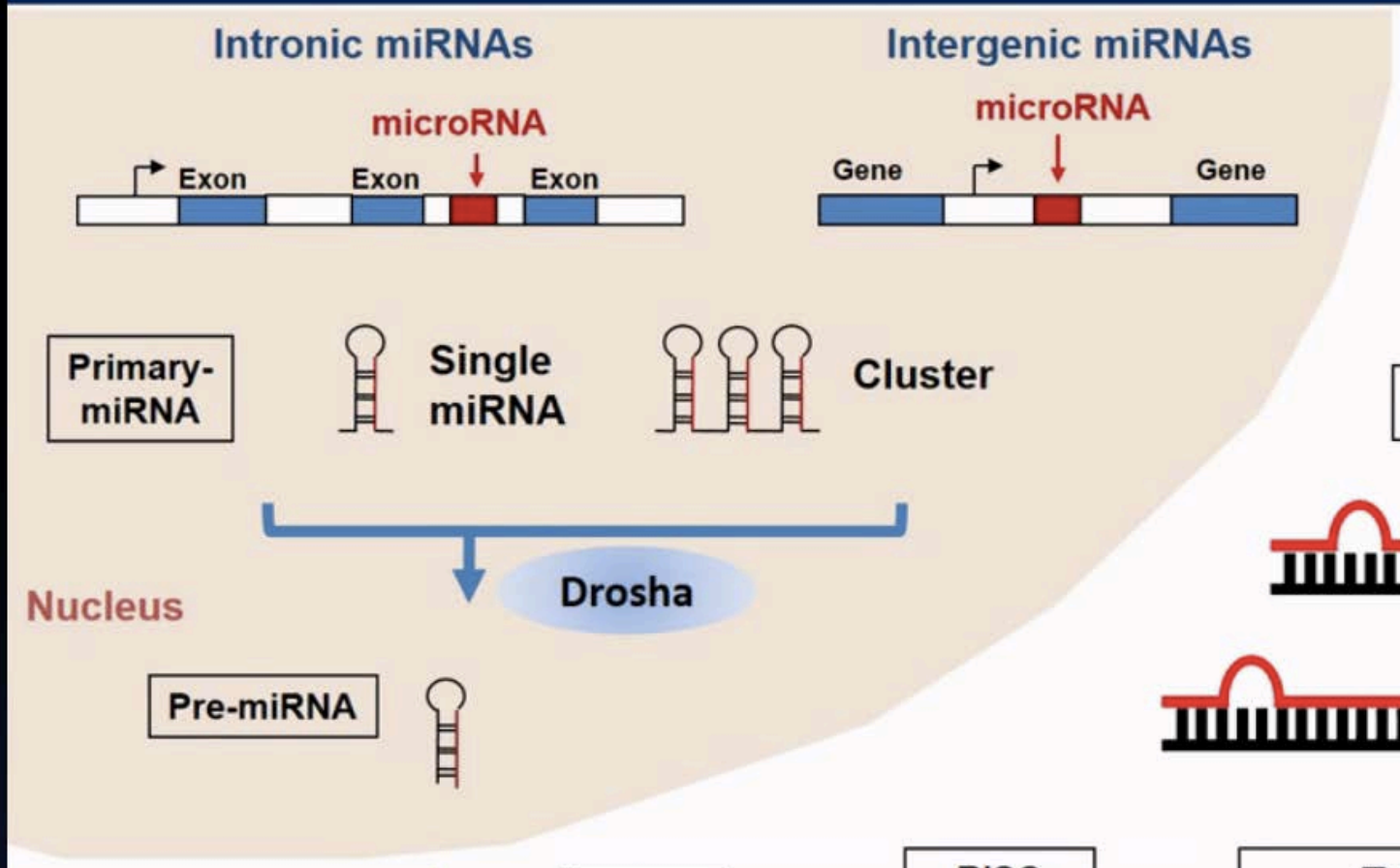
Long non-coding RNAs
"lncRNAs"

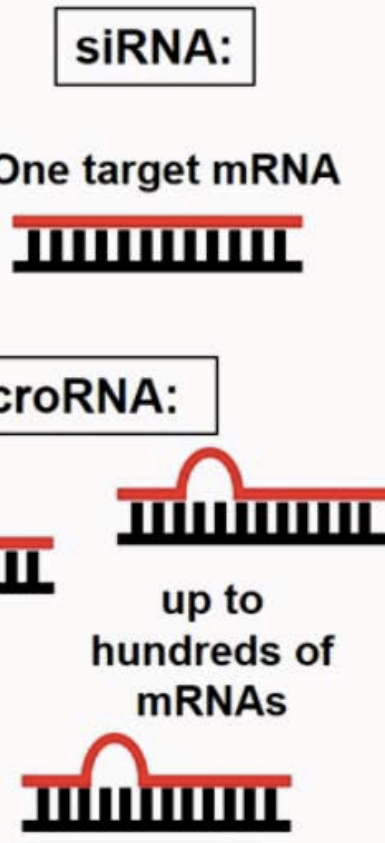
- >200nt
- transcribed by RNA Polymerase II
- mostly 5'-cap, polyadenylated (in part), spliced
- poorly conserved

> 30,000 lncRNAs

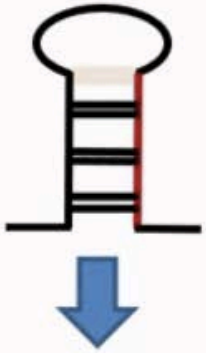
Uchida/Dimmeler Circ Res 2015

microRNAs: Processing and functions





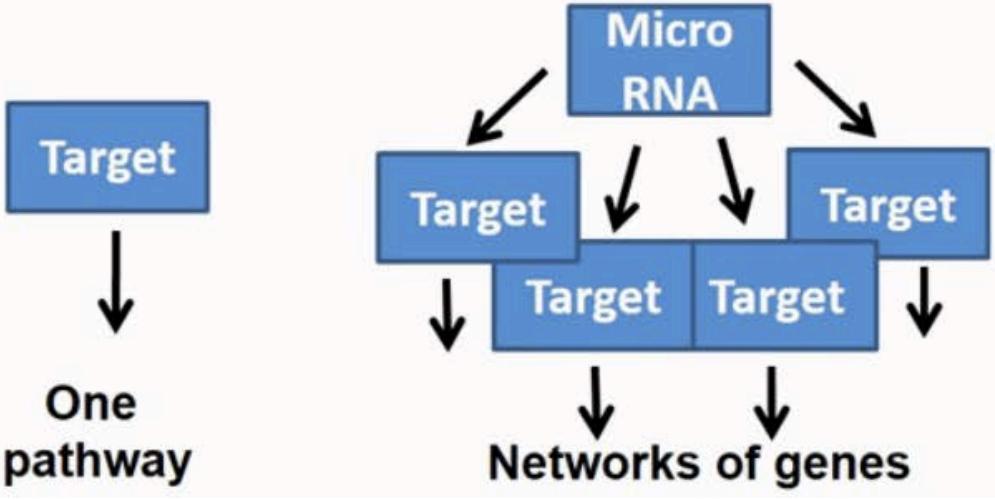
microRNA functions



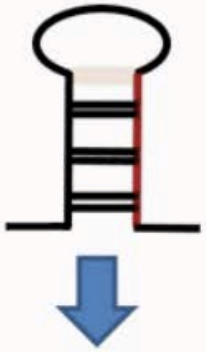
Therapeutic option

Pharmacological/ Gene therapy

microRNA therapy

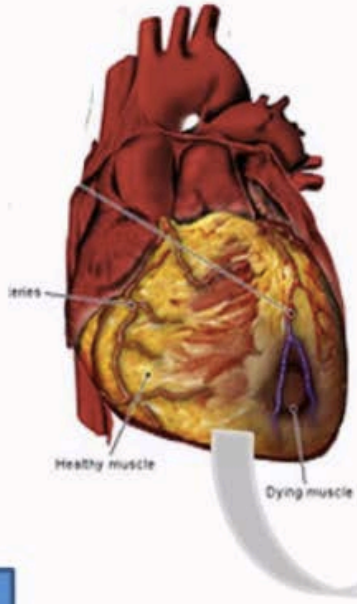


microRNA functions



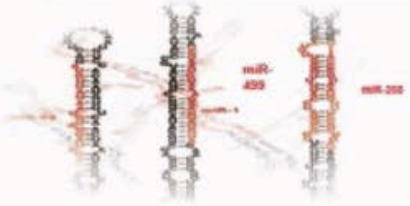
Biomarker

Heart Attack



Cardiac-specific miRNA:
miR-208a

Muscle enriched miRNAs:
miR-1
miR-133a/b
miR-499



Release of miRNAs

Therapeutic option

Pharmacological/ Gene therapy

Target

One pathway

microRNA therapy

Micro RNA

Target

Target

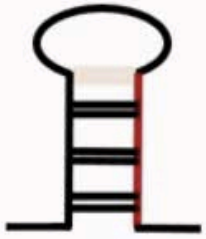
Target

Target

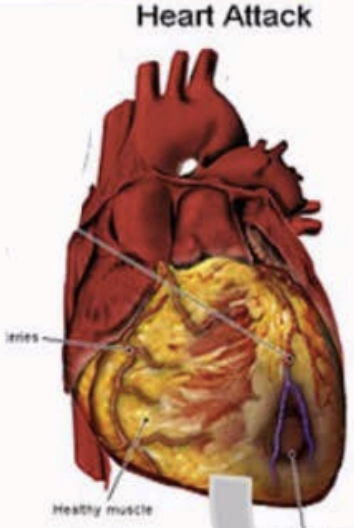
Target

Networks of genes

microRNA functions



Biomarker



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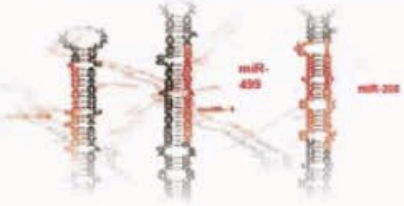


Release of miRNAs

Therapeutic option

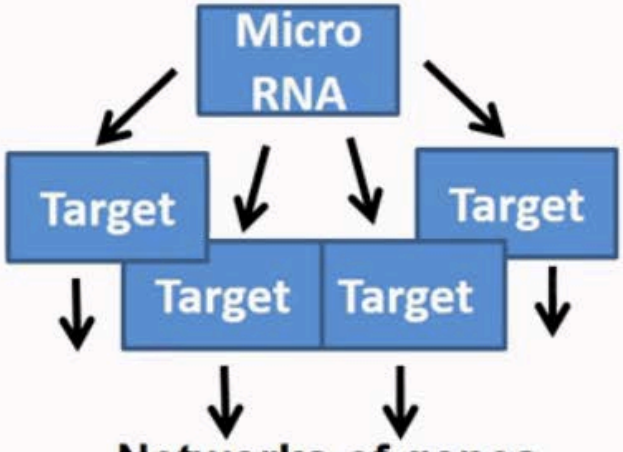
Pharmacological/ Gene therapy

Target

↓

One pathway

microRNA therapy

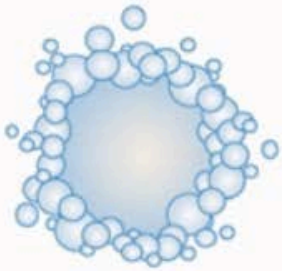


Networks of genes

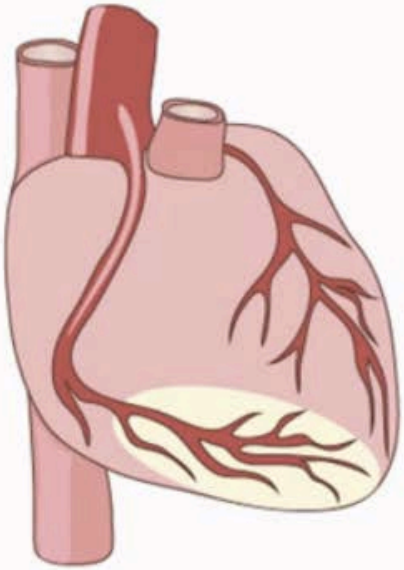
- Circulating miRNAs can be platelet-derived
- Heparin and anti-thrombotic therapies influence (the detection of) circulating miRNA levels

MicroRNAs and postinfarction repair & regeneration

Cell death



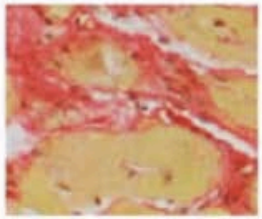
miR-15
miR-24
miR-34
miR-214
(calcium overload)



Cardiomyocyte proliferation

miR-15
miR-195
miR-199
miR-590

Fibrosis

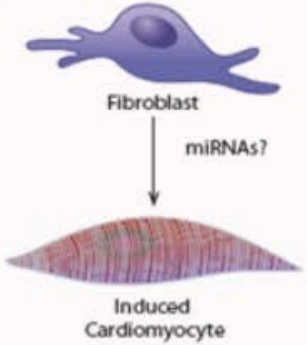


miR-21
miR-29
miR-101

Angiogenesis

miR-15
miR-24
miR-92a

Cardiac reprogramming



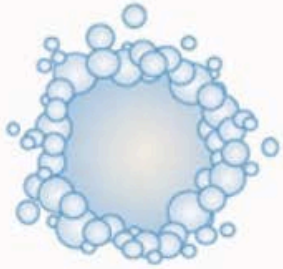
miR-1
miR-208
miR-499

Seeger et al, ATVB 2013

MicroRNAs and postinfarction repair & regeneration

Targeting the vascular niche for repair (and regeneration?)

Cell death



miR-15
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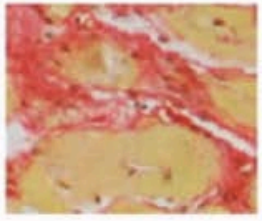

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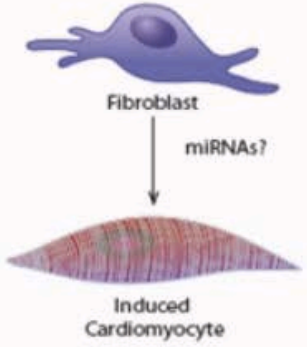
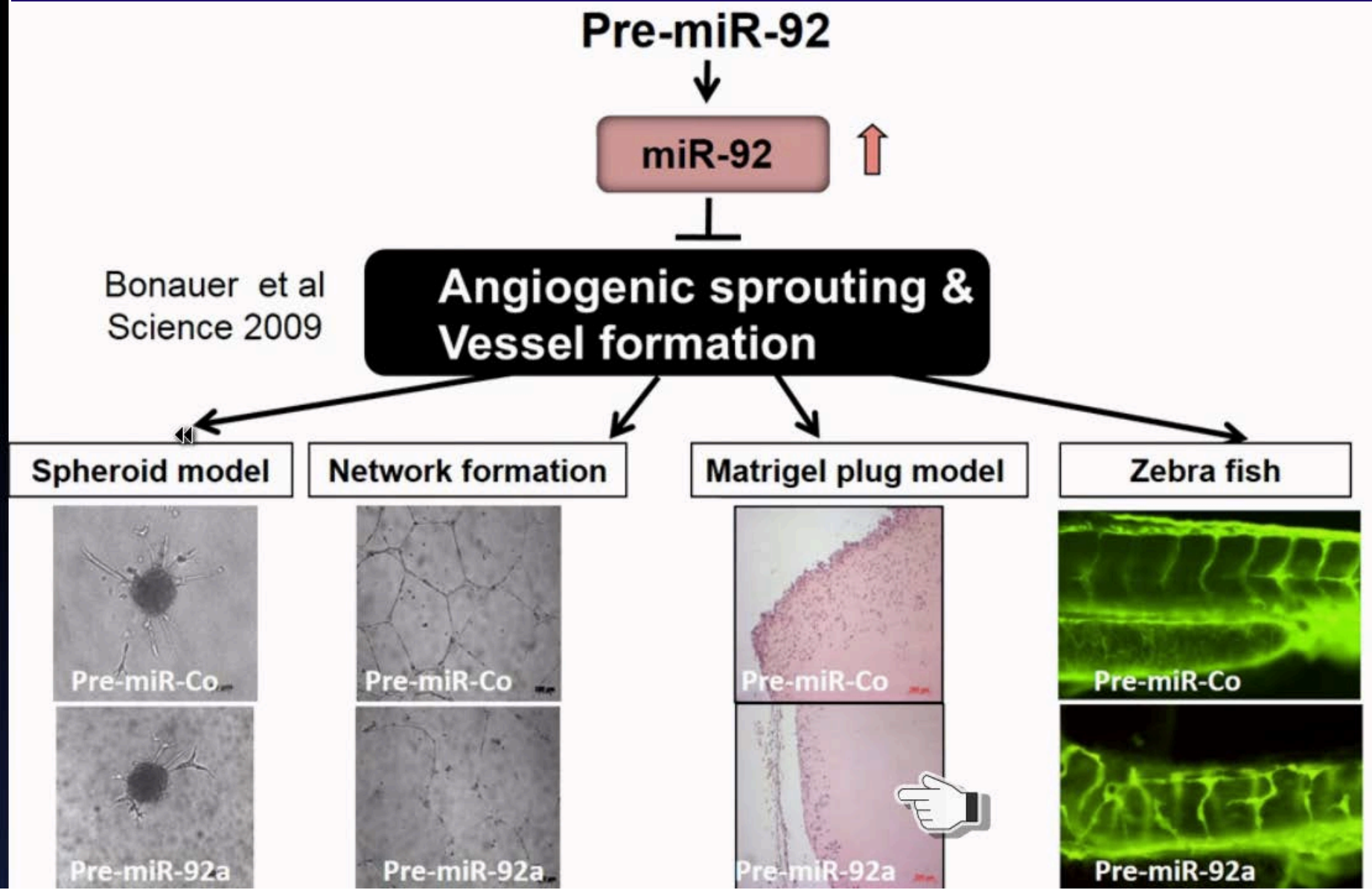


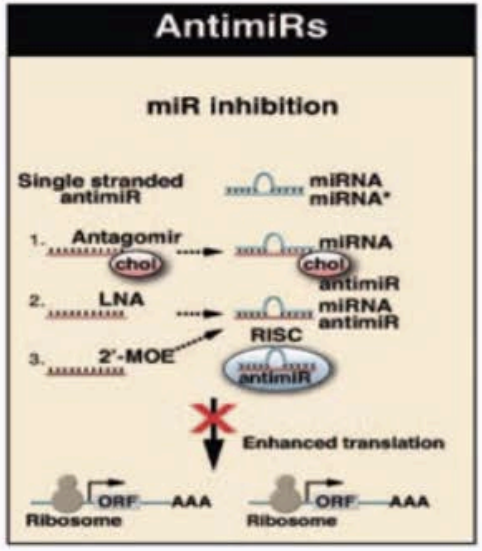
miR-1
miR-208
miR-499

Seeger et al, ATVB 2013

miR-92a downregulates angiogenesis and negatively affects vessel patterning

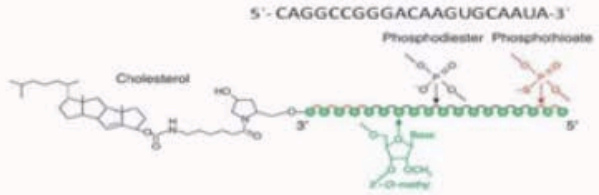


miR-92a inhibition by antimiRs

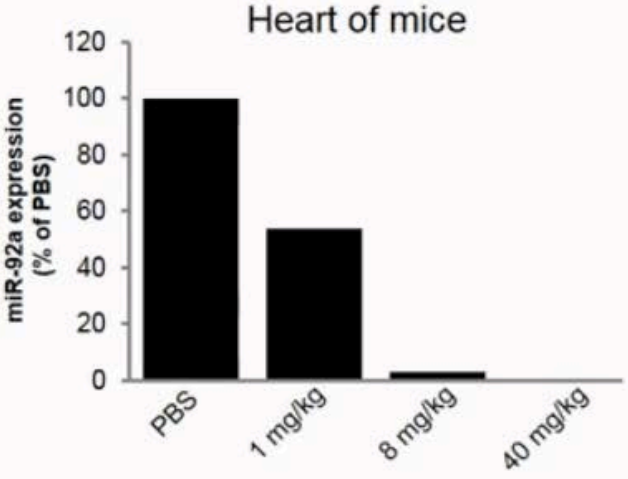


(van Rooij et al, *Circ Res*, 2008)

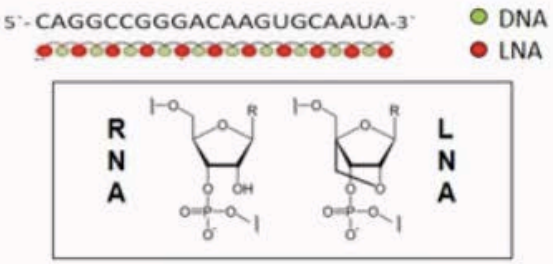
Antagomir-92a

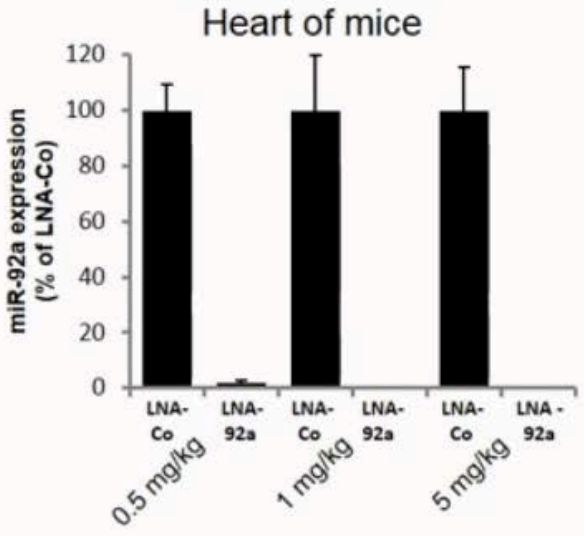


According to Krutzfeldt,et al. *Nature* 2005



LNA-92a

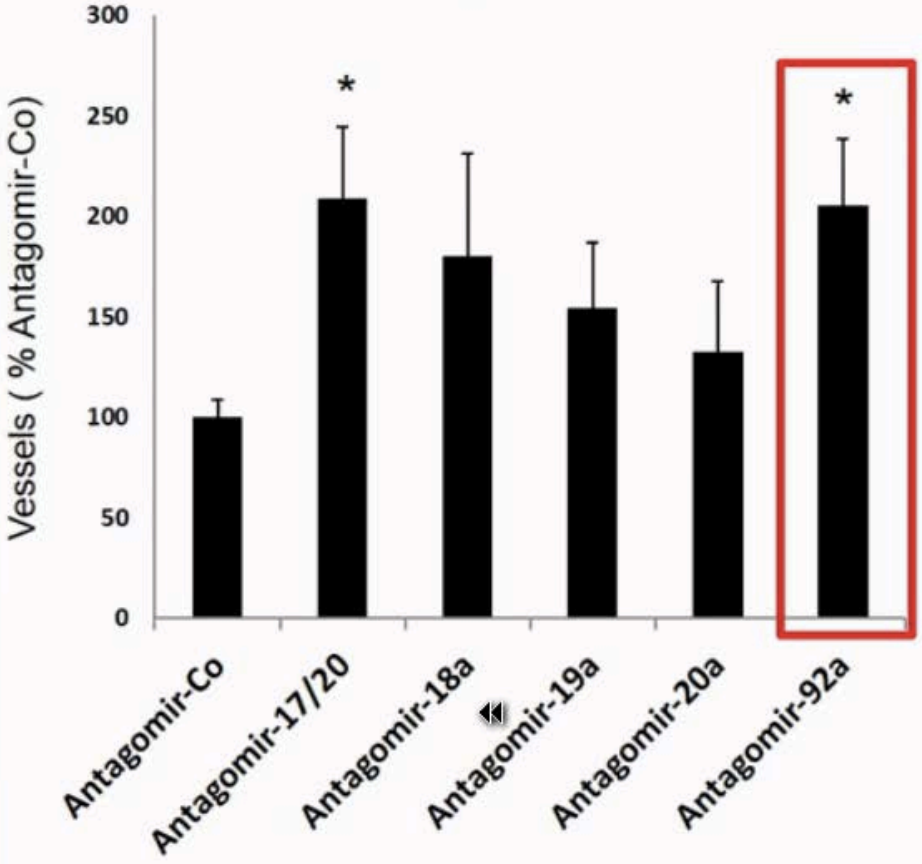




➔ **LNA –modified antimiRs have been tested in non-human primates and were successful and safe in a phase II study (NEJM 2013)**

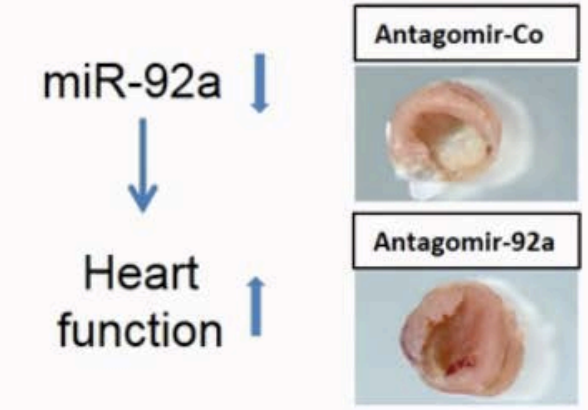
Inhibition of miR-92a enhances neovascularization & recovery after ischemia

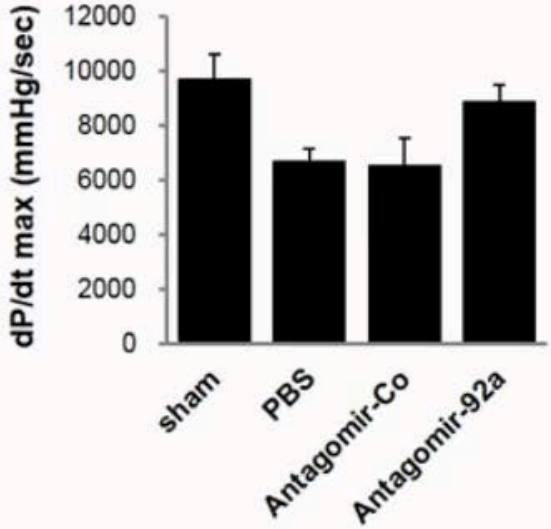
Angiogenesis (Matrigel-Model)



Doebele et al, Blood 2010

Recovery after myocardial infarction

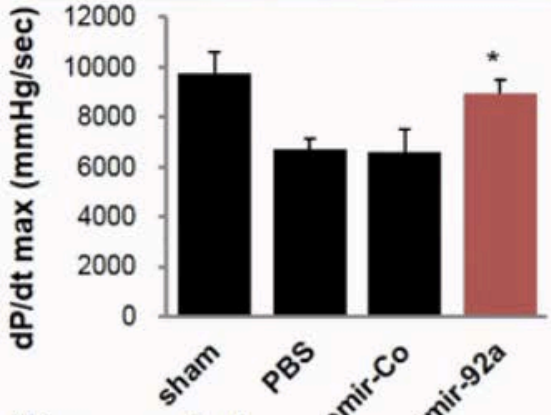




Bonauer et al, Science 2009

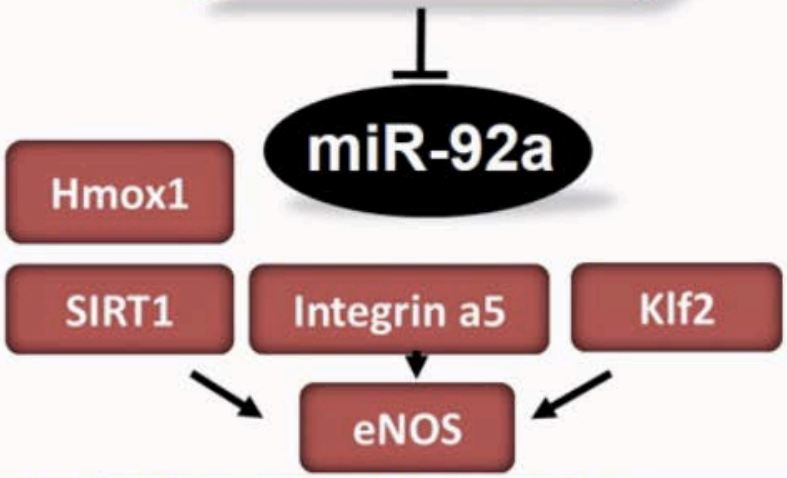
miR-92a effects in the cardiovascular system

Acute myocardial infarction

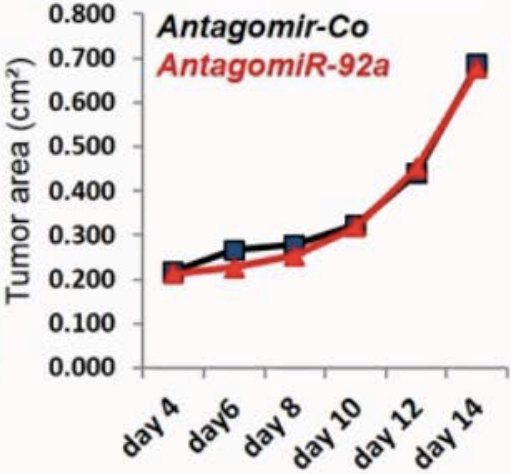


(Bonauer et al, Science 2009)

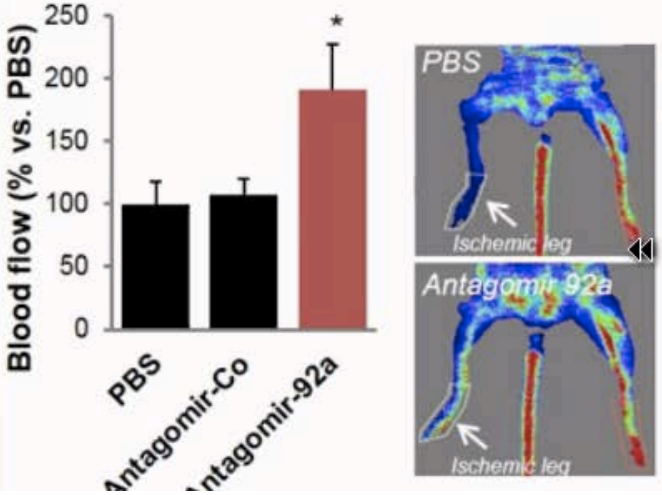
AntimiR-92a



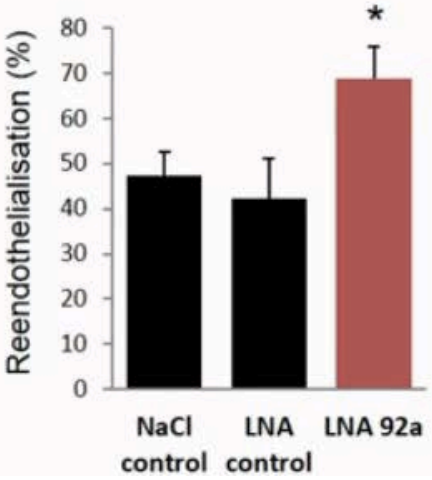
Tumor growth



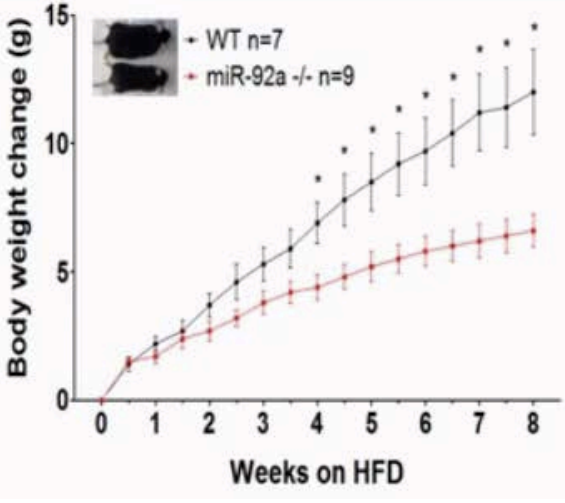
Hind limb ischemia



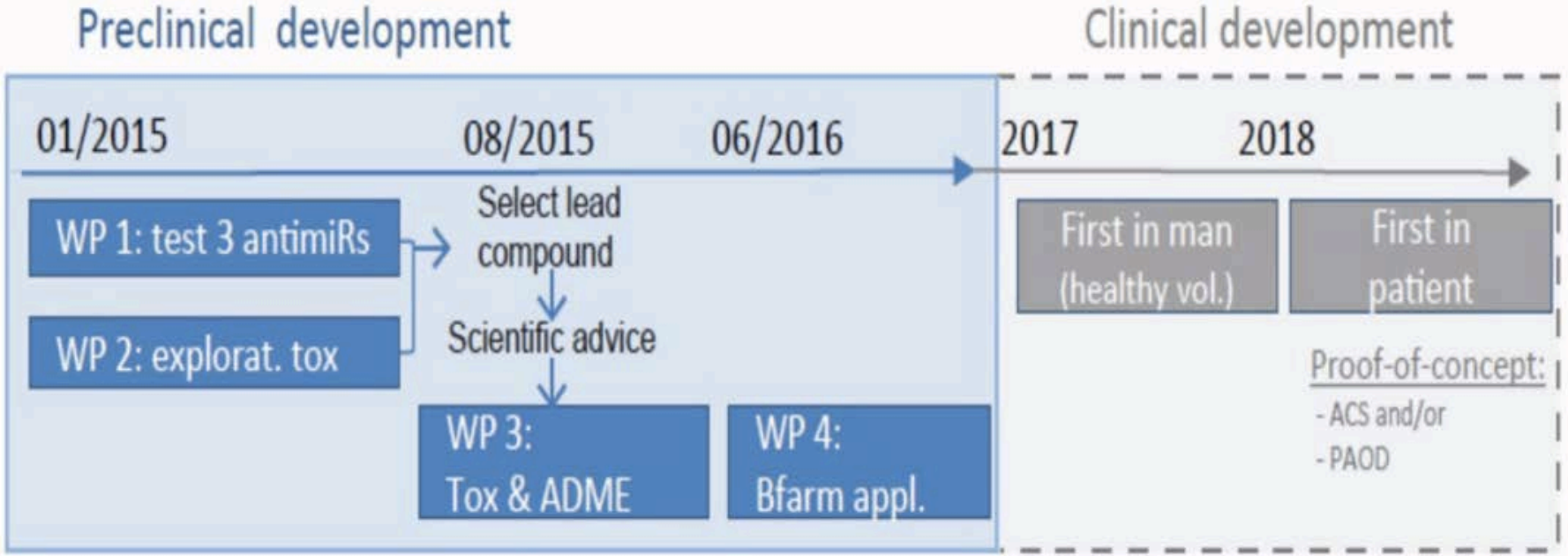
Vasculoprotection/ Atheroprotection



Metabolism



AntimiR-92a- the path to the clinic



DZHK
DEUTSCHES ZENTRUM FÜR HERZ-KREISLAUF-FORSCHUNG E.V.

Identification of cardiovascular disease specific genes and their regulating miRNA's

Deshpande V[1], Ghatge M[2], Mundkur L[1] and Kakkar V[1,3]

[1]Molecular Immunology; [2]Tata Proteomics and Coagulation Unit, Thrombosis Research Institute, Bangalore, India; [3]Thrombosis Research Institute, London, UK

Results: We clustered the genes expressed in Early and Intermediate stage of the disease. The main clusters enriched were MMPs, Proliferation differentials, ECM differentials and Adhesion differentials. About 16 genes were shortlisted and network was constructed with their interacting miRNAs. MicroRNA's mmu-miR-484 and mmu-miR-335-5p were found to interact with maximum number of genes. The genes targeting mmu-miR-484 are LRAT, CSF2RB2, SAA4, NACAN, MEFV, BST1, FCGR4 and SLC6A13. The genes targeting mmu-miR-335-5p are LRAT, HHIP, CSF2BR2, CYP1a1, CCL7, SAA4, NCAN and MEFV. Five genes LRAT, CSF2RB2, SAA4, NCAN and MEFV are targeting both miRNAs rendering these genes to be important ones.

Conclusion: This data identifies two novel miRNA interacting with cardiovascular disease specific genes which could be potential targets for cardiovascular therapy.

Platelet-derived matrix metalloproteinase (MMP)-2 contributes to atherosclerosis progression in hypercholesterolemic mice

Momi S, Falcinelli E, Manni G and Gresele P

Department of Medicine, University of Perugia, Perugia, Italy

Results: Femoral artery intimal hyperplasia was significantly lower in dKO and in chimeric mice compared with LDLR / mice (IM ratio: LDLR / = 1.6 0.4; dKO= 0.42 0.2, chimeras = 0.12 0.02, P < 0.001). Similarly, aortic arch area covered by lipid lesions was lower in dKO and in chimeric mice than in LDLR / mice (29 3.2%, 21 7.3% vs. 72 5%, P < 0.01). Aortic atherosclerotic area of LDLR / mice infused with activated platelets from MMP-2 / mice was strikingly reduced (LDLR / not infused: 15.2 2%; LDLR / -control platelets = 23 5%; LDLR / -MMP2 / platelets: 4.8 0.71%). Thrombin-activated platelets from LDLR / mice, but not from MMP-2 / mice, induced EC activation (VCAM-1 expression: 4.4 and 1.7 fold increase vs. baseline, P < 0.001).

Conclusion: MMP-2 expressed/released by activated platelets plays a central role in hyperlipydemia-induced atherogenesis by a mechanism that involves EC activation. The interference with the expression/ release of MMP-2 by platelets may represent a new target for anti-atherogenic therapy.

Changes in the expression of plasma and platelet microrna s in type 2 diabetes mellitus and obesity

Fejes Z[1], Póliska S[2], Penyige A[3], Káplár M[4], Debreceni IB[1], Kappelmayer J[1] and Nagy B Jr[1]

[1]Department of Laboratory Medicine, University of Debrecen, Debrecen, Hungary; [2]Genomic Medicine and Bioinformatic Core Facility, Department of Biochemistry and Molecular Biology, University of Debrecen, Debrecen, Hungary; [3]Department of Human Genetics; [4]Institute of Internal Medicine, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

Results: In DM2 there was a significantly decreased expression of platelet miR-223 compared to normal counterparts, while in contrast, obese subjects showed increased levels. Enhanced platelet P-selectin positivity (5.1 [3.9–8.4]%) in diabetics and in obesity (3.3 [2.6–4.6]%) showed a negative association with altered platelet miR-26b expression vs. controls (1.5 [1.1–2.4]%). Furthermore, downregulated plasma miR-424 and miR-24 in DM2 and obese subjects significantly correlated (P = 0.014) with elevated vWFAg levels. MiR-223 content of microparticles (MPs) was also investigated, and a significant inverse relationship (rho = 0.428; P = 0.033) was found between this miRNA level in platelets and that of MPs suggesting the release of miRNAs from platelets into MPs in DM2.

Conclusion: Altered miRNA levels may contribute to abnormal platelet and endothelial function in metabolic disorders.

Dicer, the key enzyme of RNA interference, is regulated by thrombin stimulation in human platelets

Manni G, Bury L, Piselli E and Gresele P

Deptartment of Internal Medicine Section of Internal and Cardiovascular Medicine, University of Perugia, perugia, Italy

Results: Dicer protein and mRNA are present in resting human platelets. Activation with thrombin induces a rapid increase in the expression of Dicer protein in platelets, already evident at 5 min and steadily increasing until 60 min. The increase of Dicer was blocked by pretreatment with puromycin. S35-methionine was incorporated into Dicer in activated platelets, confirming de novo synthesis. Thrombin induced a down-regulation of P2Y12 which correlated with Dicer increase.

Conclusion: Thrombin induces a rapid and marked *de novo* synthesis of Dicer in human platelets. Newly synthesized Dicer is functional because it modifies the expression of a target platelet mRNA (P2Y12 mRNA). Our results add an additional level of complexity to the control of gene expression in human platelets.

Platelets release extracellular vesicles in an agonist dependent manner but release a consistent profile of microrna

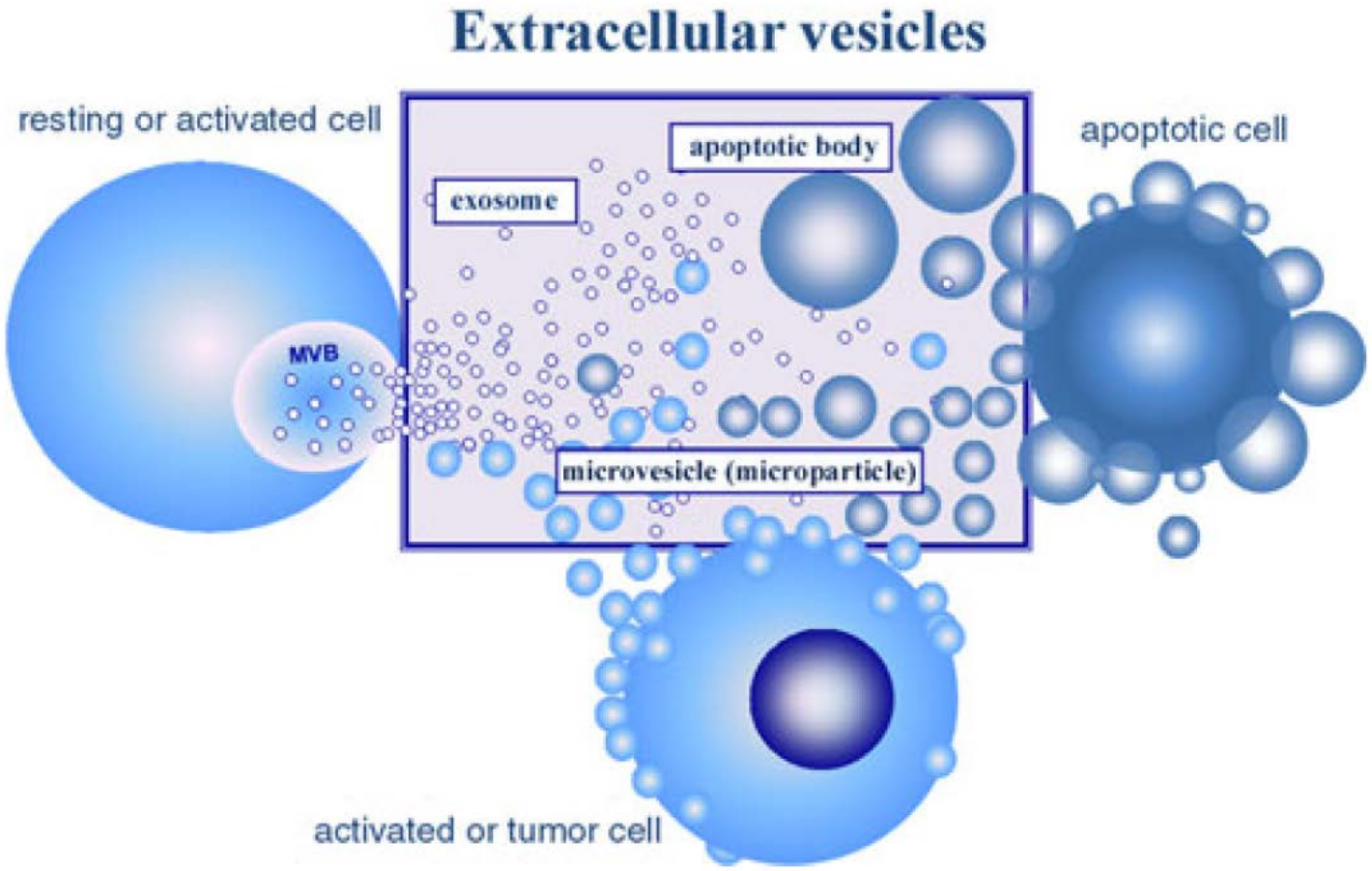
Ambrose AR[1], Pringle JH[2] and Goodall AH[1]

[1]Department of Cardiovascular Sciences; [2]Department of Cancer Studies, University of Leicester, Leicester, UK

Results: Stimulation through GPVI produced a mixed population of MPs and exosomes, while all other agonists released predominantly exosomes. Of the inhibitors, only apyrase reduced EV release for all agonists. The EVs contained between 57 and 79 different miRNA with a core of 36 miRNA observed with all agonists. There is high correlation of agonist profiles (r2 > 0.98 for all), and also with the total platelet miRNA content (r2 > 0.98). The 36 miRNA seen in all samples are predicted to have significant effects on the translation of proteins involved in endocytosis, cell cycle control and differentiation. miR-223 was most highly expressed in all samples and has previously been shown to affect myeloid lineage development and have anti-inflammatory and cardioprotective effects.

Conclusion: These data suggest that while the EV profile released from platelets is agonist-dependent, all agonists release exosomes with similar miRNA content. ADP plays an important role in the release of exosomes. The data also suggest exosomes as the most likely vehicle for miRNA release from platelets.

Extracellular vesicles: exosomes, microvesicles and apoptotic bodies

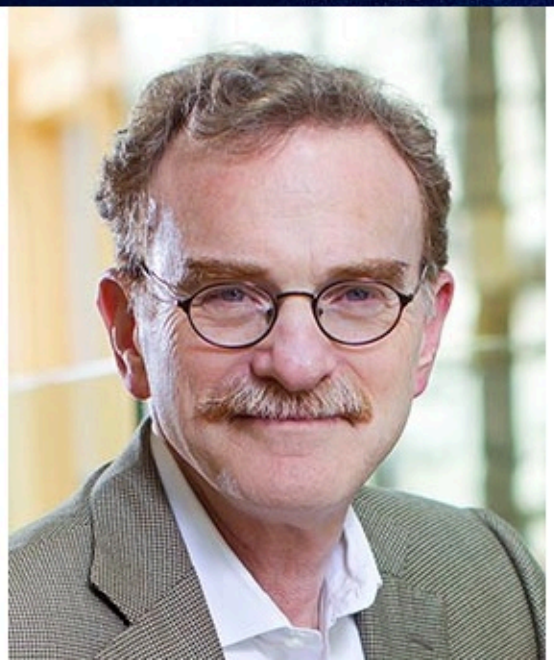


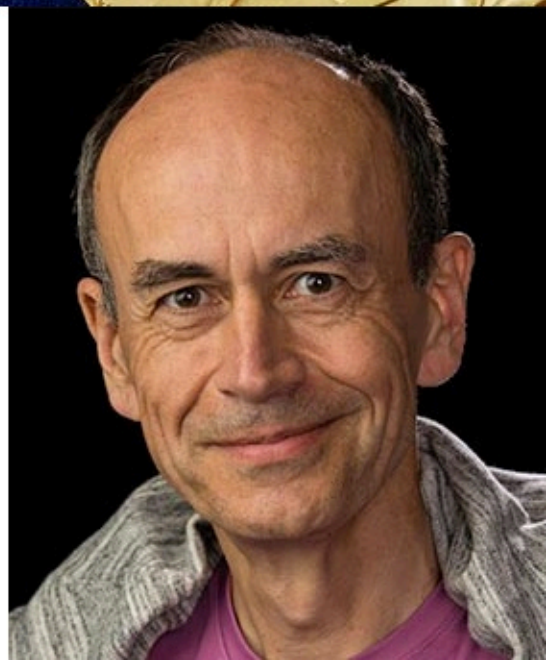
Extracellular vesicles are physiologically released by almost all cell types and represent endogenous cargos that are able to participate in cell-to-cell communication.

Gyorgy B et al, Cell Mol Life Sci 2011

2013 NOBEL PRIZE IN PHYSIOLOGY OR MEDICINE

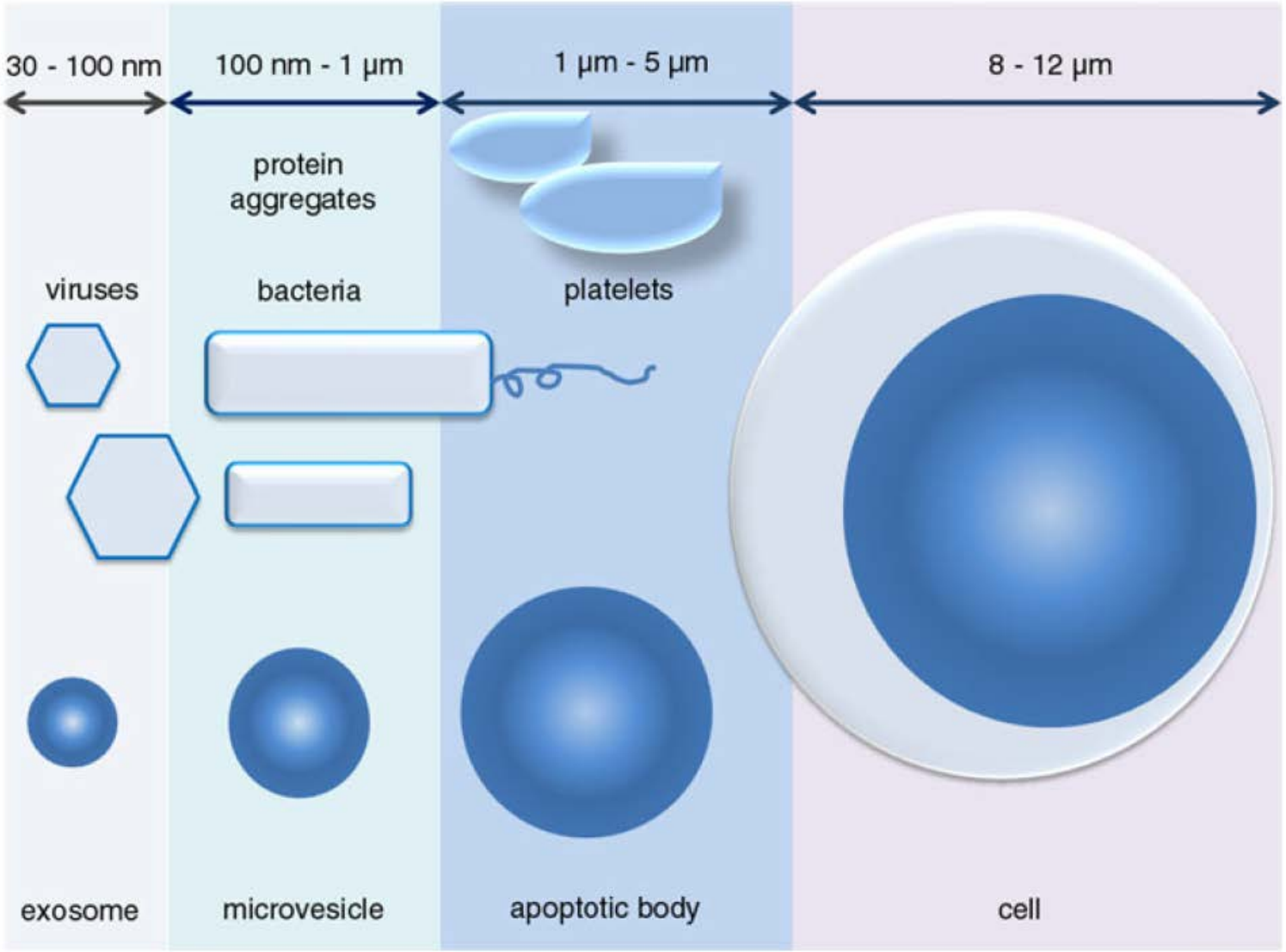
James E. Rothman
Randy W. Schekman
Thomas C. Südhof





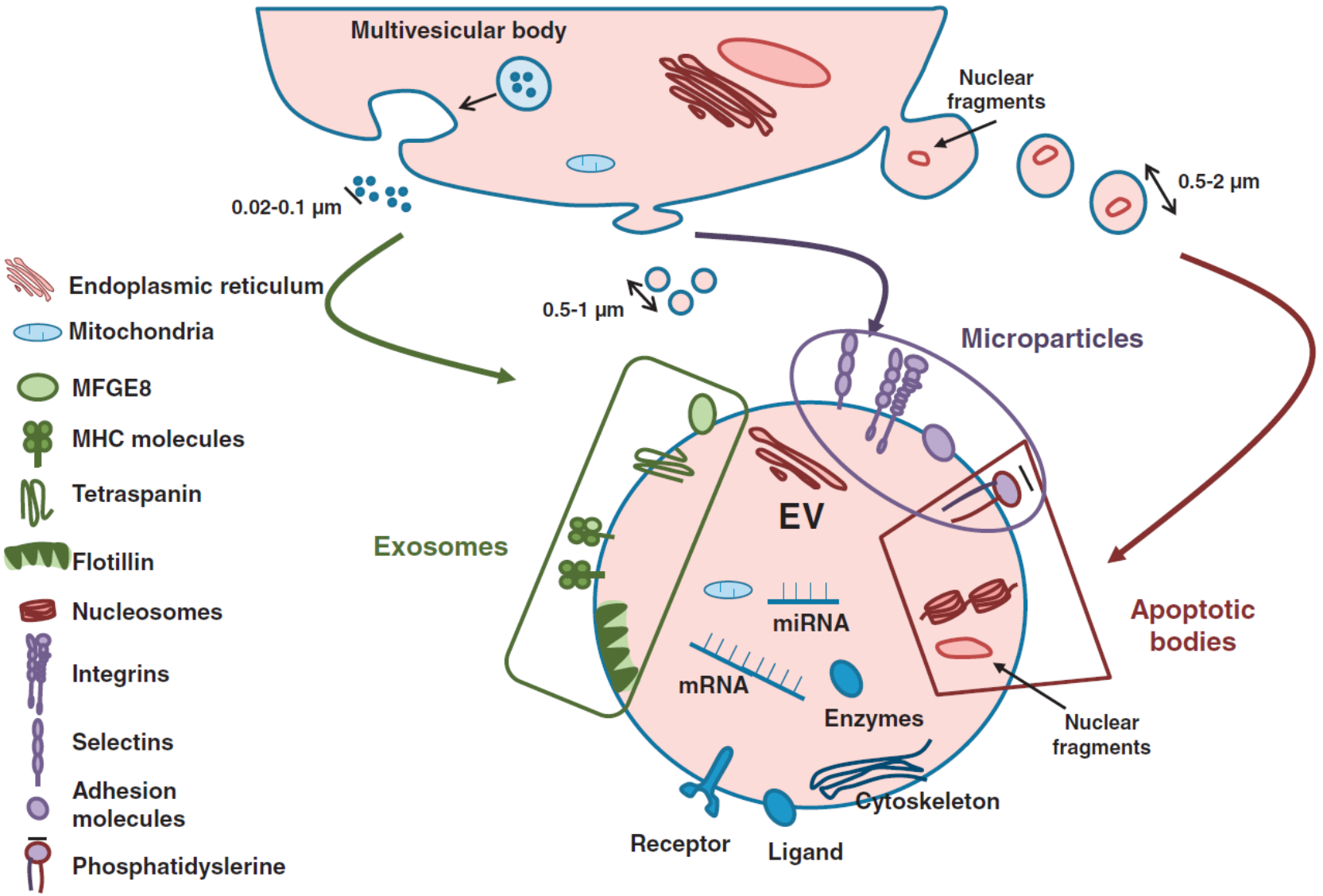
"for their discoveries of machinery regulating vesicle traffic, a major transport system in our cells".

Size ranges of major types of membrane vesicles



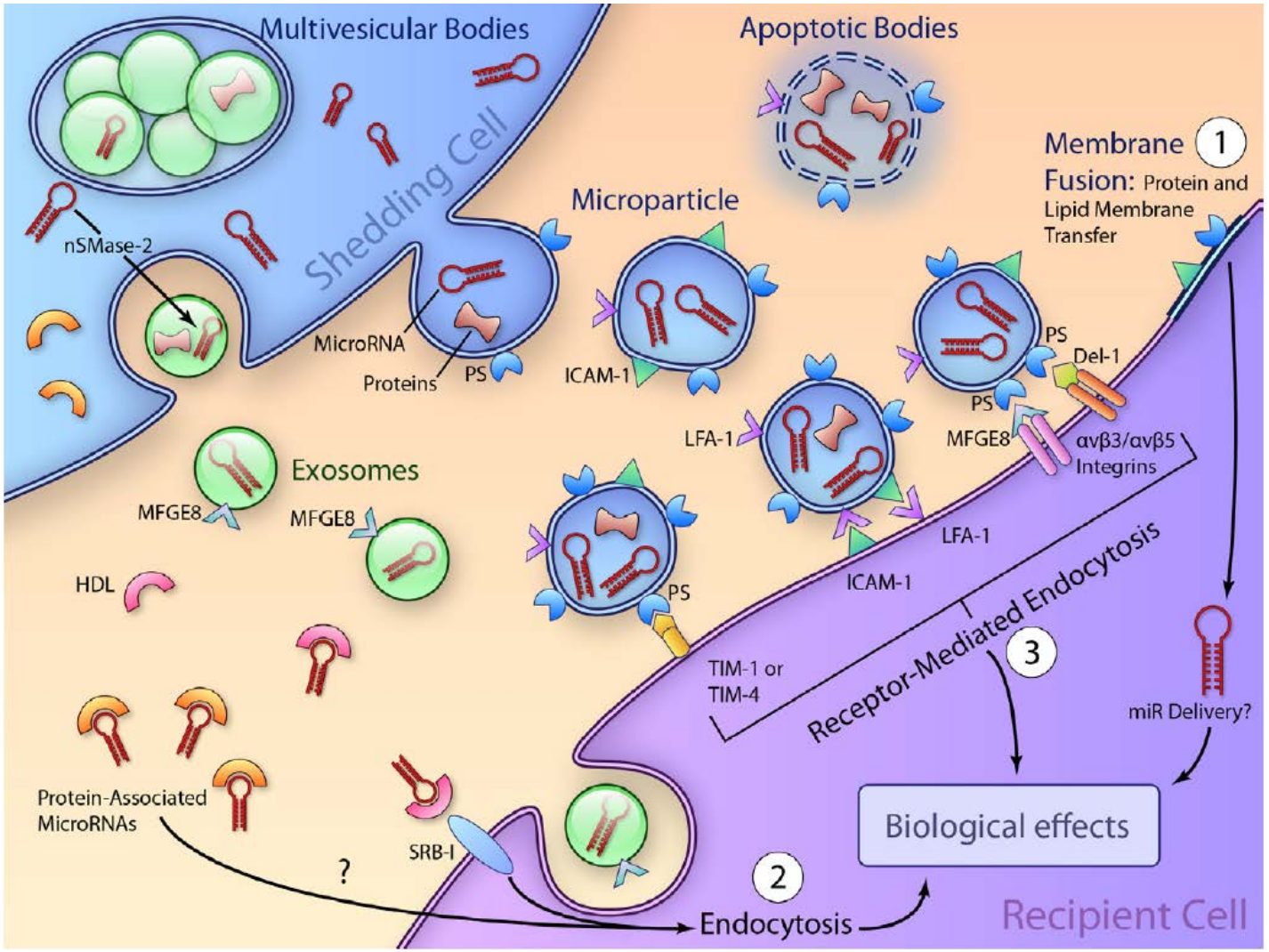
Gyorgy B et al, Cell Mol Life Sci 2011

Biogenesis and content of extracellular vesicles



Gaceb A et al, Int J Bioch & Cell Biol 2014

Exchange of receptors and RNA between eukaryotic cells

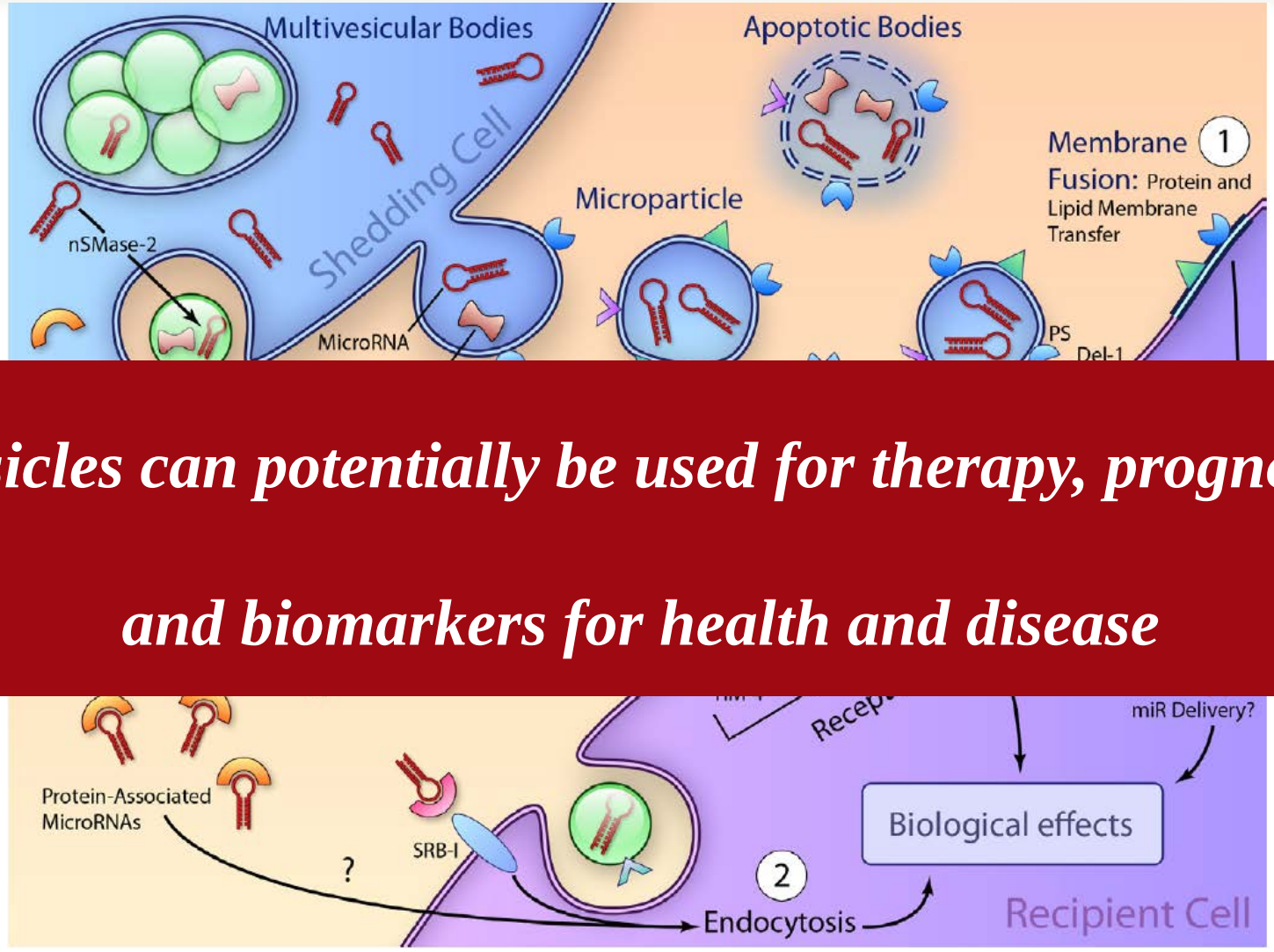


Loyer X et al, Circ Res 2014

Functions ascribed to extracellular vesicles

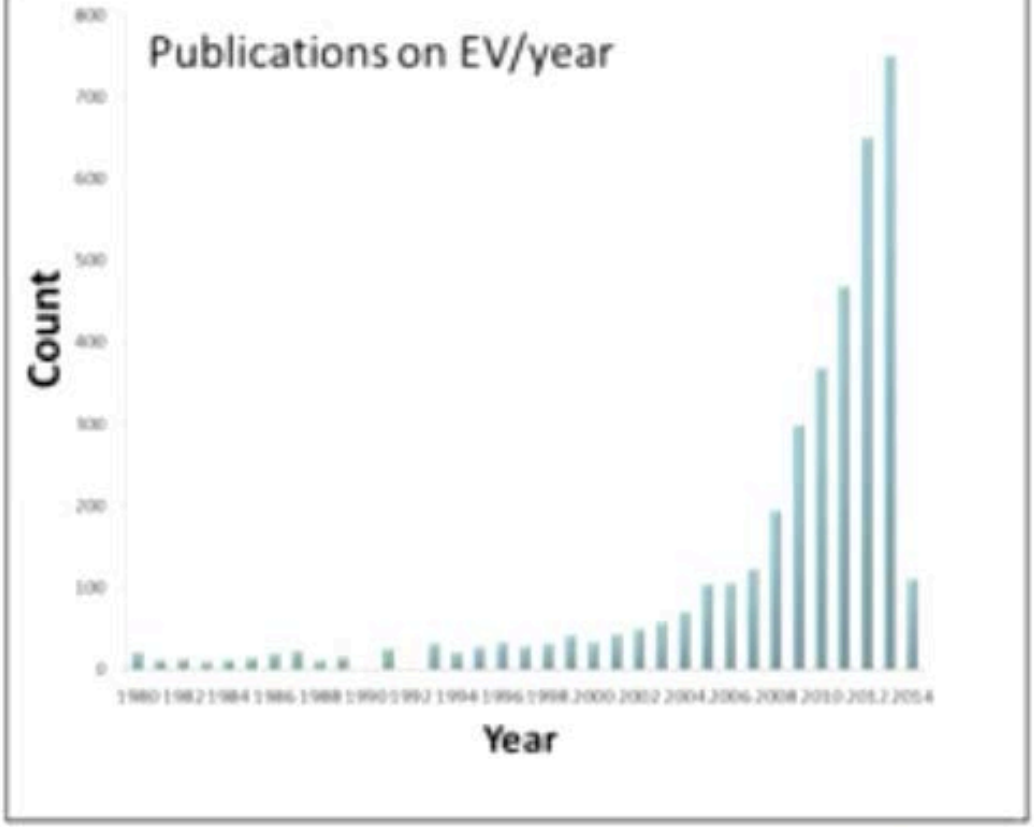
- Coagulation
- Angiogenesis
- Cell survival
- Modulation of the immune response
- Inflammation
- Maintenance of stem cell niche
- ….

Mechanisms involving extracellular vesicles in cell-to-cell communication.



Vesicles can potentially be used for therapy, prognosis and biomarkers for health and disease

Clinical Relevance



- Body fluids (10^{10}-10^{15}/L)
- Numbers, cellular origin, composition and function disease-dependent
- Clinical interest
  - Biomarkers
    - Diagnosis, monitoring of therapy
  - Drug delivery (e.g. siRNA)
  - Prognosis
  - Therapy

http://evpedia.info

Platelet Microparticles and Calcium Homeostasis in Acute Coronary Ischemias

John N. Katopodis,[3] Luciano Kolodny,[1] Wenche Jy,[1] Lawrence L. Horstman,[1] E.J. De Marchena,[3] Jian G. Tao,[2] Duncan H. Haynes,[2] and Yeon S. Ahn[1]*

American Journal of Hematology 54:95–101 (1997)

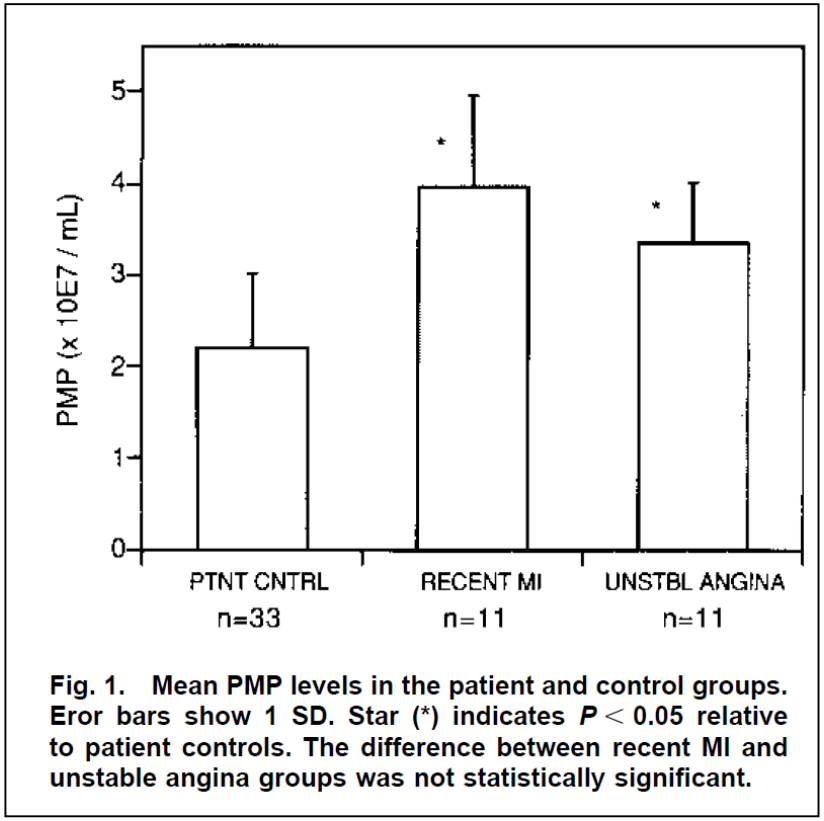


Fig. 1. Mean PMP levels in the patient and control groups. Eror bars show 1 SD. Star (*) indicates $P < 0.05$ relative to patient controls. The difference between recent MI and unstable angina groups was not statistically significant.

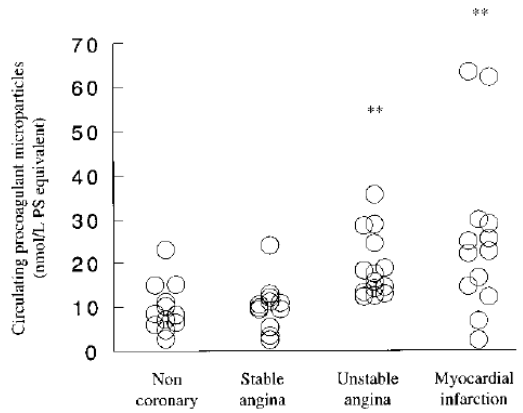
PMP and [Ca^{++}]cyt appear significantly elevated in UA and recent MI…

Further study is needed to refine these methods for possible clinical applications…

Elevated Levels of Shed Membrane Microparticles With Procoagulant Potential in the Peripheral Circulating Blood of Patients With Acute Coronary Syndromes

Ziad Mallat, MD, PhD; Hakim Benamer, MD; Bénédicte Hugel, PhD; Joëlle Benessiano, PhD; P. Gabriel Steg, MD, PhD; Jean-Marie Freyssinet, PhD; Alain Tedgui, PhD

(*Circulation*. 2000;101:841-843.)



Individual values of circulating procoagulant microparticles expressed as nmol/L PS equivalent. **$P<0.01$ vs noncoronary patients or stable coronary patients.

Antigenic Characterization of the Circulating Microparticles in Patients With SA, ACS or Noncoronary Heart Disease (NC)

Capture Antibody	SA (n=5)	ACS (n=11)	NC (n=6)	*P*, 1-Way ANOVA
CD146	3.3±0.9	8.2±1.4	2.6±0.8	<0.01
CD31	1.6±0.4	8.8±3.0	1.2±0.3	0.06
GP Ib	2.6±0.5	6.9±1.8	6.5±1.5	NS
CD3	0	0.2±0.2	0	NS
CD11a	0.6±0.3	1.7±0.9	0.2±0.2	NS

Values are expressed as nmol/L PS equivalent.

…this observation suggests that the level of circulating microparticles could be useful as an indicator of the persistence or recurrence of thrombus and therefore as a <u>prognostic marker</u> of the recurrence of ischemic events….

Microparticles from patients with acute coronary syndrome and heart transplanted patients induce premature endothelial senescence and thrombogenicity: role of oxidative stress and of the local angiotensin system

Abbas M[1], Jesel L[1,2,3], Nguyen PN[3], Auger C[3], Messas N[4], Ribeiro T[3], Silva GCC[3], Ohlman P[5], Morel O[5], Schini-Kerth V[1,3] and Toti F[1,3]

[1]Faculty of Pharmacy, Université De Strasbourg, Illkirch-Graffenstaden; [2]Service de cardiologie, Hopitaux universitaires de Strasbourg, Strasbourg; [3]UMR7213, CNRS, Illkirch-Graffenstaden; [4]Hôpitaux universitaires de Strasbourg, Faculty of Medicine; [5]Service de Cardiologie, Faculty of Medicine, Hôpitaux Universitaires de Strasbourg, Strasbourg, France

Results: Compared to HV, circulating MPs from ACS and HT prompted premature senescence in young ECs and up-regulated the expression of NADPH oxidase (gp91phox, p47phox, p22phox), COX2,senescent markers p53, p21, p16, actors of the local angiotensin system (AT1 angiotensin receptor, angiotensin converting enzyme) and of TF. MPs also down-regulated eNO synthase. Losartan, an AT1 receptor antagonist, prevented MPs-induced premature endothelial senescence. Senescence markers in plaques and mammary arteries were upregulated when the number of risk factors increased.

Conclusion: MPs isolated from ACS and HT patients induced premature endothelial senescence and thrombogenicity through enhanced oxidative stress. Moreover, these data shed light on the importance of the local angiotensin system in MPs-mediated induction of endothelial senescence.

The change of the amount of circulating microparticles and their association to the general atherosclerotic burden after acute coronary syndrome

Christersson C[1], Jonelid B[1], Thulin A[2] and Siegbahn A[2]

[1]Cardiology; [2]Clinical Chemistry, Department of Medical Sciences, Uppsala, Sweden

Results: The mean age was 68 10 years in the ACS group and 48% had a non-ST-elevation myocardial infarction as the index event. 97% and 95% were treated with aspirin and P2Y12-receptor blocking agent, respectively. The type of ACS was not related to the concentrations of platelet, endothelial or monocyte derived MPs. The majority of the circulating MPs, 2894 (2262–3950)/10,000 platelets, expressed CD41 and their concentration did not change during the first year after the ACS. The concentrations of CD62P+, CD144 + and CD14 + MPs were low and did not change significantly during the first year. 2 years after the ACS the levels of CD41 + and CD62P+MPs were increased compared to the other time-points ($P < 0.0001$). Patients with manifest atherosclerosis in the coronary, carotid and peripheral arteries had higher concentrations of platelet derived MPs ($P = 0.01–0.03$) despite treatment with double platelet inhibition after the ACS.

Conclusion: In an ACS population the majority of MPs are platelet derived and there is no dynamic change of concentrations during the first year after the acute event. Patients with an increased general atherosclerotic burden have higher amounts of circulating platelet derived MPs and further research regarding optimal antithrombotic treatment in this group of patients is warranted.

Circulating microparticles in deficiency of the natural anticoagulants

Campello E[1], Spiezia L[1], Radu CM[1], Bulato C[1], Gavasso S[1], Tormene D[1], Woodhams B[2] and Simioni P[1]

[1]*Department of Medicine, University of Padua, Padua, Italy;*
[2]*Haemacon Ltd, Bromley, UK*

Results: Carriers of natural anticoagulants deficiencies had higher median levels of annexin V-MP, EMP, PMP, TF+MP and PPL activity than healthy controls ($P < 0.001$, < 0.001, < 0.01, 0.025 and 0.03, respectively). The carriers of AT and PC defects had significantly higher levels of annexin V-MP, EMP and PMP than controls. Carriers of PS defects had significantly higher levels of annexin V-MP, EMP and TF+MP than controls. The carriers with high levels of annexin VMP, EMP and PMP had an adjusted OR for VTE of 3.36 (95% CI, 1.59–7.11), 9.26 (95% CI, 3.55–24.1) and 2.72 (95%CI, 1.16–6.38), respectively.

Conclusion: We showed higher levels of MP in AT, PC and PS defects compared to controls. We confirm a role of circulating MP in the development of VTE in severe hereditary thrombophilia, possibly by a mechanism of endothelial dysfunction (the highest OR for VTE was seen in EMP). Further studies are needed to better define the role of MP as triggering factors for the thrombotic complications characterizing hereditary thrombophilic defects.

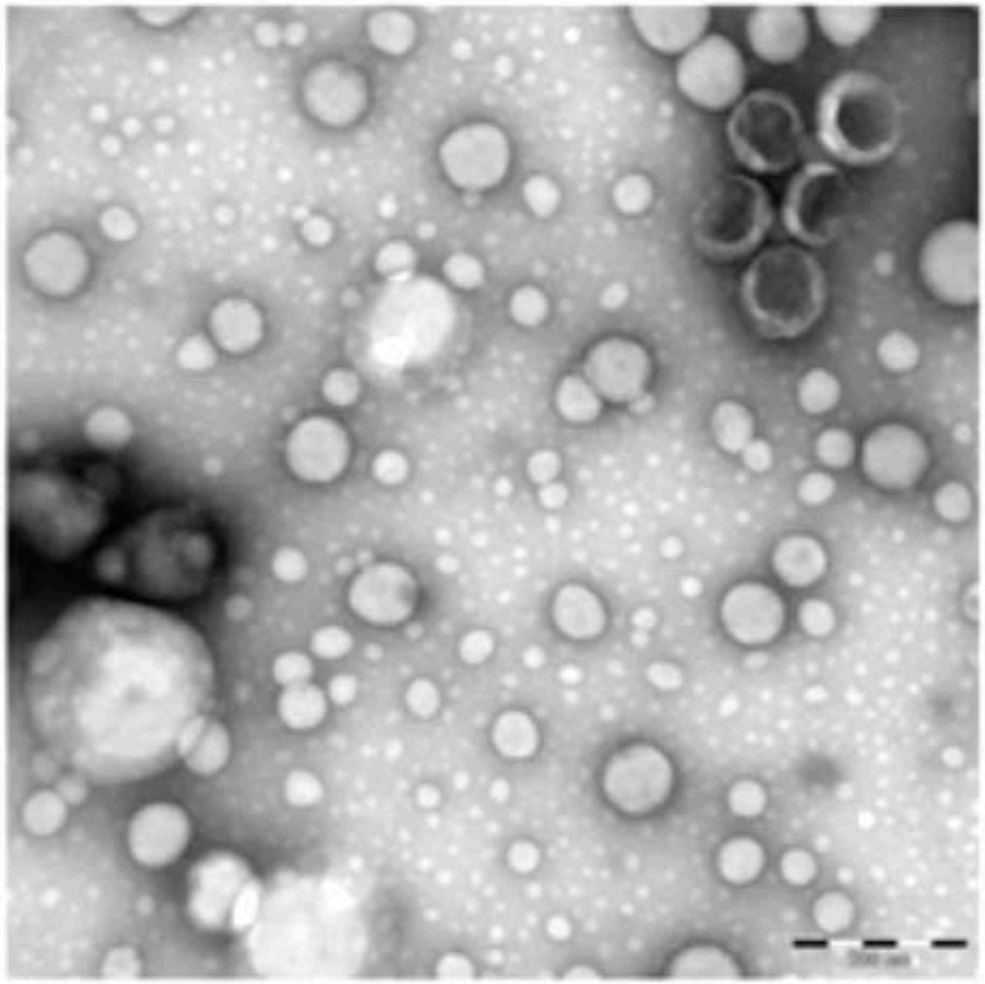
Microparticles in health and disease

Nieuwland R

Clinical Chemistry, Academic Medical Centre, Amsterdam, The Netherlands

….Furthermore, changes in EV composition contribute to disease development, e.g. bleeding and thrombosis are now associated with the presence of EV exposing coagulant tissue factor in blood. At present, isolation and detection of EV have become a 'hot topic'. Recently, we (re)introduced size-exclusion chromatography to isolate EV from body fluids such as plasma. So far, this methodology is very promising and has several important advantages compared to other methods. With regard to detection, to extract relevant clinical information such as cellular origin from single EV, ideally every single EV should be detected. Clearly, this is a huge challenge and 'work in progress' because most EV are extremely small, heterogeneous in size and shape, contaminants are present in the size range of EV, the refractive index of EV is low, 'swarm detection' may occur, etc. Taken together, the presence and relevance of EV has now been firmly established. The next steps will be to further standardize pre-analytical conditions, to develop novel and reliable detection platforms for measurement of single EV, and to standardize functional assays.

Challenges with MV isolation and measurement



- Pre-analytical variables represent the largest single source of variability
- Despite many methods many basic questions on size, morphology, phenotype and concentration remain
- Problems with their size range, heterogeneity and methodologies
- Standardization in protocols and methods ongoing
- Biological Standards required

Journal of Thrombosis and Haemostasis, **11**: 1190–1193 DOI: 10.1111/jth.12207

OFFICIAL COMMUNICATION OF THE SSC

Standardization of pre-analytical variables in plasma microparticle determination: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop

R. LACROIX,*† C. JUDICONE,‡ M. MOOBERRY,§ M. BOUCEKINE,¶ N. S. KEY,§ F. DIGNAT-GEORGE*† and ON BEHALF OF THE ISTH SSC WORKSHOP[1]

Standardised protocol for preparing platelet free plasma

2500 ×*g* for 15 min at RT (x 2) within 1 hour of collection prior to snap freezing in liquid nitrogen and storage at -80°C

Efficient removal of residual platelets reduces inter-laboratory variation by preventing ex-vivo formation of MV during freeze/thawing.

Excellent for intended use, i.e., multicentre flow-cytometric studies of plasma MV

But double centrifugation reduces plasma MV by ~80% compared with whole blood (by FCM)

Not suitable for isolating rare populations (e.g. Tumour vesicles) or larger microvesicles

Platelets may act as a sink for rare vesicle populations

Isolation Methods :- Pros and Cons

Method	Pros	Cons
Ultracentrifugation	Relatively straightforward	Purity/EV integrity
Density centrifugation	High purity	Slow, sucrose toxicity
Ultrafiltration	Quick	Low purity
Immuno-magnetic bead	Specific, high purity	Prior knowledge of EV characteristics required
Affinity purification	Relatively specific, high purity	Prior knowledge of EV characteristics required
Chromatography	High purity	Specialised equipment
Microfluidics	Specific, high purity	Prior knowledge of EV characteristics required
Field flow fractionation	Quick	? purity
Precipitation techniques	High yield	Low purity

Considerations in selecting isolation method

- Type of sample

  Blood, plasma, Cell Culture Media (exosome free serum)

  Complexity – protein, lipoproteins

  Viscosity
- What are you going to do with the MV?
- How important is yield?
- How important is purity?
- How important is processing time?
- What is your starting sample volume?

SISET

Workshop

SISET

Società Italiana
per lo Studio dell'Emostasi
e della Trombosi

POST-ISTH:
Novità dal meeting di Toronto 2015

29-30 Gennaio 2016

Ospedale Papa Giovanni XXIII
Bergamo

Grazie per

l'attenzione!

Università degli Studi di Milano
Centro Cardiologico Monzino IRCCS, Milano