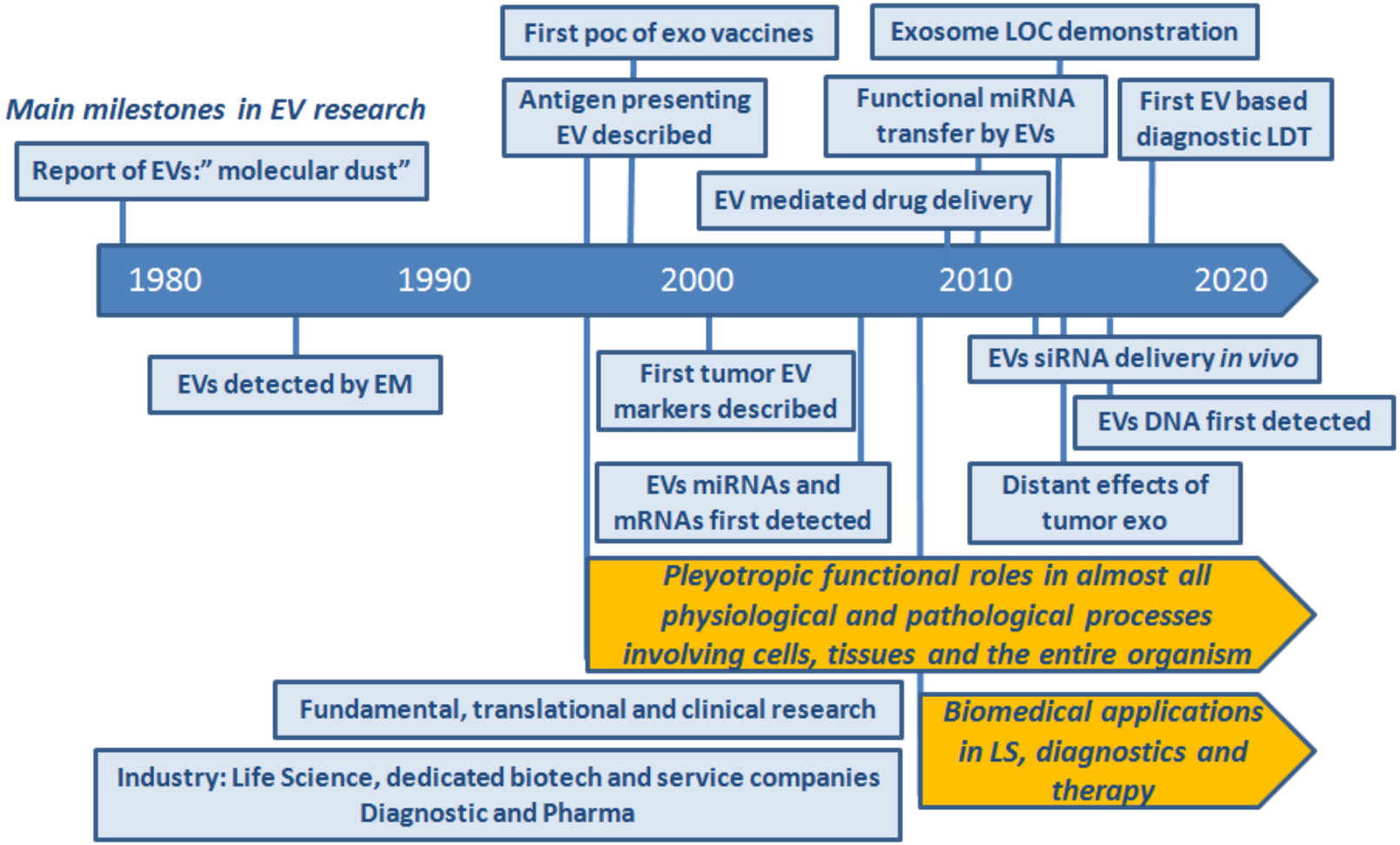
The roadmap towards the industrial and clinical applications of extracellular vesicles

Natasa Zarovni, HBM-LS, October 11°, 2017

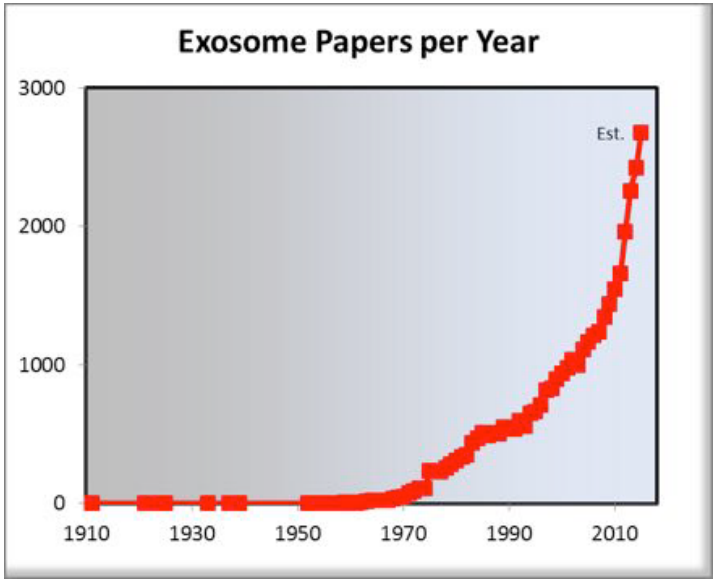
Exosomes and EVs have come a long way: major scientific milestones timeline



** POC: proof-of-concept; LOC: lab-on-chip; LDT: laboratory developped test (companion diagnostics in particular)*

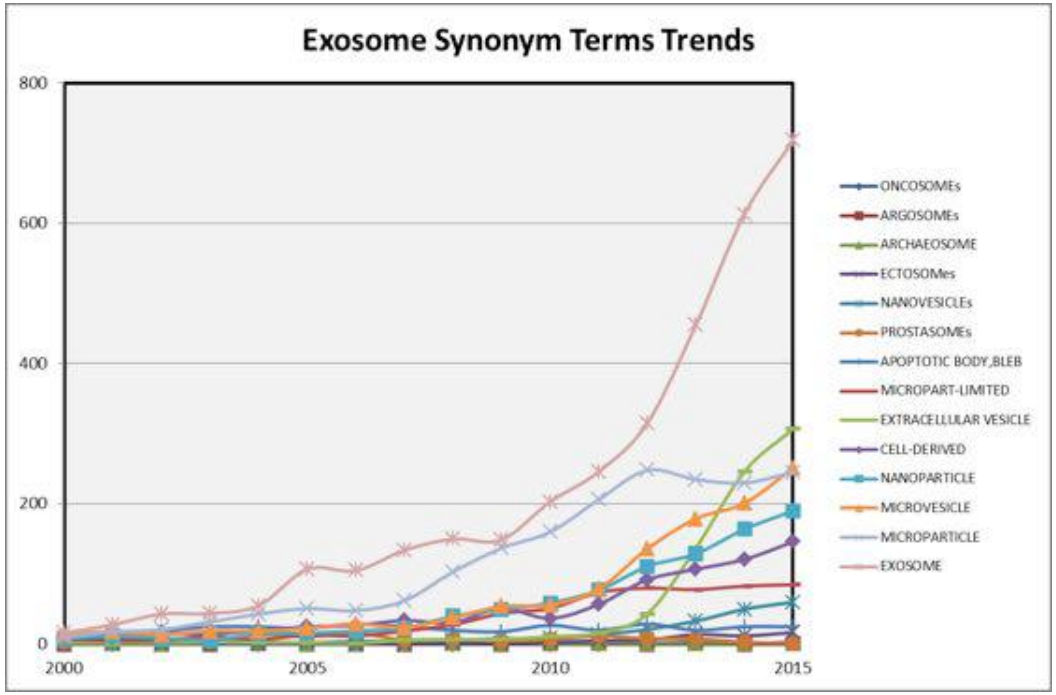
Means to frame the size and evolution of the EV field

1. *Growth of peer-reviewed publications on exosomes/EVs*



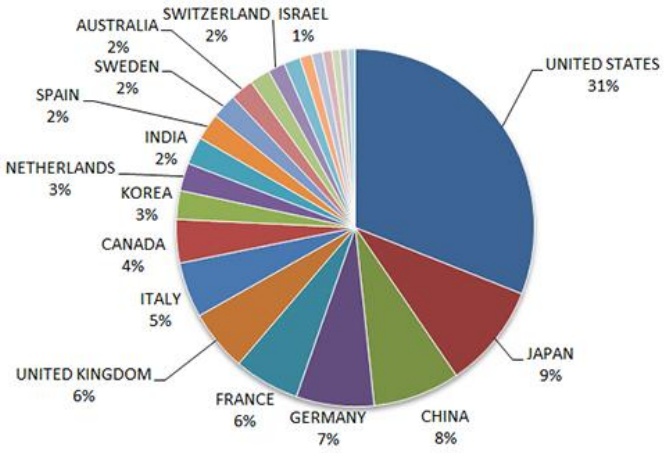
One of the metrics for the evolution and maturation of the fields is the consensus on nomenclature (overlapping→clarity)

More than 1/3 of all EV pubblications ever date to last 5 years



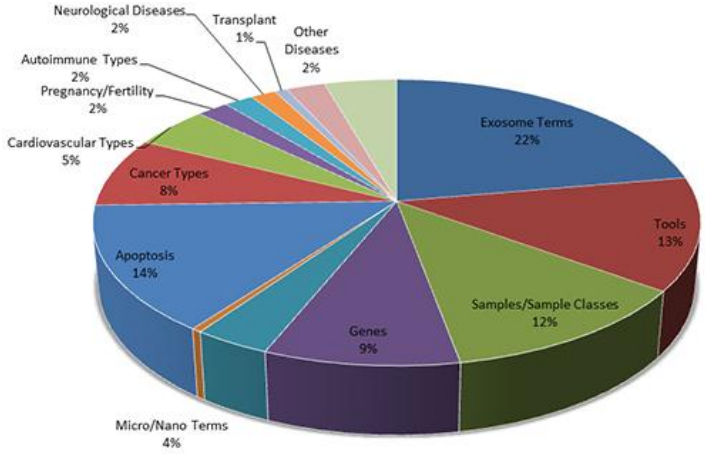
Means to frame the size and evolution of the EV field

2. Geographical dispersion

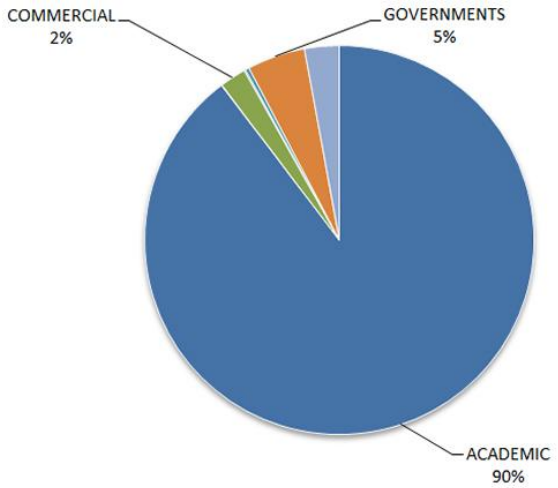


Breakout by the country of source

3. Fields of interest



Breakout by areas of focus

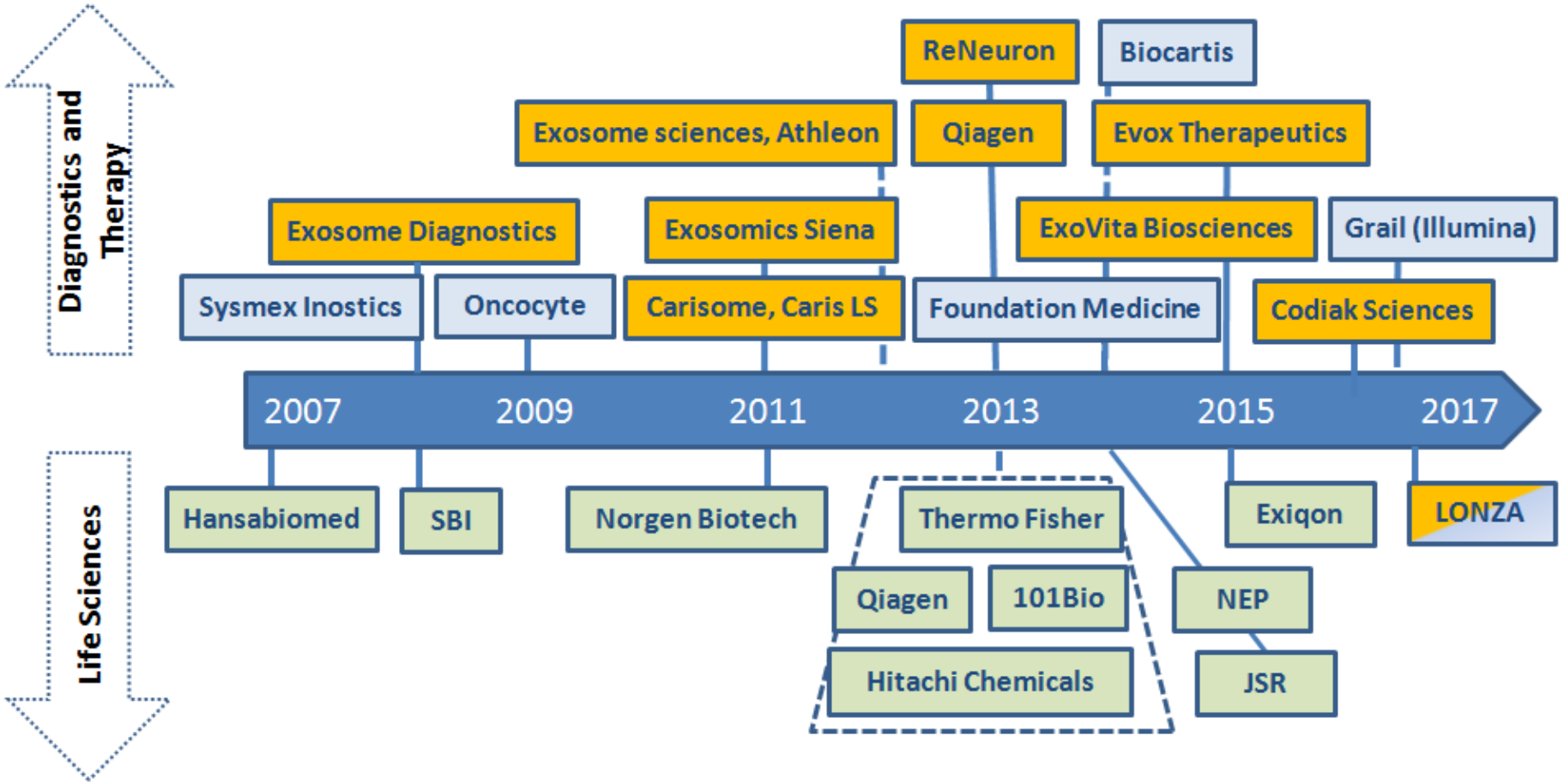


NIH: Program to Assess the Rigor and Reproducibility of Exosome-Derived Analytes for Cancer Detection, Extracellular RNA Communication, Role of Exosomes in HIV Pathogenesis

ClinicalTrials.gov: biomarkers, gene delivery, regenerative medicine

EU: COST, MC-ITN, EuroNANOMED, RIA, IMU

Industrial EV ®evolution

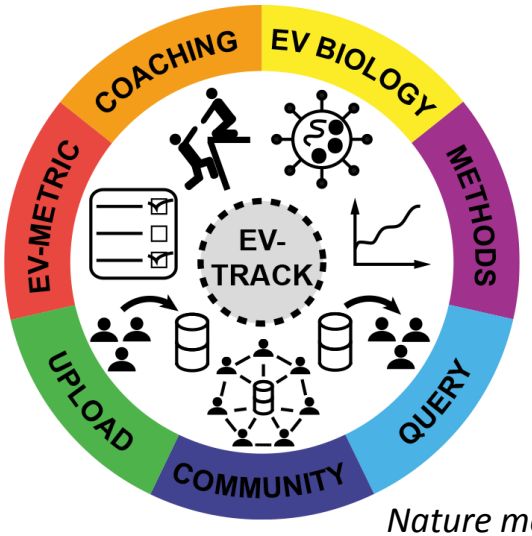


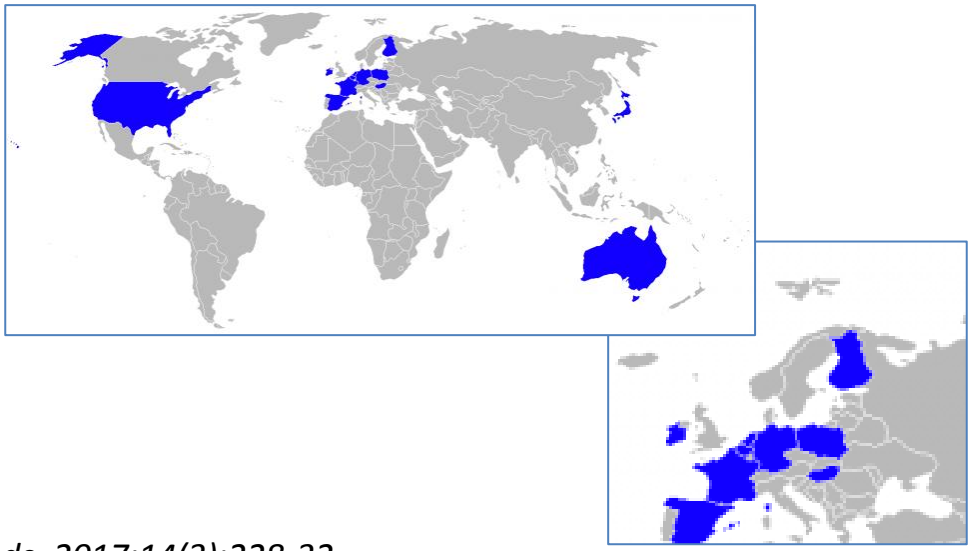
From “lab-specific” protocols to standards

EVQC (GEHNT UNI) : Academic EV isolation methods vs Providers of EV isolation methods

Yield - Purity - Repeatability - Heterogeneity - Accuracy / precision - Efficiency - Detection limit - Cost / hands-on time / hardware investment / turnaround time / sample input amount requirements

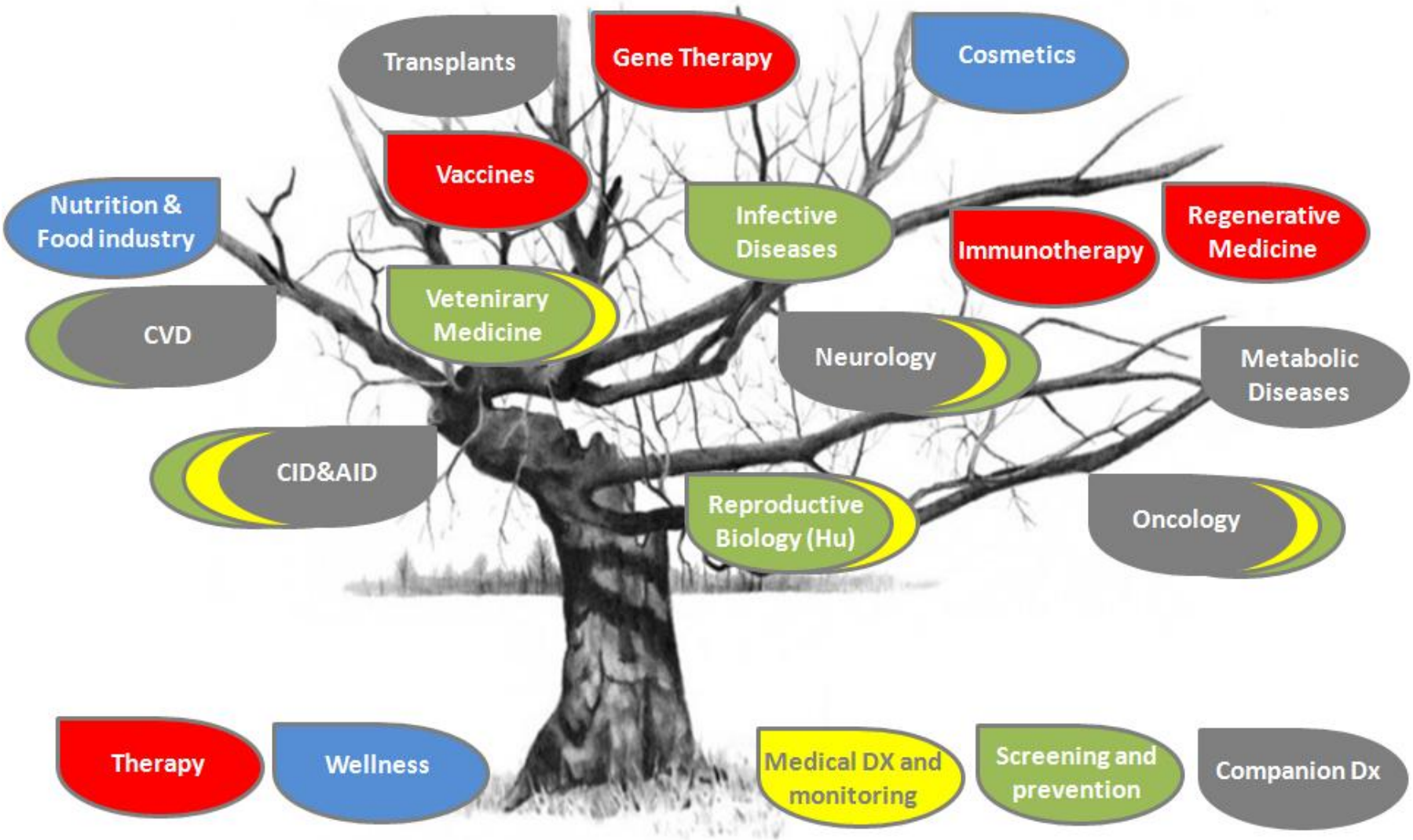
EV-TRACK: *transparent reporting and centralizing knowledge in extracellular vesicle research*



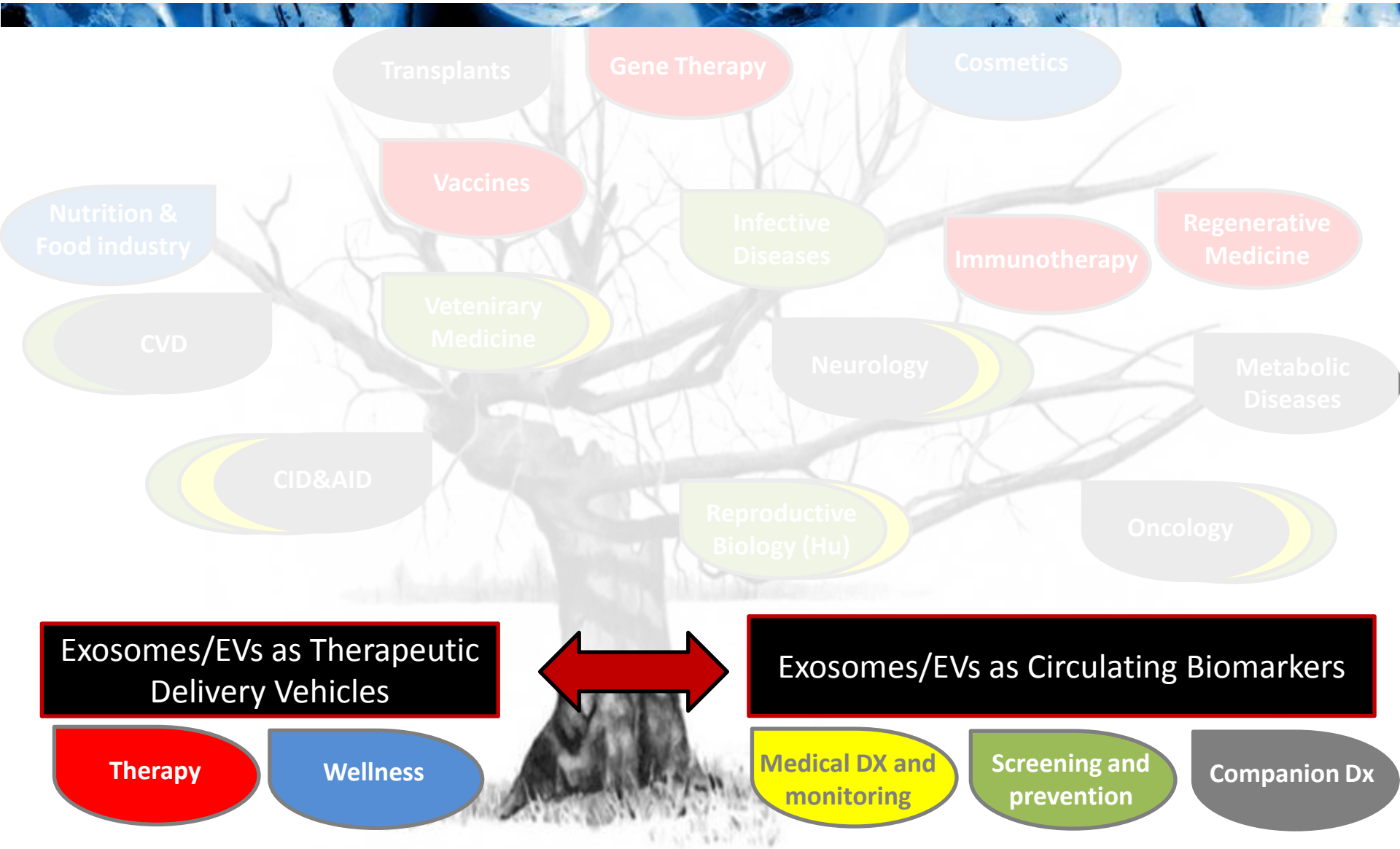


Nature methods. 2017;14(3):228-32.

One Root different directions

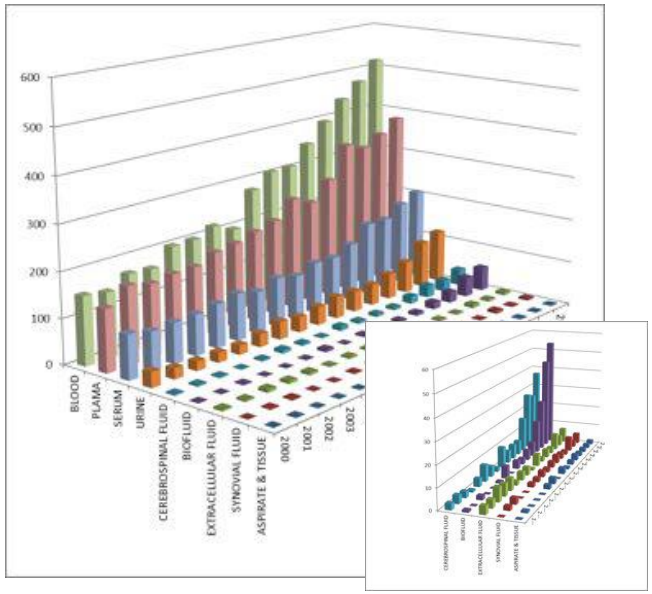


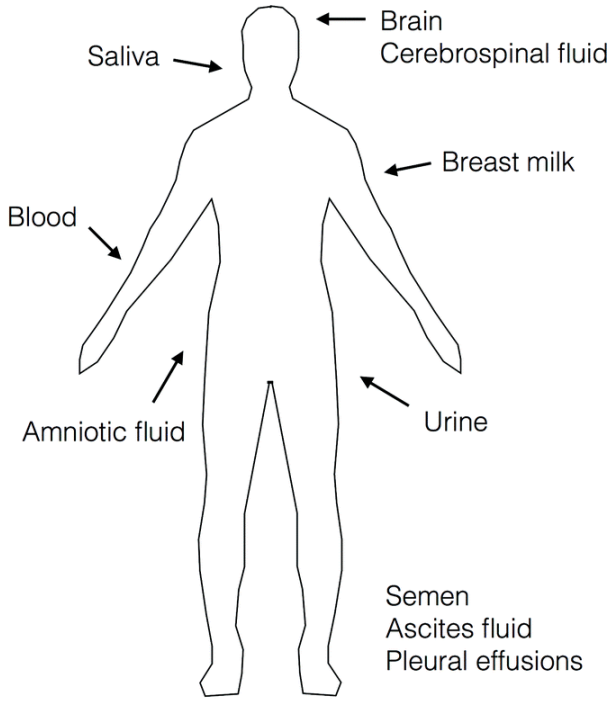
Two major exploitation paths



EV/Exosomes as circulating biomarkers

- **Exosomes circulate in periferal blood and in different biological fluid**

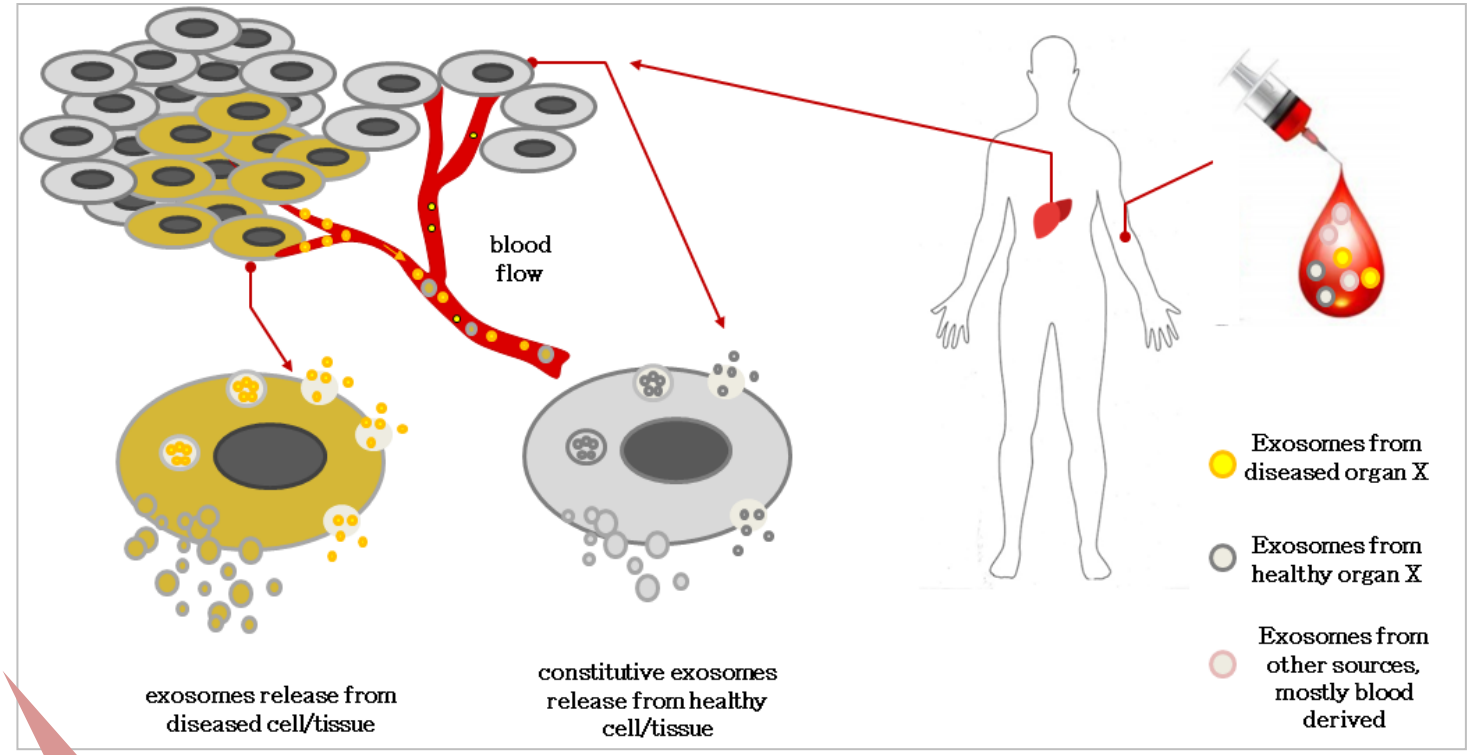




- **Exosomes as concentrators of biomarkers:**

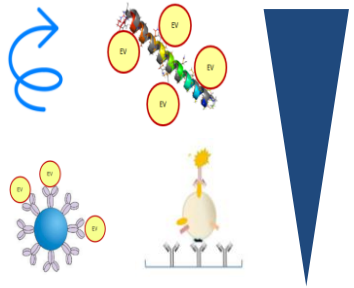
✓*Reflect the proteic and genomic content of the parent cell **
✓*Enable secretion of otherwise non-soluble molecules*
✓*Enrich in low-abundant molecular species*
✓ *Can be selectively isolated*
✓*Enrich in some acknowledged cancer markers*

EV/Exosomes as circulating biomarkers



Preanalytical protocols and Devices

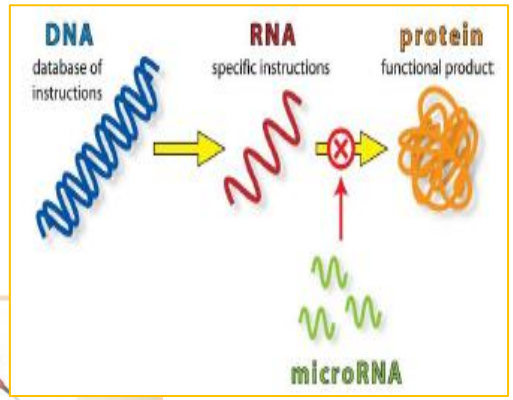
EV/Exosomes Isolation from complex matrix : Different isolation protocols ensure different stringency /yield/purity

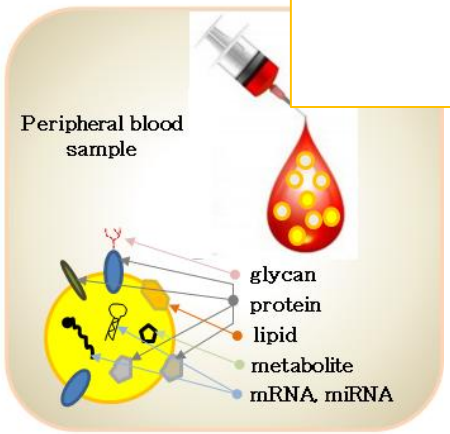


Total EVs (exosomes + MVs)

Organ specific EVs (exosomes + MVs)

Disease specific EVs (exosomes + MVs)





EV/Exosome binding antibodies & agents, surface chemistry

EV/Exosomes biochemical analysis, counting and sizing

EV/Exosomes physical properties, mass, coloidal, stiffness

EV/Exosomes quantification, quality control markers and normalizers for different categories of informative markers, QC

Preanalytical protocols and Devices

Isolation

Isolation of target EVs

Isolation of EVs and/or cell-free DNA/RNA

Extraction

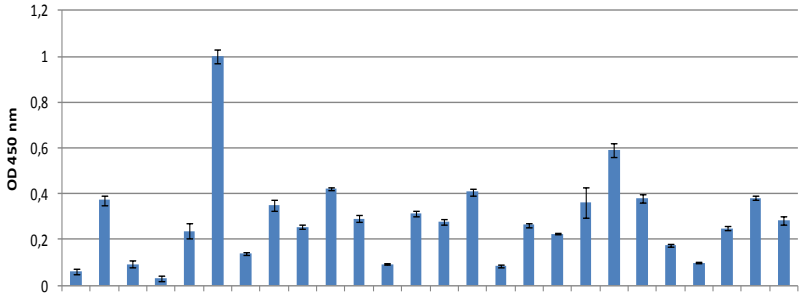
Optimized extraction for high quality proteins /DNA/RNA

Detection

PREANALYTICAL VARIABILITY
Major source of variability and error in diagnostics

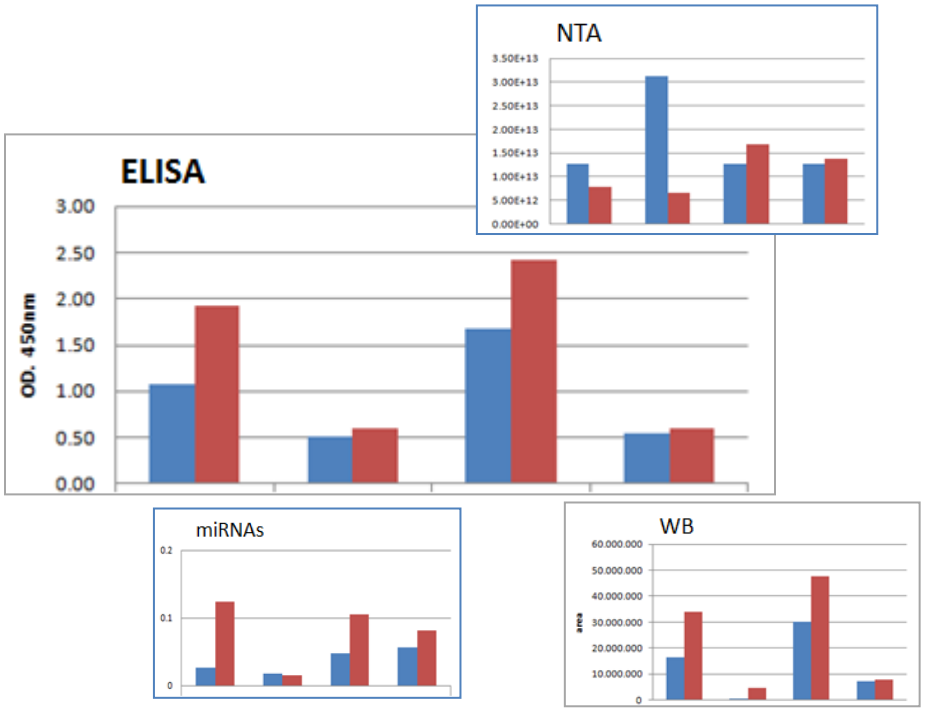
biological variation
can be controlled or at least recorded

Quantification of CD9+ exosomes in plasma



Different healthy donors →

technical variation
is introduced by sample collection&processing.



Same healthy donors – different plasma separation protocols

- **Selective focus on EVs of interest**
- **Definition of baseline levels variations**
- **Normalisation strategies**

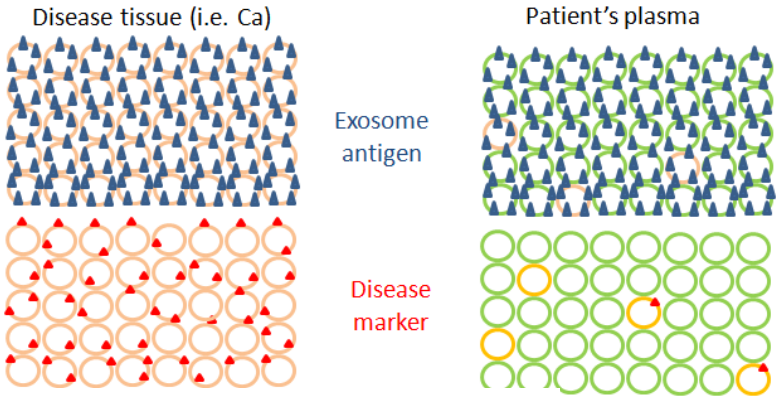
Preanalytical protocols and Devices

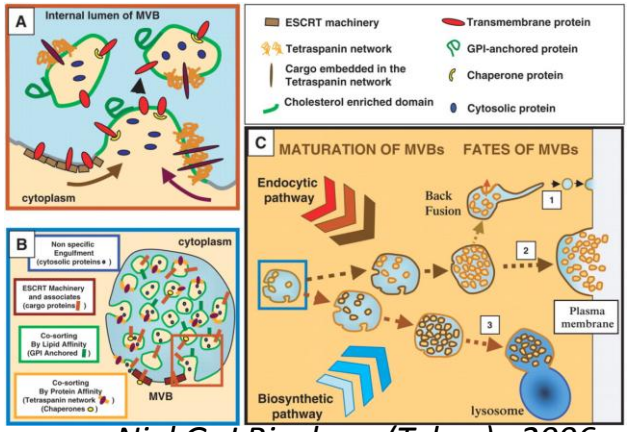
- **Harmonisation of preanalytical protocols**

EV/EXOSOMES HETEROGENEITY :
Major challenge in detection of relevant EV markers

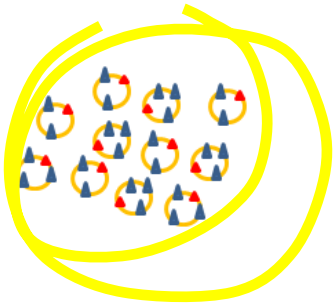
1. Theoretically, every exosome could have unique molecular composition *Sverdlov ED, Bioassays (2012*).

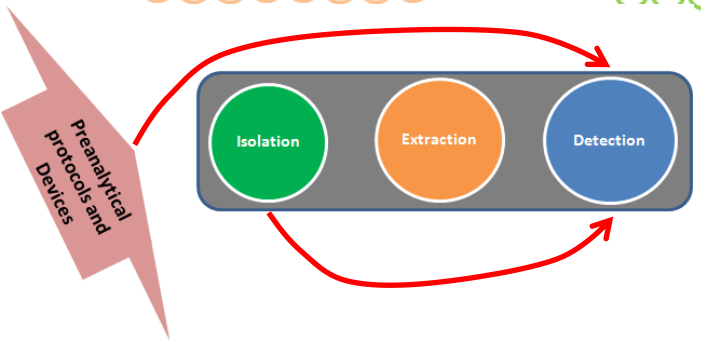
2. Specific tissue derived exosomes are drastically diluted in complex biogluids (likely to <0.01%)





van Niel G: J Biochem (Tokyo) . 2006

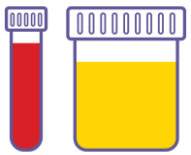




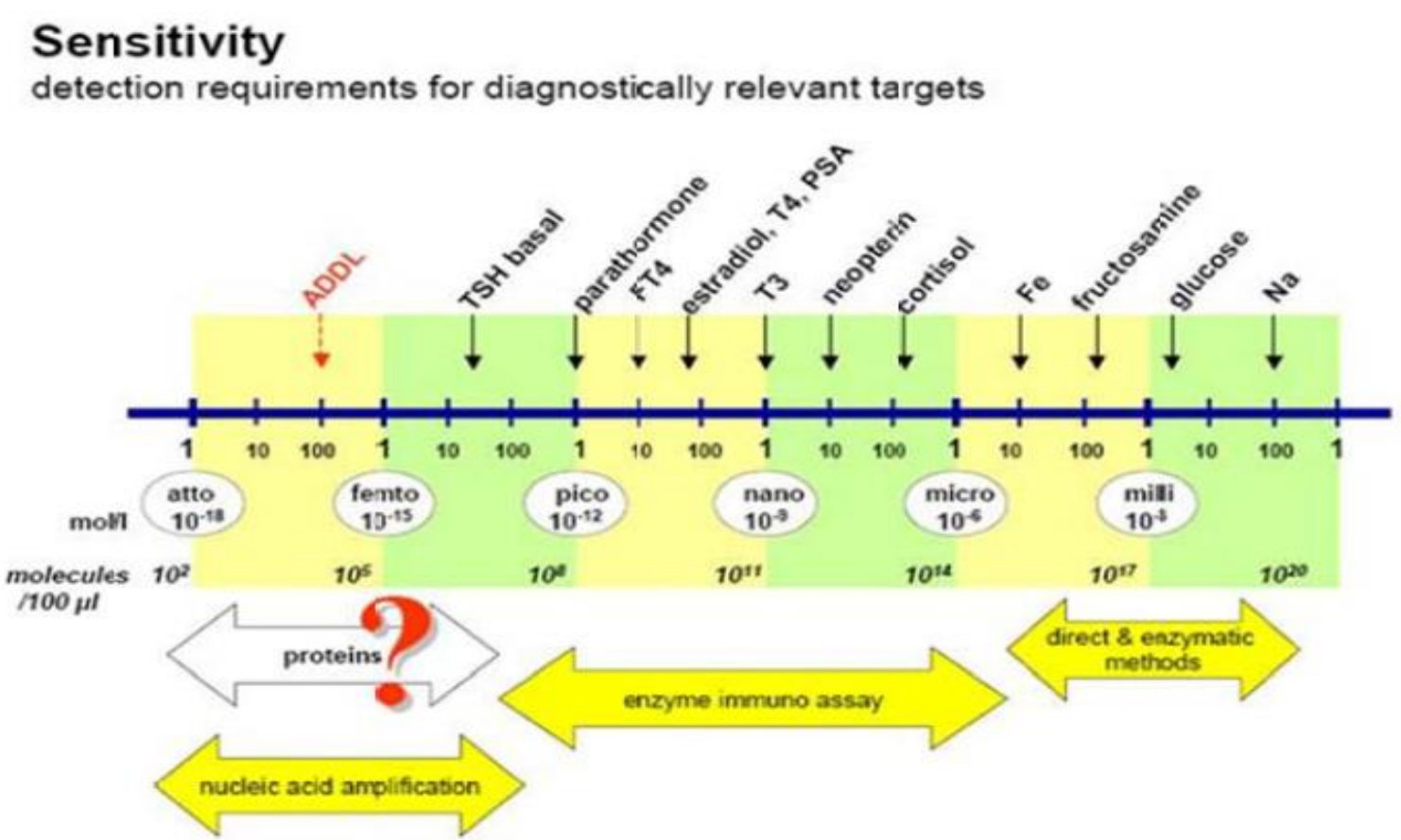
3. EVs can be selectively recovered from biofluid samples
-size, charge, density
- immuno-affinity isolation

True requirement for EV based diagnostics: streamlined isolation and sensitive quantification of associated molecules

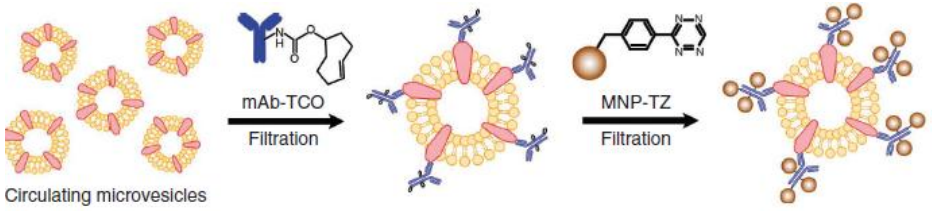
Assumptions:		**Calculations:**
Detection Ab K_D	1nM	For a picomolar assay minimum concentration of exosomes is **6 10^{11} molecules/ml** *(10^{-9}molar x 1litre/1000ml x 6*10^{23} molecules/mole)*
Total concentration of exosomes in blood	10^{10}-10^{12} in heathy 10^{12}-10^{14} in cancer exosomes/ml	Cancer exosomes in 1 ml of blood is ~ **$10^{9\text{-}10}$ cancer exosomes** *number of circulating exosomes/ml (10^{12}-10^{14}) x cancer exosomes/totall exosomes(0.1-5%) x separation efficiency (0,5))*
% cancer exosomes over total exosomes	0.1%	
Density of protein marker per exosome	1 marker/exosome	Concentration factor needed **100 x**
Efficiency of extraction	50%	Sample volume needed 1-10 ml for reaction volumes of 10-100 microliters

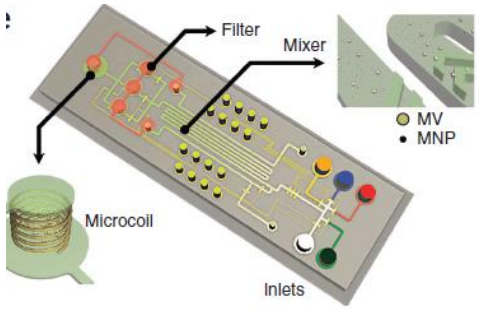


True requirement for EV based diagnostics: streamlined isolation and sensitive quantification of associated molecules

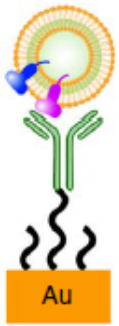


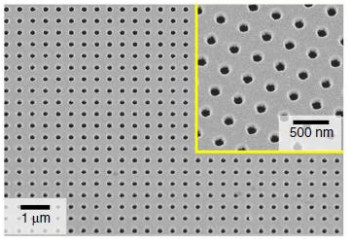
Do we need advanced tecnhnologies for detection of EV protein markers?



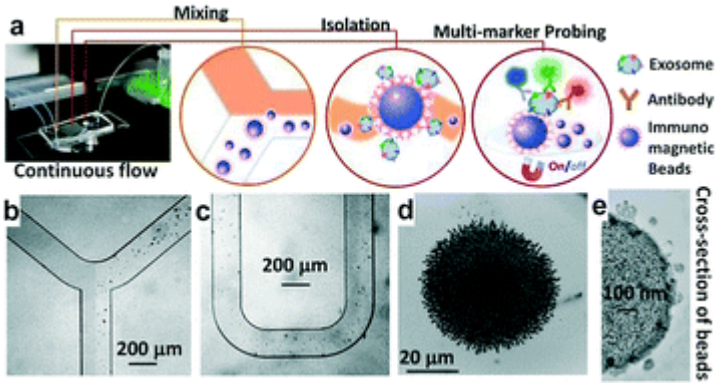


Shao et al. Nat Med 2012

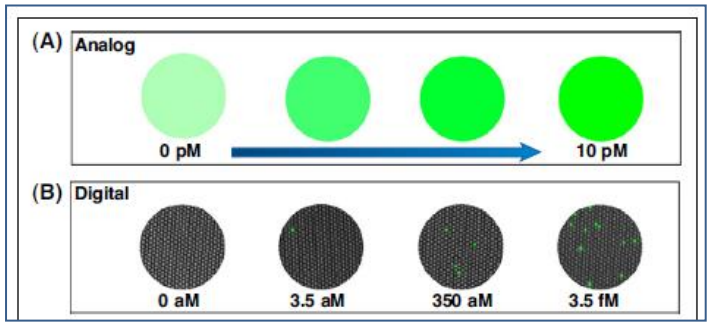




Im et al. Nat Biotech 2014

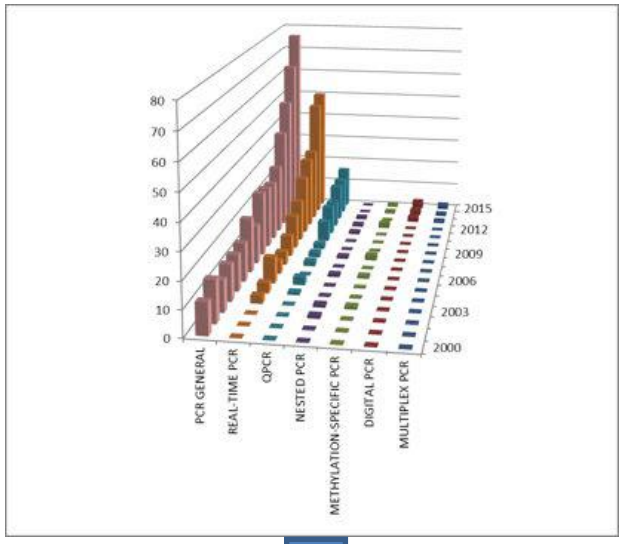


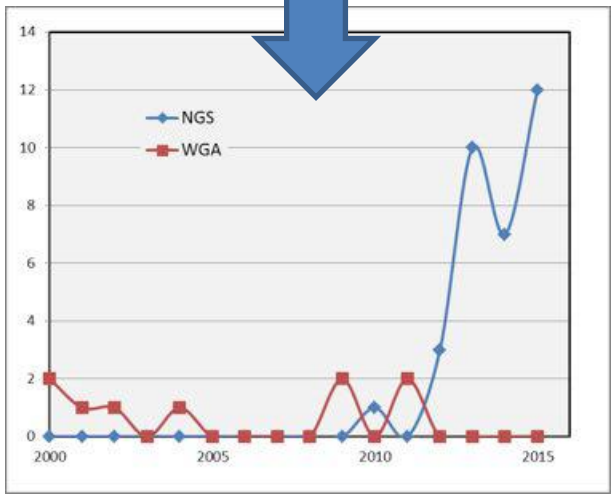
Zhao et al. Lab Chip 2016



Cretichet al. Trend Biotech 2015

PCR remains a Key Tool with outreach vs. NGS

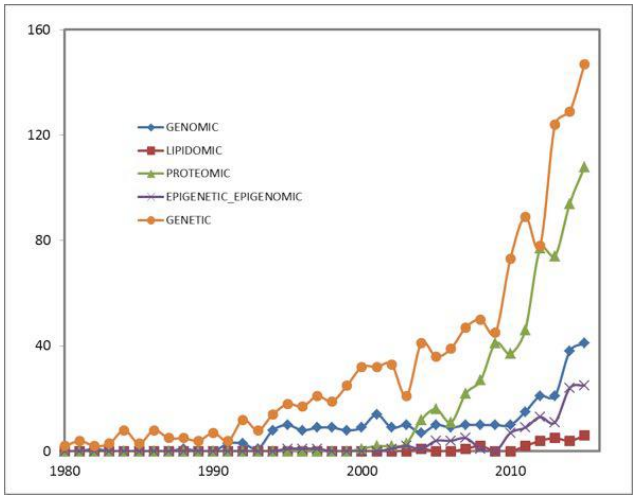




Technique	Sensitivity
Sanger sequencing	> 10%
Pyrosequencing	10%
Next-generation seqeuncing	2%
Quantative PCR	1%
ARMS	0.10%
BEAMing, PAP, Digital PCR, TAM-Seq	0.01% or lower

1. Driver: methodological issue

What are the most exploited EV biomarkers today?

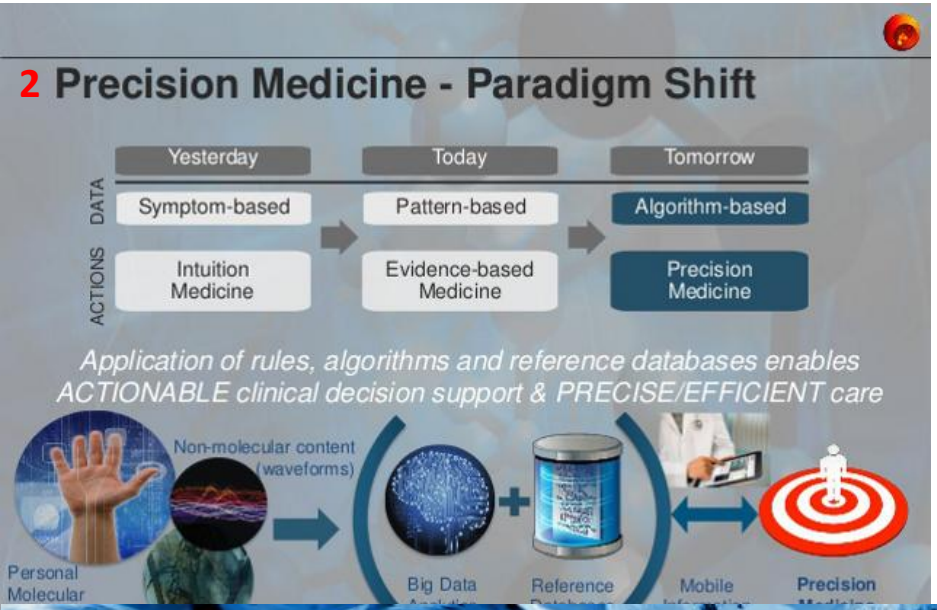


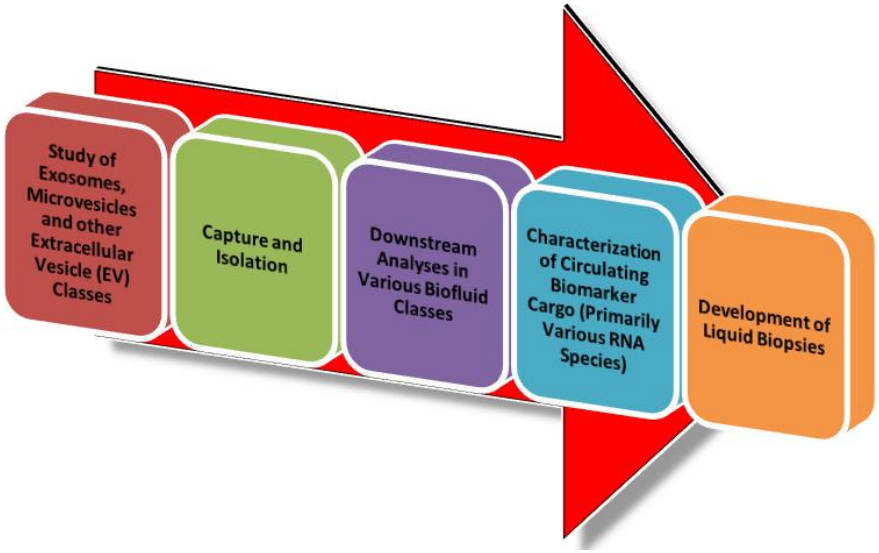
- Most work has been done on miRNAs

- EV DNA based assays are the first ones that hit the clinical use:

First LDT for Alk in Lung Cancer lunched by Exosome Dx in 2015

First IVD for CDx (EGFR) by Roche Dx FDA approved in 2016

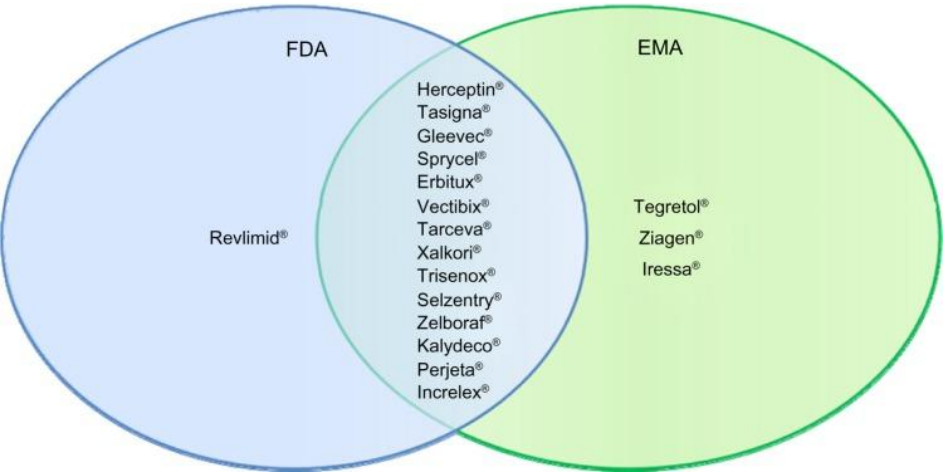




First dowel in the puzzle – Companion Diagnostics

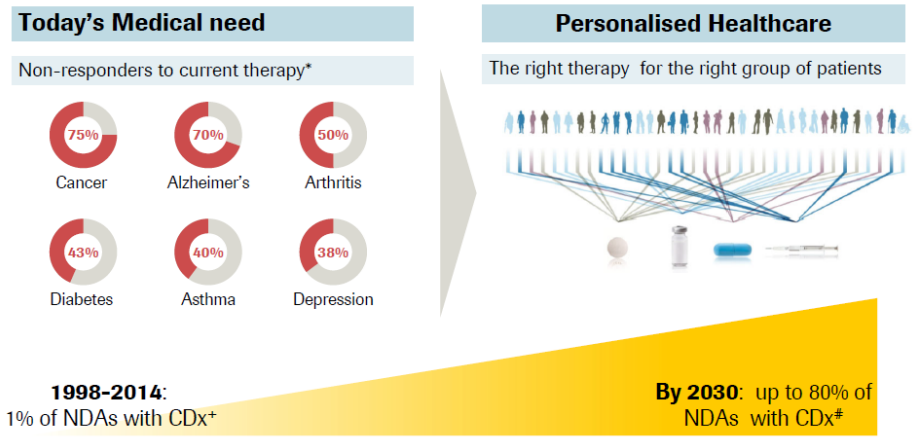
Targeted therapies are *already* standard of care

Companion Dx pipeline today



Drugs with required pharmacogenetic companion diagnostic testing according to EMA and FDA

Companion Dx pipeline evolution

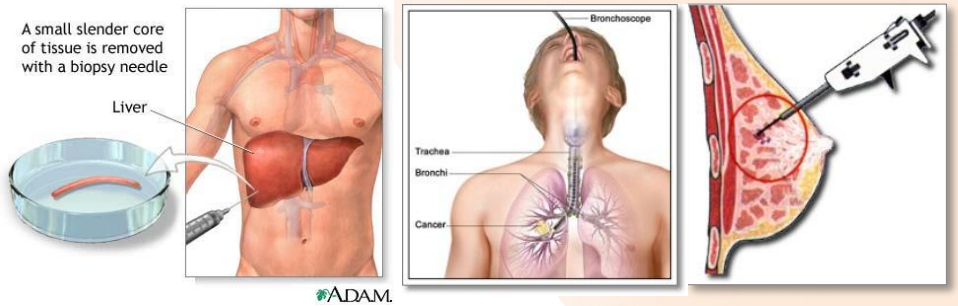


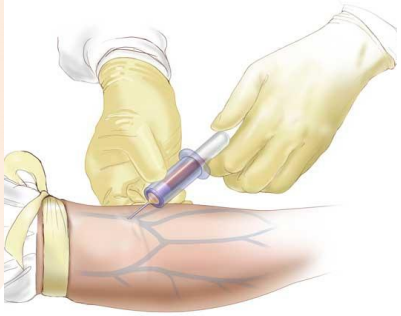
From Roche report

3. Driver: Market need

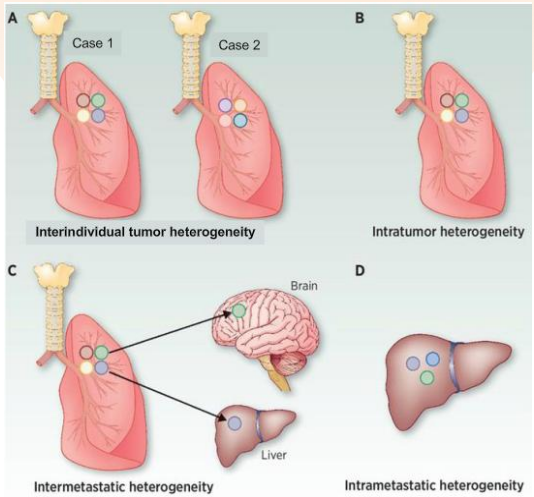
The impact of liquid biopsy for precision medicine in cancer: alternative and complementary to conventional tissue biopsy

Tissue Biopsy *vs.* *Liquid Biopsy*





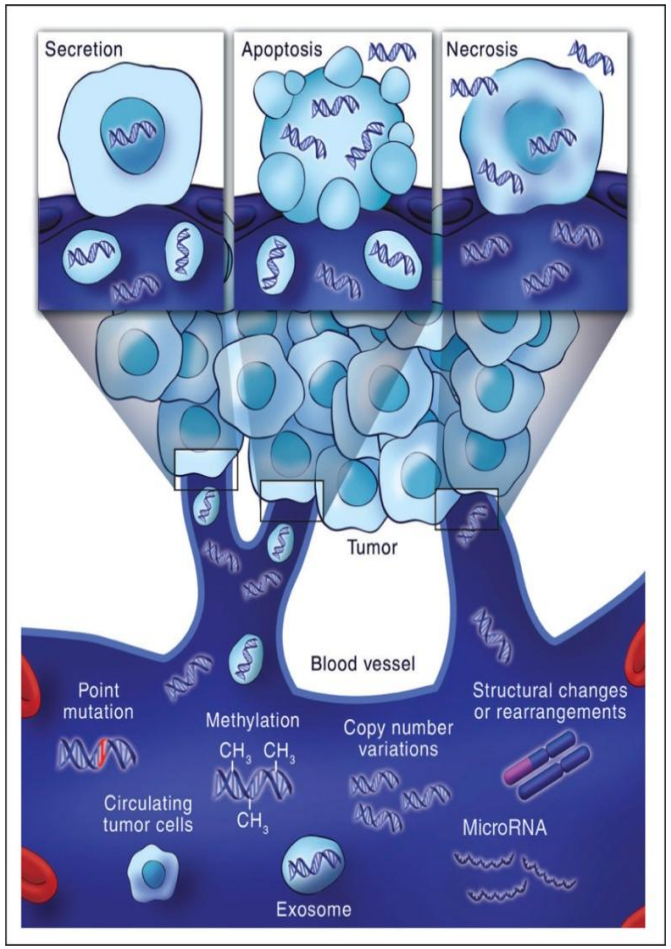
- Invasive, painful
- Expensive & time consuming
- Re-biopsy often not possible or accepted
- Not suitable for cancer monitoring
- Does not address tumor heterogeneity



Jamal-Hanjani et al Clinical Cancer Res 2015

- Minimally invasive, no risk for patients
- Cheap and quick
- Re-biopsy is not a problem
- Trully suitable for cancer monitoring
- Addresses tumor heterogeneity

Major sources of cancer biomarkers for liquid biopsy

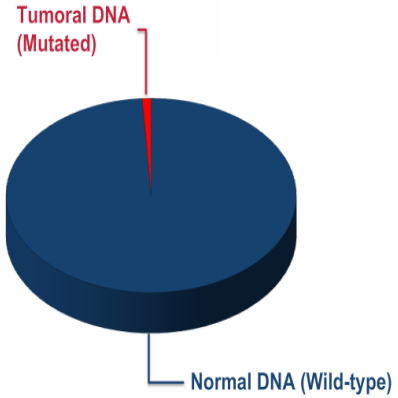


Diaz & Bardelli Journal of Clinical Oncology

Circulating cell-free nucleic acids

Most studied source of genetic alterations today but has serious limitations

- ctDNA ≠ cfDNA!
- Huge background
- Can not be selectively isolated



Circulating tumor cells

Cancer cells released by the primary tumor in circulation.

Great investments but expectations not fullfilled

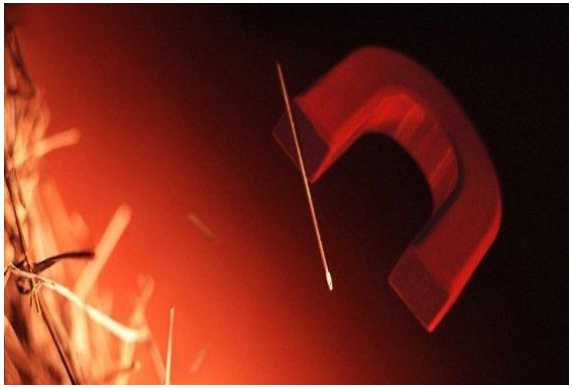
Extracellular vesicles

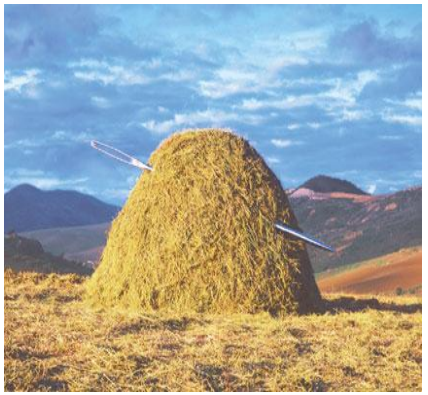
Offer unprecedented advantages (see next slides)

Technology Comparison

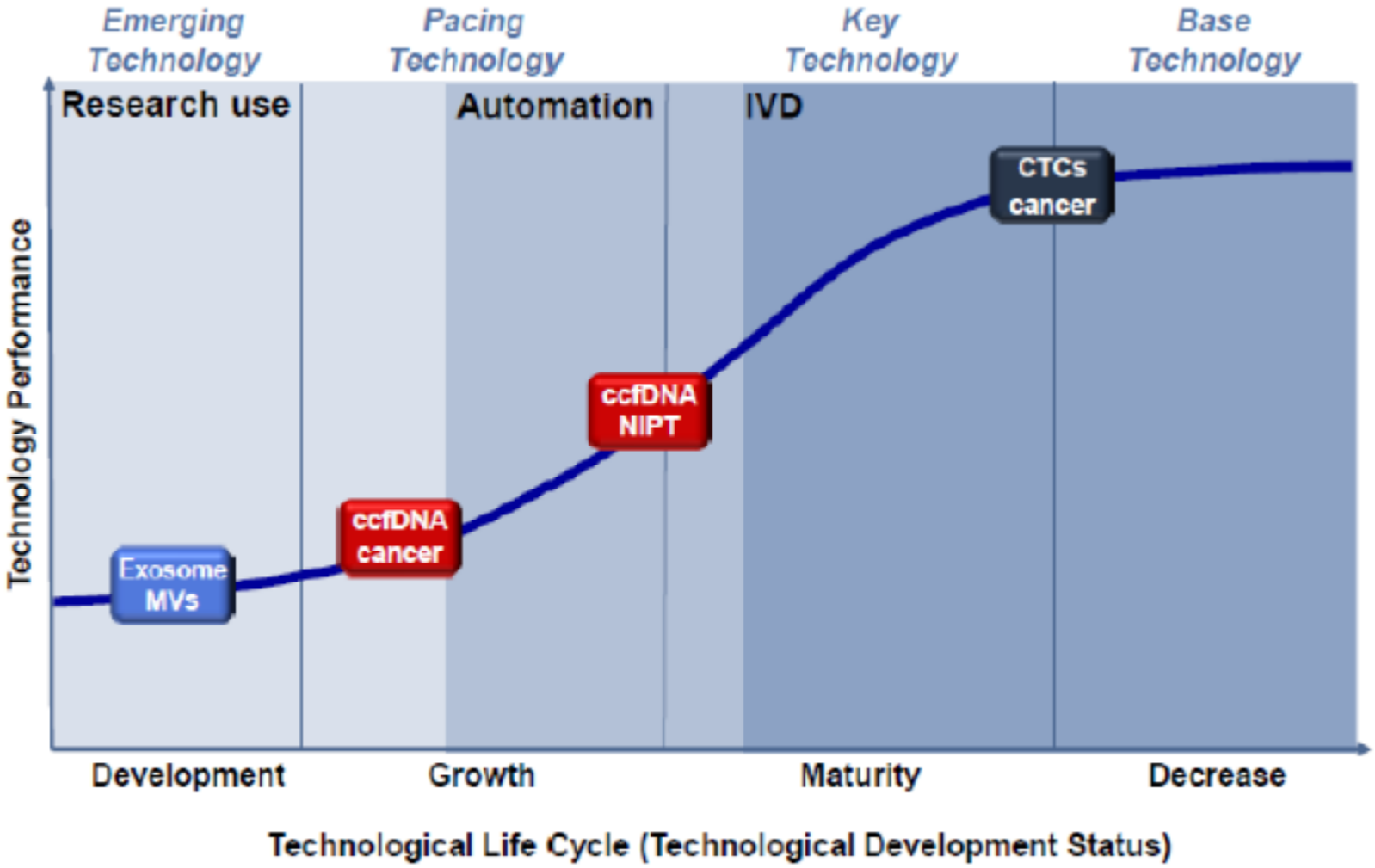
Exosomics Approach vs Current Technologies

Important Attributes	CTC	ctDNA	Exosomics Approach
Abundance of Biomarkers in the body fluid	✗	✓	✓
Ability to Enrich Selectively the Tumor-Associated Biomarkers	✓	✗	✓
Ability to reach Low Limit of Detection and high Accuracy	✗	✗	✓
Ability for Early Cancer Detection with Low Concentration of Biomarkers	✗	✗	✓
Ease of Use	✗	✓	✓
Avoidance of Complex and Expensive Equipment	✗	✓	✓



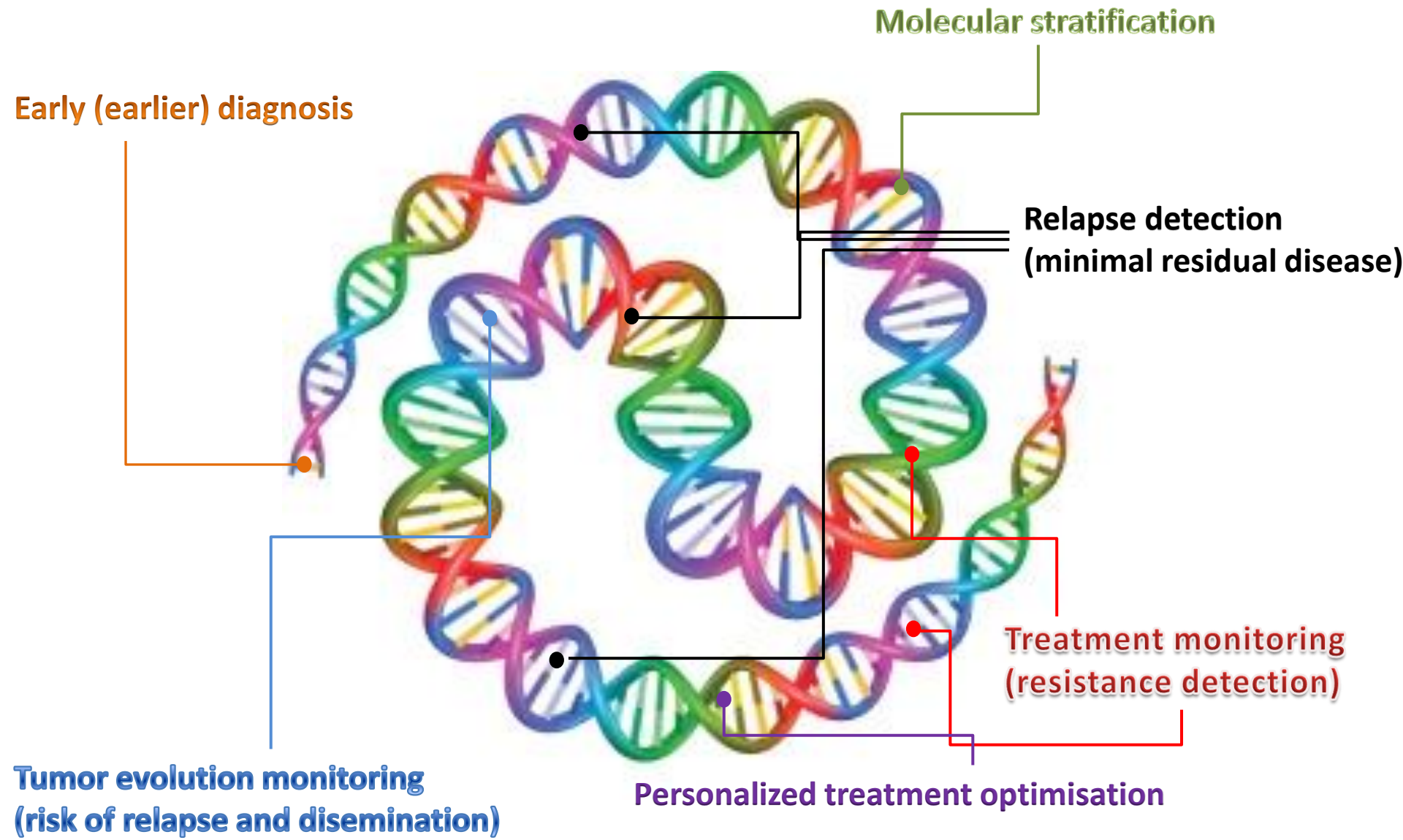


Different timelines, performance and readiness level



http://www.ecmcnetwork.org.uk/sites/default/files/ECMC%20cfDNA%20Qiagen%20Frietsch%2020141124.pdf

Lot of dowels are still missing across the patient management cycle and all can be prompted by EV/exosomes based liquid biopsy



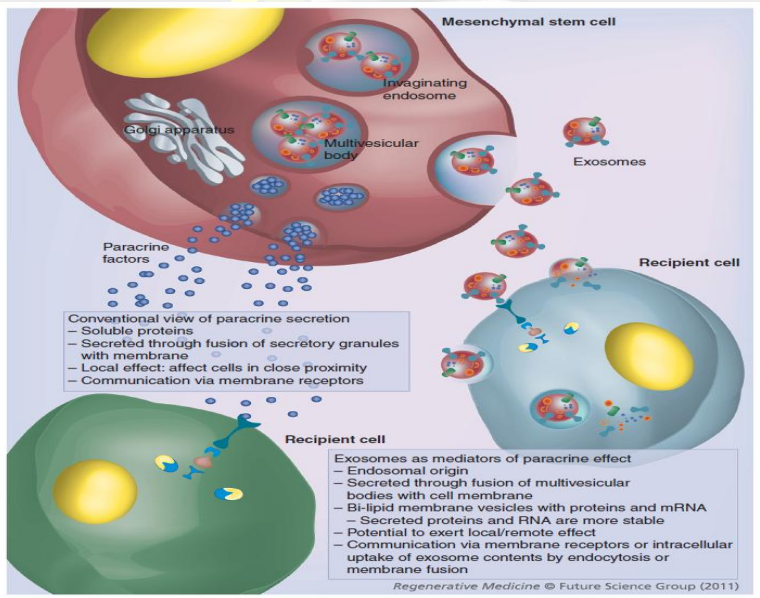
EVs in Human Healthcare -cell-free regenerative medicine

Transplants
Gene Therapy
Cosmetics
Vaccines
Infective Diseases
Immunotherapy
Regenerative Medicine
Veterinary Medicine
CVD
Neurology
Metabolic
Biology

EXOSOMES PROs

- Not immunogenic if extracted from proper source
- Very stable -long circulating life and long shelf life
- Defined tropism to specific tissue or microenvironment
- Intrinsic biological activities
- Intrinsic ability to cross biologic barriers
- Specific sorting mechanism of proteins to exosomes that helps predicting or engineerizing surface moieties
- Loadable with nucleic acids and small molecules
- Less complex then viable cells

BioRegenerative SCIENCES
ReNeuron
INTREXON
esperite you owe it to your family
zenbio
Capricor Therapeutics
ANSYS
evox THERAPEUTICS
Agency for Science, Technology and Research SINGAPORE
Università di Torino MBC Molecular Biotechnology Center
Universitätsklinikum Essen Transfusionsmedizin



Emerging areas with unmet needs and huge markets

-Veterinary testing
-Animal reproduction
-Food safety

zoetis

Gene Therapy

Cosmetics

Vaccines

Nutrition & Food industry

navhetec

Infective Diseases

Immunotherapy

Regenerative Medicine

Neurology

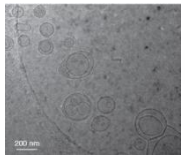
NANOSOMIX

Exosome Sciences

CID&AID

Reproductive Biology (Hu)

Hansa BioMed



Edible exosomes:
-Easily available
-Well tolerated
-Intrinsic activities

MERCK

GFI Grant for Fertility Innovation

exosomics SIENA

CNS exosomes:
-Early screening tests
- Surrogate markers

morinaga MORINAGA MILK

DANONE

MeadJohnson Nutrition

-Assisted reproduction
- Prenatal testing

Companion Dx

EVs in cell-free regenerative medicine

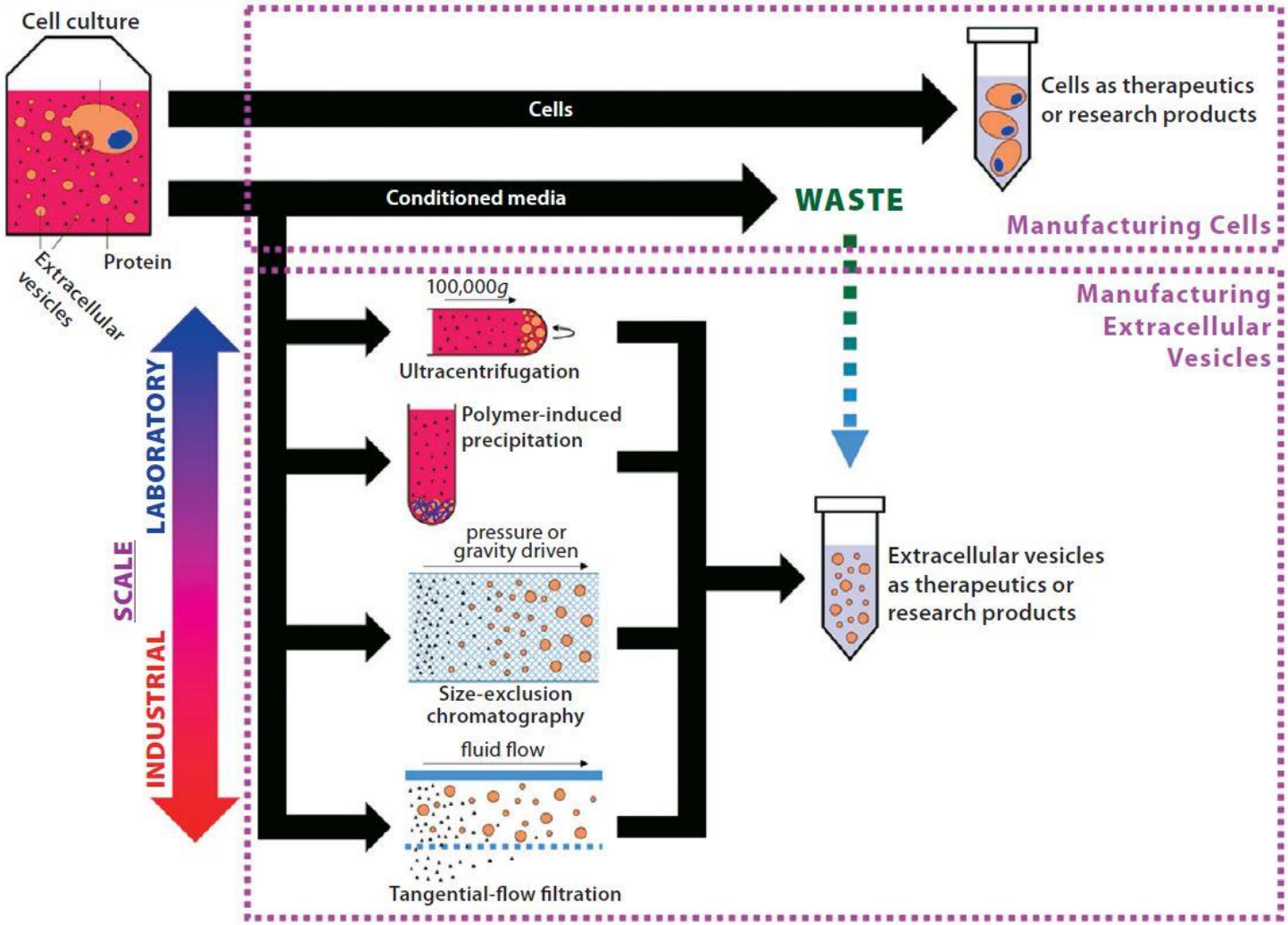
CONs

- Complexity of exosome cargo and exosome activities
- Possible wide tropism and off-side effects
- Lack of reliable and efficient methods for obtaining well defined and pure exosome preparations

OPPORTUNITIES:

- Ex vivo and in vitro modification
- Fit-to-the purpose purification methods
- Path to scalable and GMP manufacture
- Compliance with regulatory reqirements
- QC testing through manufacturing (DX and potency assays)
- New sources (plants, platelelts etc)
- Synergy with drugs/agents
- Advanced preclinical testing
- Clinical trial capacity with experienced physicians

Large scale production of exosomes from cell culture



Smith et al.

True enabling technical requirement: fit to the purpose EV/ exosome isolation and…..

Method	Available technologies	Pros	Cons
Ultracentrifigation (UC)	Ultracentrifuge with speed >100000g	Widely accepted and standardized protocol	EV aggregation; labor and time consuming
Optiprep-Density Gradient	Optiprep + ultracentrifuge	Only method that uses both size and density to separate EVs; highest EV purity	EV aggregation; labor and time consuming Multiple steps that require technical skills
Chemical precipitation	Exoprep (HBM), Exoquick (SBI), Total Exosome Isolation (Thermo Fisher)	Simple procedure; Faster than UC	Contaminats co-precipitation; poorly and wide molecule size range; need to remove polymer after isolation
Size-exclusion chromatography (SEC)	Sepharose 2B and CL2B, qEV (Izon), HBM columns	Single step process; high purity isolation; affordable reagents and tunable processing time	Colums must be optimized for large scale volumes
Tangential flow filtration	TFF capsules with 100kDA MWCO	Scalable; faster than UC; high enrichment potential	May require additional steps including UC
Immuno-affinity	Exosome-targeting antibodies (HBM, EXS)	Scalable, faster than UC, high enrichment potential including for EV subpopulations	Antibodies need to be removed after EV isolation for applications that require intact EVs

.....and quality control: what do we need to check?

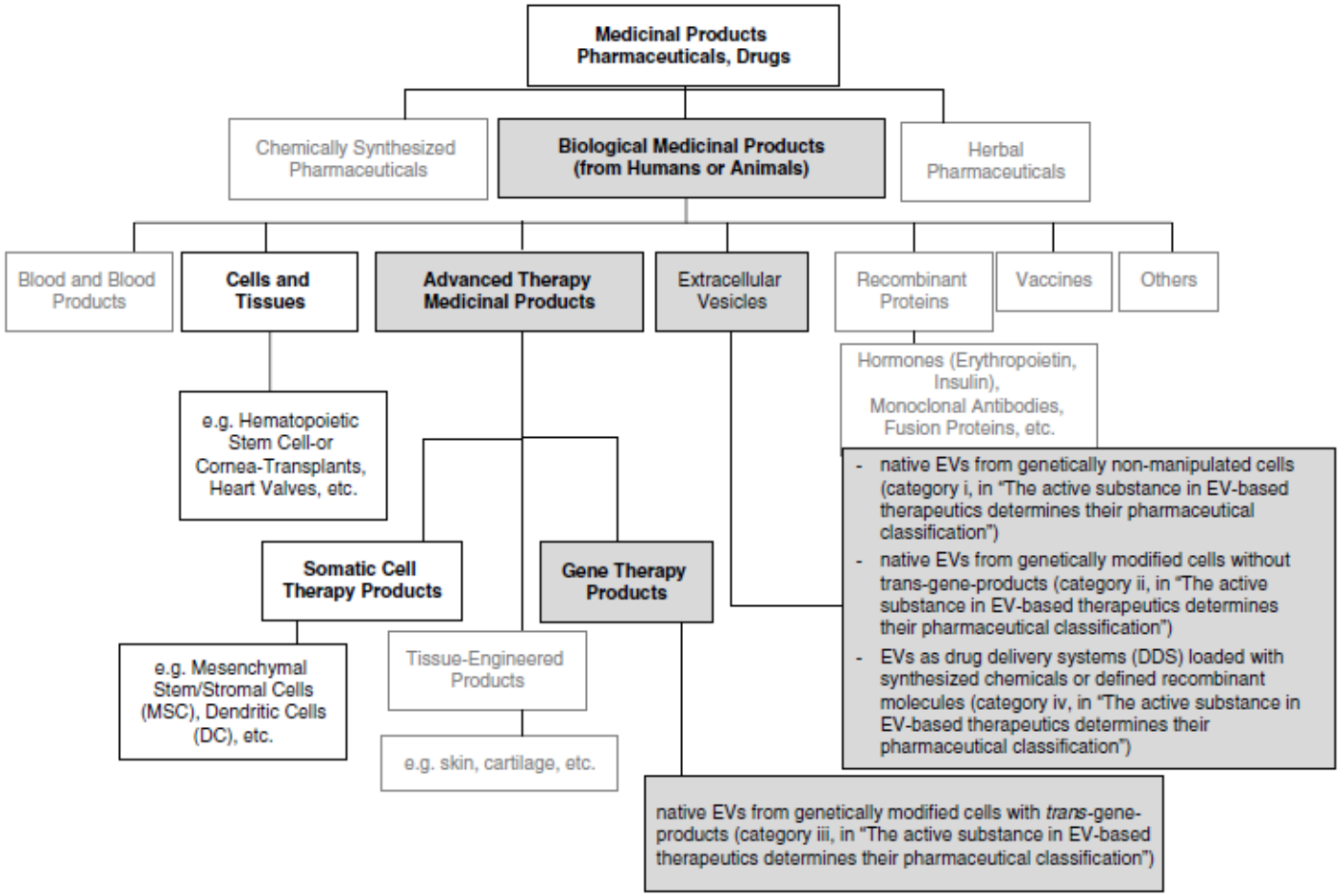
Task	Objective	Technology
EV (exosome) size	Monitor size consistency	• TEM, light scattering, NTA
EV markers	Check for both generic and cell specific EV markers.	• Western blot or mass spec for protein biomarker • PCR and/or NGS for nucleic acid biomarkers • Tandem HPLC and mass spec for lipids and metabolites
Purity markers	Check for intracellular markers (eg Golgi proteins) and serum markers (albumin)	• Immunoassays • PCR for nucleic acids
Integrity and funcionality	Check for integrity and activity, expression profiles and assays	• Protection assays • potency assays or surrogates
Sterility (eg. EVs for therapeutic use)	Check for microbiological contaminants	• As laid down in regulations for blood products, ATMPS and pharmacopeia
EV stabilization	Chemical stabilization	• Under development
Formulation	Define optimal EV storage and formulation	• Additives • Lyophilization

Clinical trials are a reality

Identifier	Source	delivered drug	disease	purpose	phase	size of cohort	Location
NCT01294072	Plant exosomes	Curcumin	Colon Cancer	Plant exosomes delivery of curcumin to normal colon tissue and colon tumors	I	35	USA
NCT01668849	Grape-derived exosomes		Head and neck cancer	Reduce the incidence of oral mucositis during radiation and chemotherapy treatment	I	60	USA
NCT01854866	Treated tumor cell-derived microparticles	Chemotherapeutic drugs	Advanced cancer	Treat malignant ascites and pleural effusion in patients with advanced cancer	II	30	China
NCT02507583	Treated tumor cell-derived Evs	Antisense oligonucleotide against IGF1R	Malignant Glioma	Trigger immune responses	I	32	USA
NCT02657460	Methotrexate-autologous tumor derived microparticles		Lung cancer	Treat malignant pleural effusion	II	90	China
NCT01159288	Tumor antigen-loaded dendritic cell-derived exosomes		Non Small Cell Lung Cancer	Activate the innate and adaptive immunity	II	47	France

Kooijmans et al. 2016

While regulatory status still needs to be defined, at least in Europe



ISEV position paper 2016

EVs have gone commercial already……

EXOFACE

Exosome moisturizer

MISSION PERFECTION SERUM

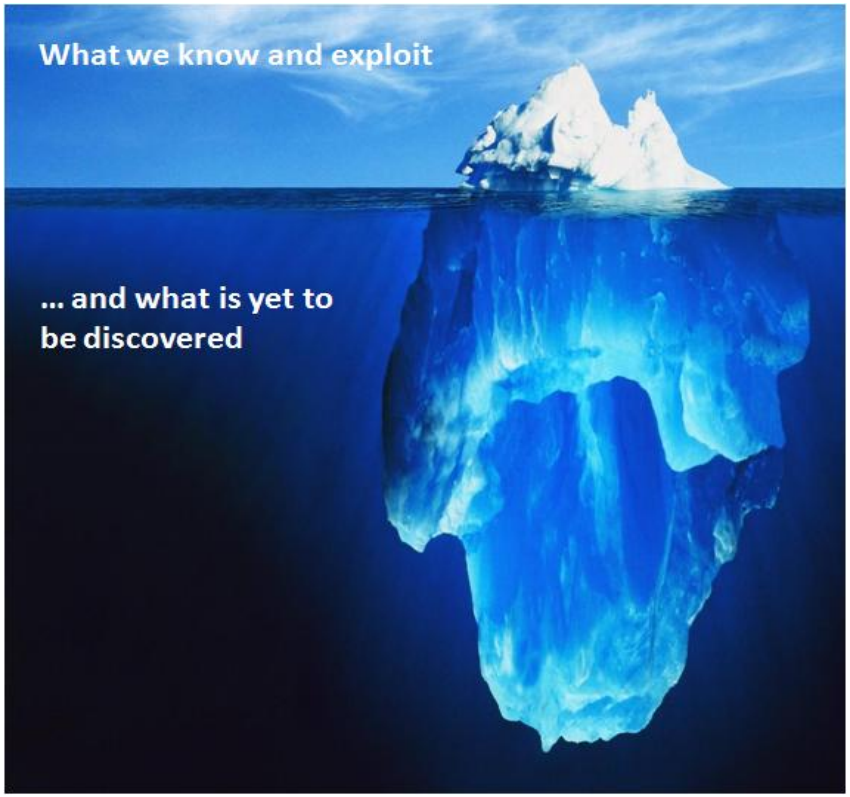
Clarins

….or are about to

NUTRICIA
Advanced Medical Nutrition

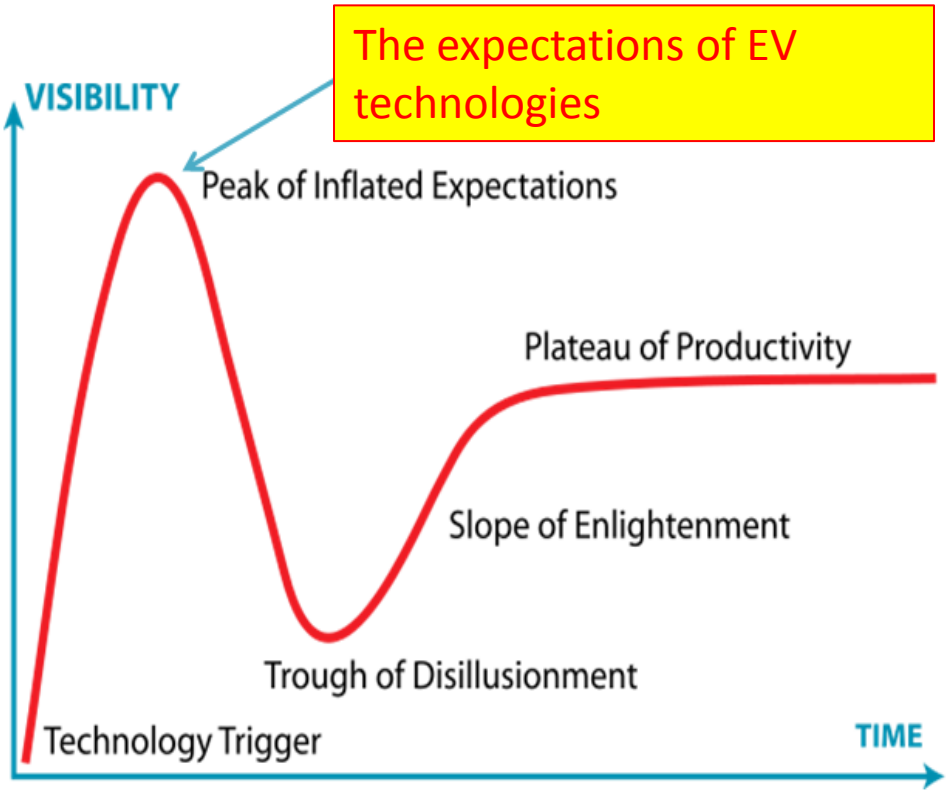
DANONE

There is still a long way to run



The Hype Cycle

(representing the maturity, adoption and application)



The expectations are high, some technological components are not ready on the market – great opportunity for who comes first to fulfill the niches.....

EVs in medicine: oversold or underappreciated?

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