European Society for Liquid Biopsies

Cerebrospinal Fluid: Pre-analytical Considerations & Unknown Variables

Ursula Sandau, PhD

Instructor

Oregon Health & Science University

May 28, 2024

Acknowledgements

21 Members

Scientists and Physicians from
North America
Central America
Europe
Australia

Expertise

- CSF Collection, Processing & Storage
- CSF EV Enrichment
- CSF EV Analysis
  - Size and Number
  - Protein
  - RNA
  - Other Cargo

International Society for Extracellular Vesicles (ISEV)
Cerebrospinal Fluid (CSF) Task Force

- Julia Costa
- Beth Coyle
- Juan M. Falcon-Perez
- Andrew Hill
- Tsuneya Ikezu
- Hannah Jackson
- Setty Magana
- Trevor McFarland
- Lissette Retana Moreira
- Rienk Nieuwland
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- Paola Loreto Palacio
- Joanna Palade
- Joseph Quinn
- Ursula Sandau
- Julie Saugstad
- Eric Sribnick
- Huaqi Kate Su
- Kendall Van Keuren-Jensen
- Laura Vella
- Kostas Vekrellis

Acknowledgements

CSF Collection, Processing & Storage

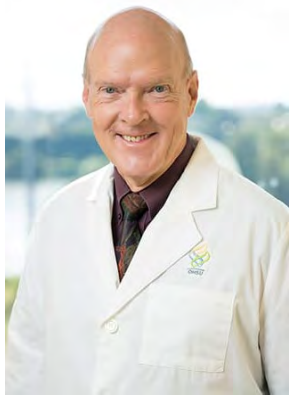
Julie Saugstad, PhD

Oregon Health & Science University

Setty Magana, MD, PhD

Nationwide Children's Hospital

Joseph Quinn, MD



Oregon Health & Science University

Eric Sribnick, MD

Nationwide Children's Hospital

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CSF Task Force

Goal: Improve reproducibility through recommendations & reporting guidelines
Expertise of CSF Extracellular vesicle (EV) community
Review of published studies

Received: 30 June 2023 | Revised: 9 November 2023 | Accepted: 21 November 2023
DOI: 10.1002/jev2.12397

PERSPECTIVE

Recommendations for reproducibility of cerebrospinal fluid extracellular vesicle studies

Ursula S. Sandau[1] | Setty M. Magaña[2] | Júlia Costa[3] | John P. Nolan[4] | Tsuneya Ikezu[5] | Laura J. Vella[6,7] | Hannah K. Jackson[8,9] | Lissette Retana Moreira[10,11] | Paola Loreto Palacio[2] | Andrew F. Hill[12,13] | Joseph F. Quinn[14,15] | Kendall R. Van Keuren-Jensen[16] | Trevor J. McFarland[1] | Joanna Palade[16] | Eric A. Sribnick[17] | Huaqi Su[7] | Kostas Vekrellis[18] | Beth Coyle[19] | You Yang[4] | Juan M. Falcón-Perez[20,21,22,23] | Rienk Nieuwland[24,25] | Julie A. Saugstad[1] | International Society for Extracellular Vesicles Cerebrospinal Fluid Task Force[1]

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DOI: 10.1002/jev2.12404

POSITION PAPER

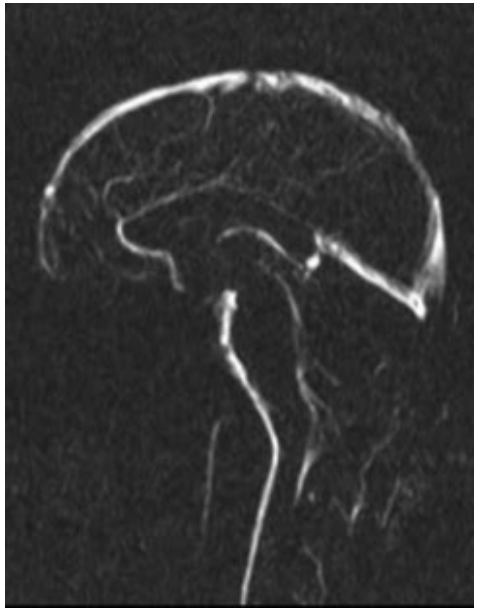
Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches

Areas of Focus

CSF Collection, Processing, and Storage
CSF EV Enrichment
CSF EV Analysis:
Size & Number
Protein
RNA
Other Omics
Functional Assays

1. Recommendations
2. Reporting Guidelines

Utility of Cerebrospinal Fluid as Biomarker Source



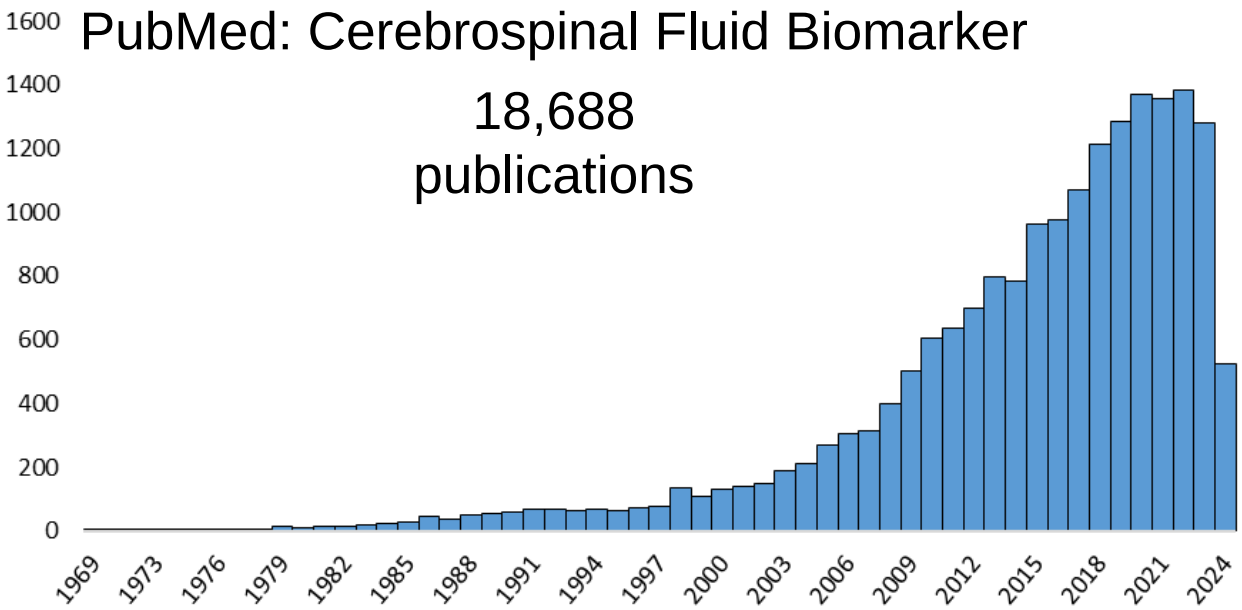
Central nervous system (CNS)

- Brain
- Spinal cord

CSF is biofluid most intimately associated with brain

Biomarker source for neurological disorders

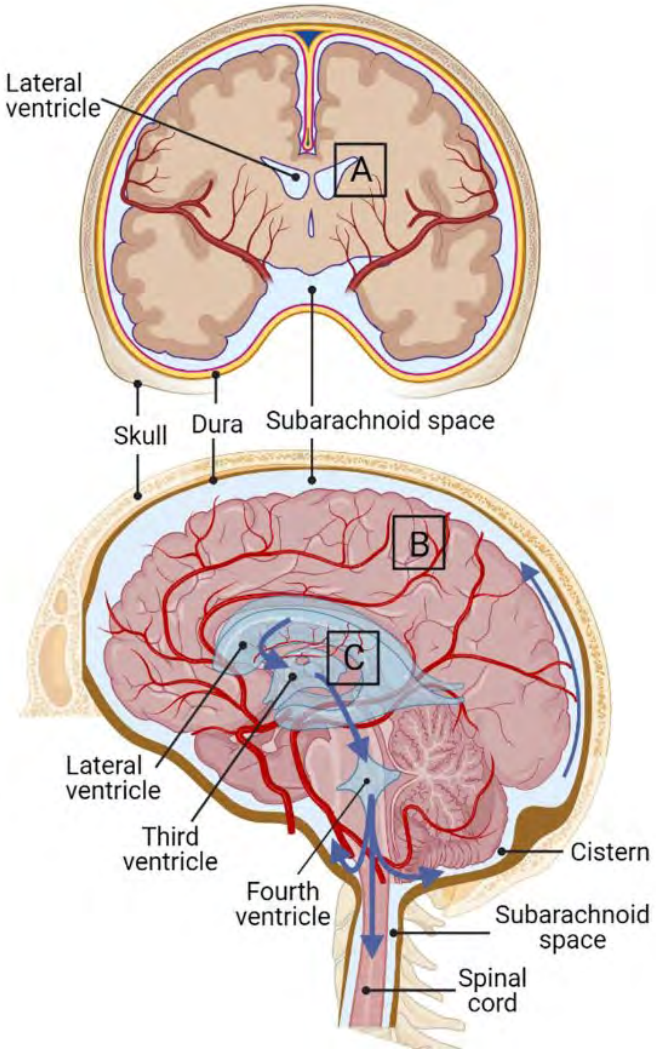
- Alzheimer's Disease
- Parkinson's Disease
- Multiple Sclerosis
- Amyotrophic Lateral Sclerosis
- Stroke
- Traumatic Brain Injury
- Cancer
- Infection – Viral and Bacterial



Differences in pre-analytical variables affect CSF biomarker measurements by up to 20%–30%

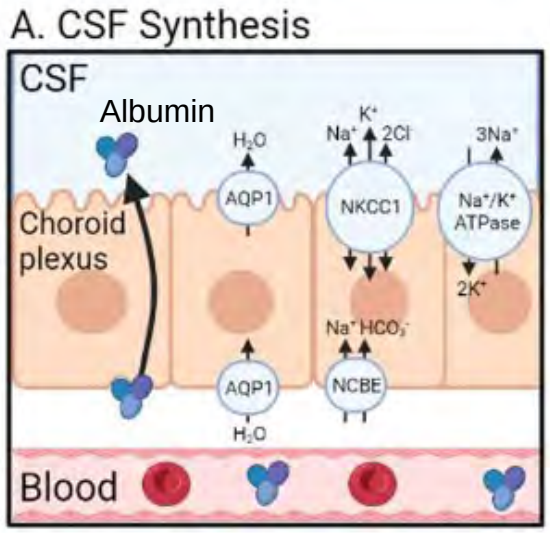
Klener et al., 2014, *Clinical Chemistry and Laboratory Medicine*; Lewczuk et al., 2006, *Neuroscience Letters*; Mattsson et al., 2011, *Alzheimer's & Dementia*

CSF Production and Flow



Extraventricular

Intraventricular



CSF location

- Intracerebral ventricles
- Spinal & brain subarachnoid spaces
  - Cisterns
  - Central canal of spinal cord

CSF is produced within the brain ventricles

- Choroid plexus epithelial cells filter circulating plasma to form an ultrafiltrate
- Ion channels generate an osmotic gradient for water influx

Average adult male has ~150 ml of CSF within the ventricular and subarachnoid space

- ~20ml/hour
- ~500 ml/day

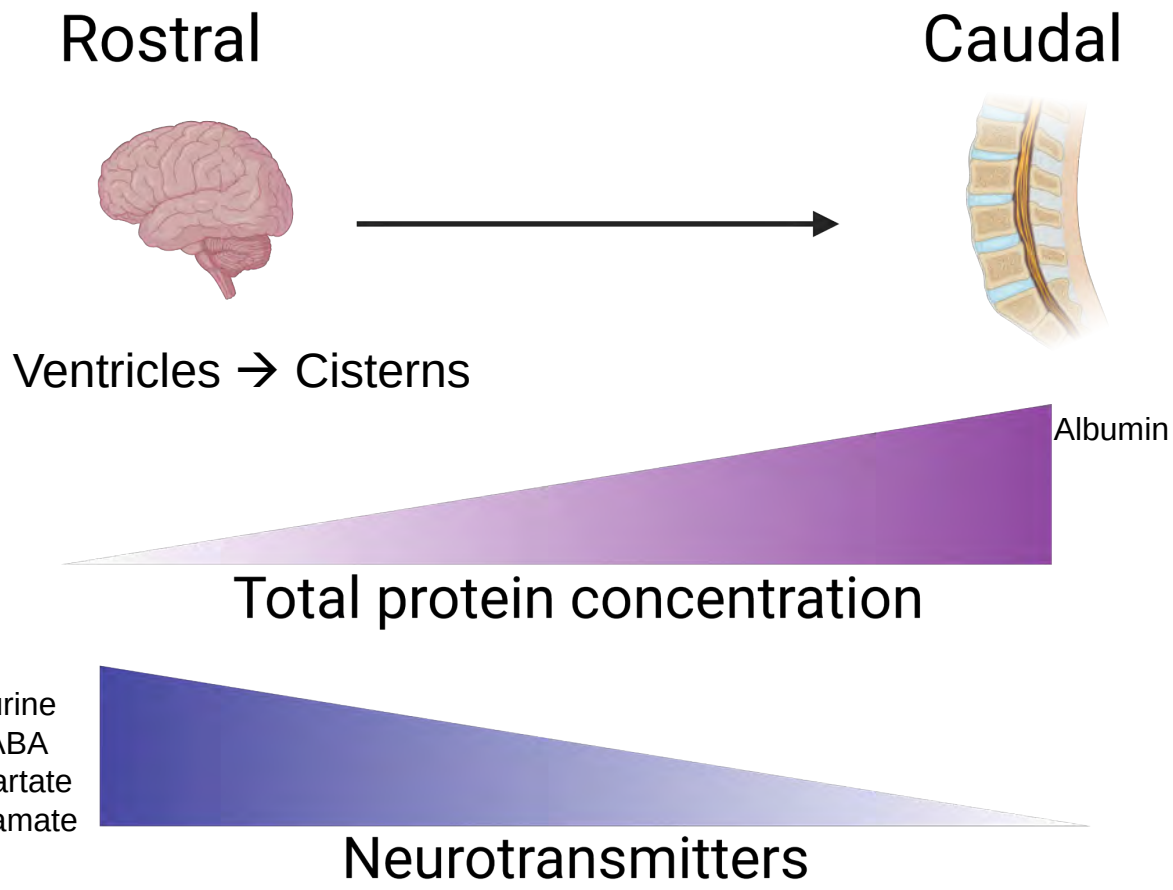
CSF circulates throughout CNS and is absorbed via:

- Arachnoid granulations
- Dural sinuses
- Venous circulation
- Cervical lymphatics

DOI: 10.1259/bjr/66206791
DOI: 10.3171/jns.1996.84.6.0982
Uptodate.com

Courtesy of Setty Magana, adapted

Rostral-Caudal Gradient



Gradient

- Higher protein and cell count lumbar vs ventricular
  - o 26mg/dL lateral ventricles
  - o 32 mg/dL cisterna magma
  - o 42 mg/dL lumbar subarachnoid space

Impacts CSF Composition

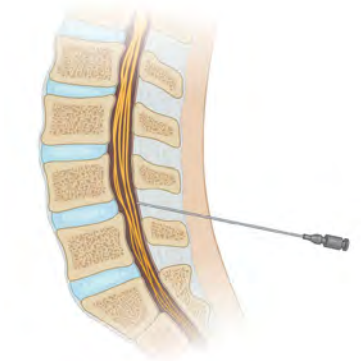
- Cells
- Extracellular Vesicles (EVs)
- Nucleic acids
- Metabolites
- Neurotransmitters

Crawford PM, et al.. 1988,. *J Neurol Neurosurg Psychiatry*
Bergman J, et al. 2020 *Fluids Barriers CNS*

Courtesy of Setty Magana

Collection for Clinical Applications & Biobanking

Lumbar Puncture



Diagnostic Applications

- Neurodegenerative diseases (e.g., Alzheimer's disease)
- Neuroimmune conditions (e.g. multiple sclerosis)
- Infection (e.g. meningitis)
- Subarachnoid hemorrhage
- Intracranial hypertension
- Neurooncologic

Therapeutic Applications

- CSF removal in cases of intracranial hypertension
- Intrathecal administration of treatments
- Spinal anesthesia

Critical Pre-analytical Variable:
❖ Site of collection

Ventricular Drain or Shunt

Diagnostic Applications

- Shunt infection
- Ventriculoperitoneal shunt blockage

Hurdle:
Establishing controls for studies using ventricular drain or shunt CSF

Therapeutic Applications

- Malignant intracranial hypertension
- Continuous intracranial pressure monitoring
- Hydrocephalus

Uptodate.com

Courtesy of Setty Magana, adapted

Lumbar Puncture Procedure

Sedated vs. Non-sedated (age, body habitus, mentation)

Biobanking: Report use of general anesthetics

Blind vs. ultrasound or interventional radiology guided

Position:

- Lateral recumbent
- Prone
- Upright

Location:

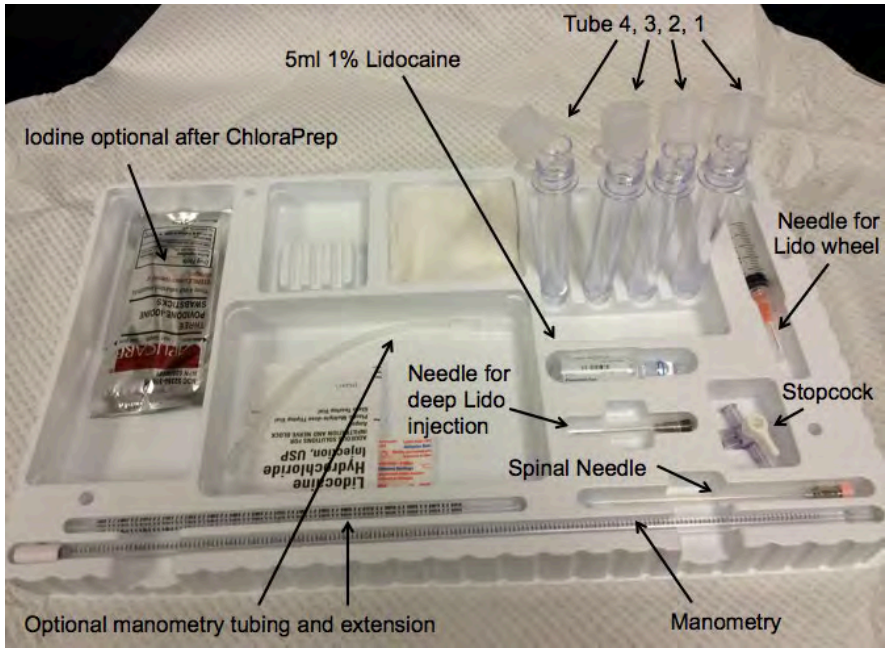
- L3/L4
- L4/L5 (superior iliac crest)

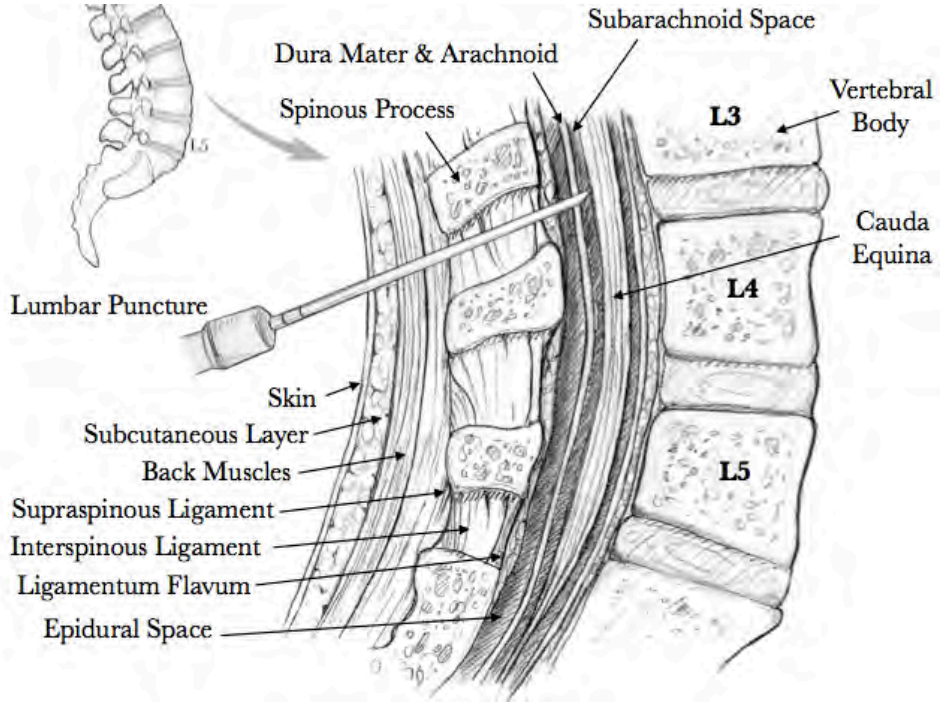
Steps:

- Local anesthesia (e.g., lidocaine) into L3/L4 intervertebral space
- 20- or 22-gauge spinal needle w/stylet inserted
- Needle advanced incrementally until subarachnoid space punctured
- Periodic removal of stylet to check for CSF flow
- Fluid is serially collected in sterile plastic tubes following specific laboratory guidelines for sample collection

Critical Pre-analytical Variables:

- **Volume of CSF collected**
- **Mixing and aliquoting**

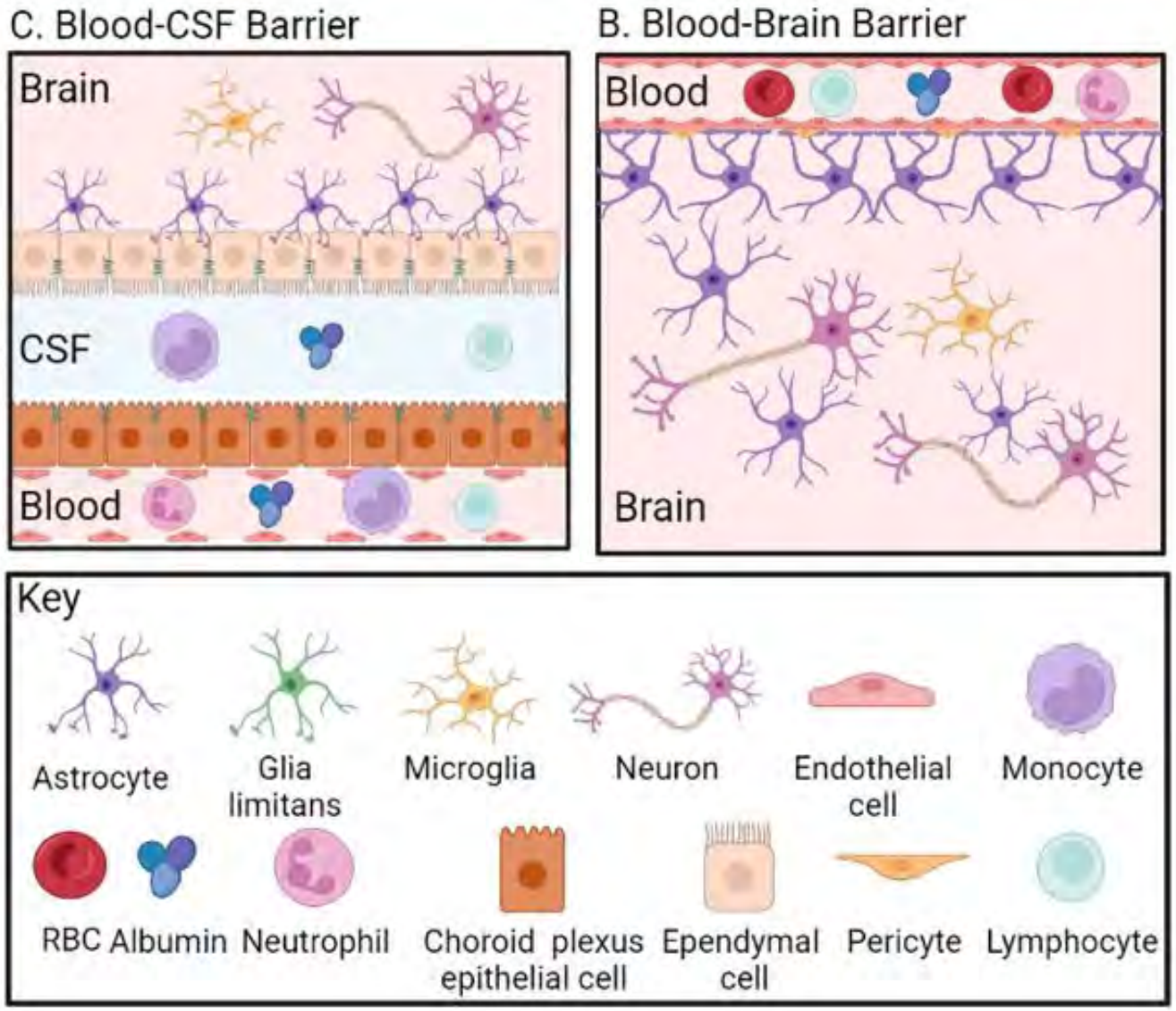




Courtesy of Setty Magana, adapted

EBM Consults
Procedurelist.org.
Fastle, RK., Bothner, J. 2022, *UpToDate*

CNS – Blood Barriers



Blood-CSF Barrier:

- Capillaries with fenestrated endothelium
- Epithelial cells in the choroid plexus

Blood Brain Barrier:

- Endothelial cells, connected by tight junction

Barrier Disruptions:

- Acute and chronic cerebral ischemia
- Brain trauma
- Multiple sclerosis
- Brain tumors
- Brain infections
- Pharmacology

Impact of CNS Barrier on CSF Composition

Table 59: Composition of cerebrospinal fluid and blood plasma in man

	Cerebrospinal fluid (mg/100 ml)	Blood plasma (mg/100 ml)
Protein	20–30	7000–8000
Glucose	50–80	68–96
Urea	10–46	16–35
Sodium	350	318–342
Potassium	8	15–20
Calcium	5	10–11
Chloride (Cl^-)	437–455	360–378
Bicarbonate (Vol. CO_2 per 100 ml)	50–75	50–75
Inorganic phosphate	1–2	2–5

CSF compared to blood plasma and serum:

- ~200–400 times lower protein levels

Lumbar CSF compared to plasma:

- ~1,000 fold less nanoparticles
- ~10,000 fold less tetraspanin+ EVs

Ventricular CSF compared to plasma:

- ~500-fold less nanoparticles

You et al., 2005, *Proteomics*
Sandau et al., 2022, *Frontiers in Cell and Developmental Biology*
Sandau et al, 2020, *Journal of Extracellular Vesicles*
Chen et al., 2013, *Molecular Therapy. Nucleic Acids*

CSF Laboratory tests

Normal Laboratory values

Appearance: clear

CSF (cerebrospinal fluid) cell counts

- Leukocytes (white blood cells): 0-5 cells/µL
- Erythrocytes (red blood cells): 0 cells/µL

CSF chloride

- 120 to 130 mEq/L; 120 to 130 mmol/L (SI)

CSF gamma globulin

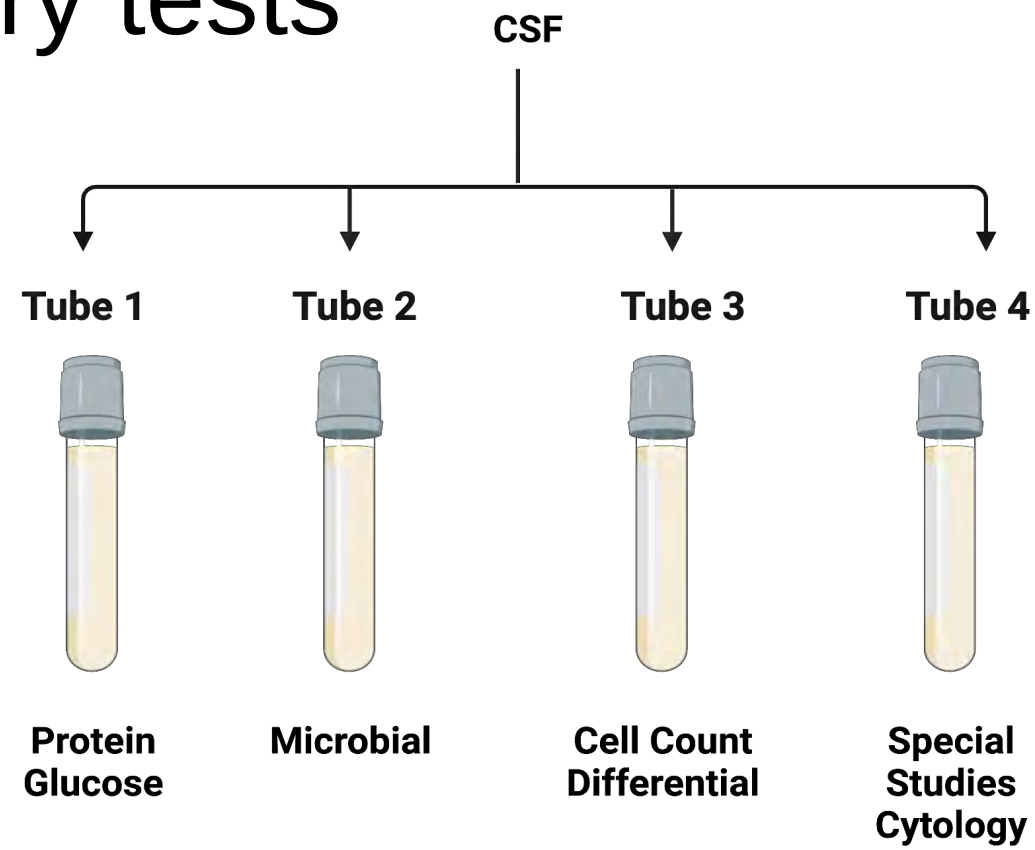
- 6.1 to 8.3 mg/dL; 0.06 to 0.08 g/L (SI)

CSF glucose

- 50 to 75 mg/dL; 2.8 to 4.2 mmol/L (SI)

CSF total protein

- 15 to 45 mg/dL



Volume per tube: 1-4ml

Least sensitive/specialized tests in first tubes

90% of patients with subarachnoid hemorrhage have red blood cell lysis and bilirubin in the CSF

Critical Pre-analytical Variable:

❖ **Establish appropriate exclusion criteria**

ABIM Laboratory Test Reference Ranges–January 2023
UK National External Quality Assessment Scheme for Immunochemistry Working Group, 2003

Courtesy of Setty Magana, adapted

Patient Demographics & Medical History

Known to Impact CSF Composition

Biological variables

- Age
- Sex
- Ethnicity

Age	Nanoparticles (NTA)
<2 years old	~5e10/mL
10–15 years old	~2e10/mL
>70 years old	~7e9/mL

Medical/social history

- Prescription medications
- Smoking status
- Alcohol use

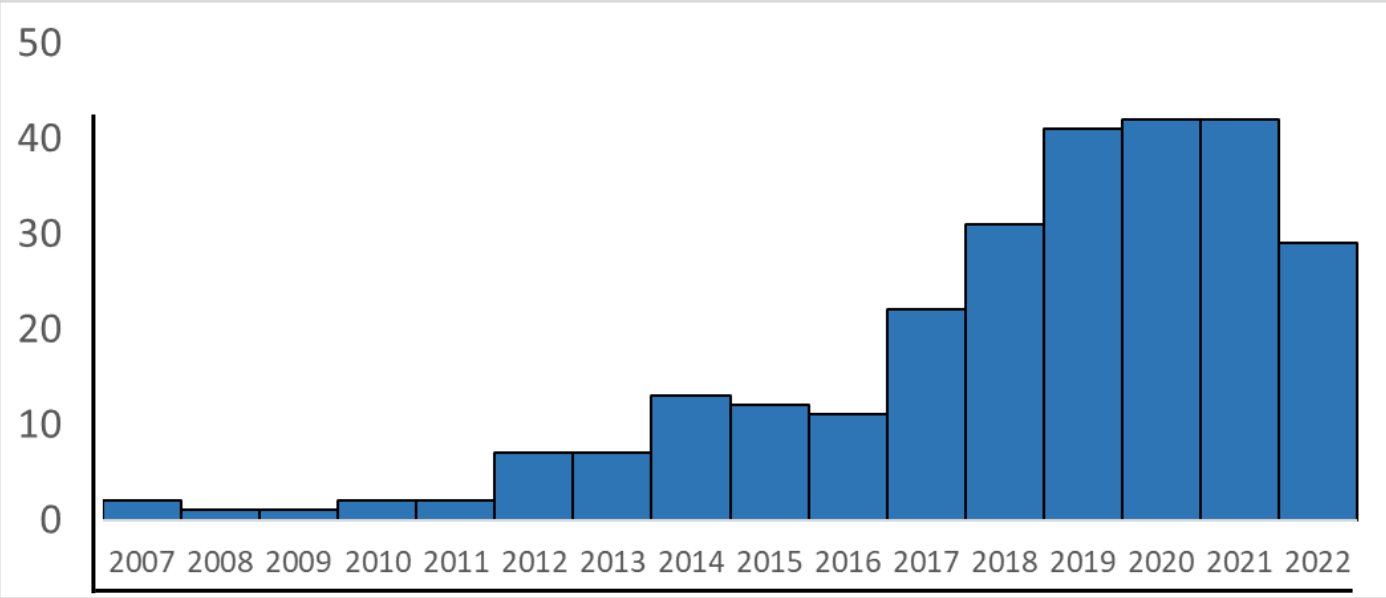
Hurdle:
Control CSF for pediatric studies

Critical Pre-analytical Variable:
❖ Record & report relevant patient demographic and medical/social history data

Tietje et al., 2014, *PLoS ONE*
Li et al., 2017, *Alzheimer's Research & Therapy*
Sandau et al., 2022, *Frontiers in Cell and Developmental Biology*
Howell et al., 2017, *Alzheimer's Research & Therapy*
Liu et al., 2020, *JAMA Network Open*
Riekse et al., 2006, *Journal of Alzheimer's Disease*
Wang et al., 2021, *Journal of Alzheimer's Disease*
Wong, 2007, *Archives of Neurology*

How is the CSF EV Community Doing?

CSF Task Force: CSF EV Literature Review



245 Publications
PubMed Conception – 09.01.22

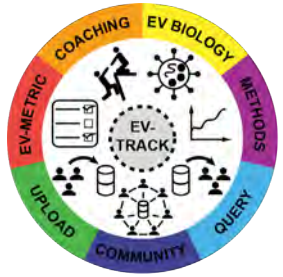
Inclusion criteria:
Published in English-language
Original research with CSF EVs

158 CSF EV Studies

+

3 EV-TRACK CSF Submissions

161 CSF EV Studies



"(Cerebrospinal fluid[Title/Abstract]) AND ((exosome[Title/Abstract]) OR (exosomes[Title/Abstract]) OR (extracellular vesicle[Title/Abstract]) OR (extracellular vesicles[Title/Abstract]) OR (microvesicles[Title/Abstract]) OR (microvesicle[Title/Abstract]) OR (ectosomes[Title/Abstract]) OR (ectosome[Title/Abstract])) NOT (Review[Publication Type])"

CSF Collection, Storage, Handling

#1 #2 #4 #4 #3

Site	Collection Vol	Clinical Assessment	Exclusion Criteria	Additives	Tubes	Centrifugation	Aliquoted	Storage	Other Collection Notes
LP	8 mL				Low protein absorption tube (PROTEOSAVE SS 15 mL)	4000 g, 10 min at 4C	0.5 mL aliquots	Collected on ice; long term -80C	Collected between 1pm-2pm; Uniever 22 G traumatic pencil point needle; L3/4 or L5/6; PMID: 26081315
Extraventricular drainage						3000 g, 10 min	Aliquoted	-80C	Sterile extraventricular drainage catheter
Subarachnoid space, cistern	30-50 mL							CSF collected at 4C	
Shunt								Liquid nitrogen	
Purchased (Precision Med Inc)					LoBind tubes (Eppendorf)				
LP			Erythrocyte counts > 50/mm3		Polypropylene tubes	2000 g, 10 min at room temp	Aliquoted	-80C within 30 min of collection	Collected between 9am-12pm
Surgical or shunt									
Ventriculostomy					Low-protein binding polypropylene tubes			-80C	CSF pooled

Recommendations for reproducibility of cerebrospinal fluid extracellular vesicle studies

Text Box 1. Recommendations and Reporting Guidelines for CSF Collection, Processing and Storage

Recommendations

- Use a standardised and detailed sample collection form, such as Supplemental Form 1.
- Collect CSF and briefly centrifuge at 2000 × *g* to remove cells.
- To minimise any gradient effects of lumbar collection, CSF can be pooled across multiple syringes prior to aliquoting.
- If not used fresh, store pre-processed CSF at −80°C as soon as possible (Saugstad et al., 2017; Teunissen et al., 2009).
- To avoid multiple freeze/thaw cycles, aliquot CSF in volumes appropriate for downstream assays.

Reporting Guidelines

- Pre-collection and collection parameters, if known:
  - (i) Institute or clinical study site where participants donated CSF
  - (ii) Institutional Review Board approval number
  - (iii) Collection site (e.g., lumbar vs. central)
  - (iv) Collection type (e.g., post-mortem)
  - (v) Collection time of day
  - (vi) Fasting or non-fasting conditions
  - (vii) Use and type of anaesthetics
  - (viii) Needle type and gauge
  - (ix) Type/supplier of collection tube
  - (x) Total volume of CSF collected
- Patient characteristics, if known:
  - (i) Neurological disorder/infection
  - (ii) Neuropathology
  - (iii) Age
  - (iv) Sex
  - (v) Medications
  - (vi) Smoking status
  - (vii) Vitals (blood pressure, body temperature, etc.)
  - (viii) Body mass index

Example of a CSF Collection Form to promote standardization of data collected

Unknown Variables that may Impact CSF and/or CSF EV Composition

Subject
- Fasting
- Circadian

Collection
- Patient position for lumbar puncture
- Tube type
- Needle size

Processing
- Centrifugation
- Additives
- Holding temperature & time

Storage
- Tube type
- Storage temperature & time

Supplemental Form 1. Standardized and Detailed CSF Sample Collection Form

CSF SAMPLE SOURCE: ☐ Lumbar puncture ☐ EVD ☐ Ventricular shunt ☐ Drain ☐ Other:

Collection Date _____ DD/MM/YY

Collection Time _____ HH/MM

Fasting ☐ Yes ☐ No
Time of Last Meal _____AM

Sedated Procedure ☐ Yes ☐ No

If Yes, which Anesthetic _____

Needle Gauge _____

Number of Tubes Collected
☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ Other _____

Volume of CSF Collected
☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ Other _____ mL

Total Volume Collected _____ mL

Collection Temperature _____°C

Transport Temperature _____°C

Type of Collection Tube _____

Were Individual Collection Tubes Pooled/Mixed Prior to Processing/Storage? ☐ Yes ☐ No

Additives to CSF ☐ Yes ☐ No Type _____

Centrifugation ☐ Yes ☐ No

Processing Time _____ HH:MM

Centrifugation Speed: _____ ☐ g or ☐ rpm

Centrifugation Duration _____ MM/SEC

Centrifugation Temperature _____ °C

Ultrafiltration ☐ Yes ☐ No Device _____

Number of Aliquots _____

Aliquot Volume _____ mL

Type of Storage Tube _____

CSF Sent for Clinical Biochemistries? ☐ Yes ☐

Visual Macroscopic Blood? ☐ Yes ☐ No

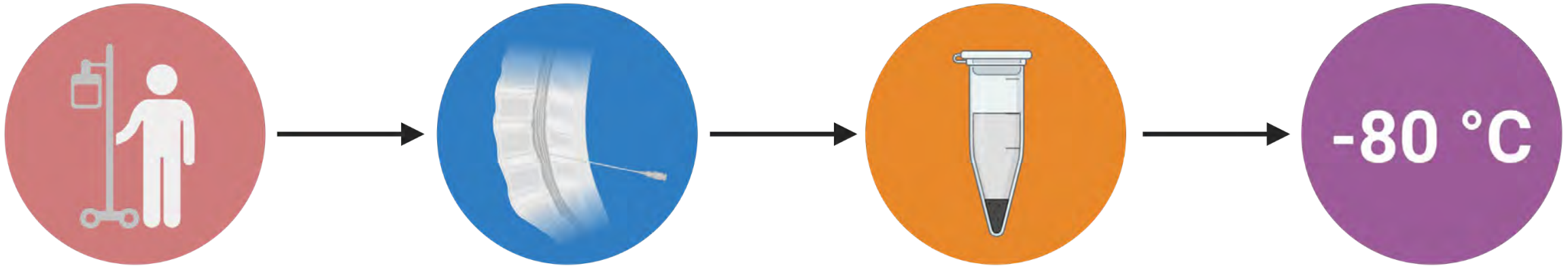
Date of Transfer to Long Term Storage
_____ DD/MM/YY

Storage Freezer Temperature _____°C

Time of Transfer to Long Term Storage
_____ HH/MM

Initials of Study Staff Completing Transfer _____

Critical and Unknown Pre-analytical Variables for Biobanking CSF



Subject

Collection

Processing

Storage

Critical Variable #1:
- Patient demographics & Medical / social history

Unknown Variables:
- Fasting
- Circadian

Critical Variables #2-3:
- Collection site
- Tube # and volume

Unknown Variables:
- Patient position
- Tube type

Critical Variable #4:
- Blood contamination / exclusion criteria

Unknown Variables:
- Additives
- Centrifugation
- Temp and duration

Critical Variable #5:
- Mixing / aliquots (gradient effect)

Unknown Variables:
- Fresh vs. frozen
- Long term storage temp and time in storage

Images created in Biorender

Acknowledgements

INTERNATIONAL SOCIETY for EXTRACELLULAR VESICLES

CSF Task Force

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- Kendall Van Keuren-Jensen
- Laura Vella
- Kostas Vekrellis

Rigor & Standardization Subcommittee

- Juan M. Falcon-Perez Co-Chair
- Rienk Nieuwland Co-Chair
- Metka Lenassi
- Kenneth Witwer
- Clotilde Thery

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UH3TR000903 (Saugstad PI)

MAYO CLINIC

Urology Research Lab

PRE-ANALYTICALS AND BIOBANKING PROCESS OF BLOOD FOR EV RESEARCH

Fabrice Lucien-Matteoni PhD
Assistant Professor
Department of Urology
Department of Immunology
Mayo Clinic, Rochester, USA

European Liquid Biopsy Society
1st EV Technology Meeting - May 28th, 2024

@flscientist

ISEV BLOOD TASK FORCE

Blood Task Force

About

This task force exists to improve the reproducibility of EV measurement results in plasma and serum.

Working Groups

1.Collection, handling and storage of blood extracellular vesicles
2.Quality controls of plasma and serum
3.Quality controls of extracellular vesicles isolated from plasma or serum
4.Anticoagulants
5.Blood stabilization tubes
6.Reference plasma samples

Output:

- *Considerations towards a roadmap for collection, handling and storage of blood extracellular vesicles* JEV 2019
- *MIBlood-EV: Minimal information to enhance the quality and reproducibility of blood extracellular vesicle research* JEV 2023

- ISEV Blood EV workshop September 2022 in Helsinki
- ISEV Blood EV workshop September 2024 in Seoul

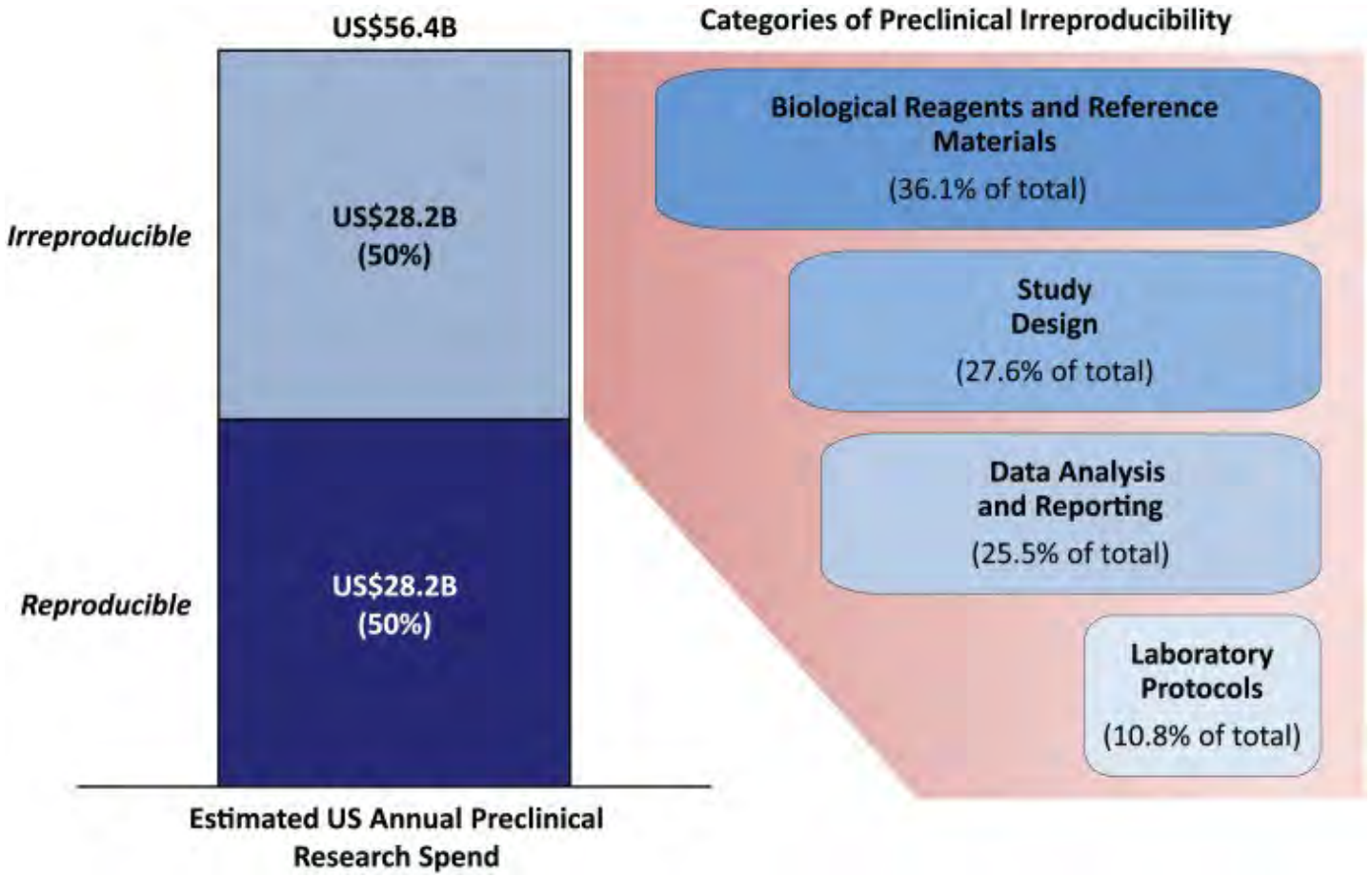
Members

Federica Anastasi
Eric Boilard
Edit Buzas
Lesley Cheng
Aled Clayton
Katharina Clemm von Hohenberg
Juan M. Falcon-Perez
Paola Gasperini
Alice Gualerzi
Dakota Gustafson
An Hendrix
Andrew Hoffmann
Jennifer Jones
Lucia Languino
Cecilia Lässer
Charlotte Lawson
Metka Lenassi
Fabrice Lucien-Matteoni
Kendra Maass
Irina Nazarenko
Rienk Nieuwland
Lorraine O'Driscoll
Ryan Pink
Annie Reale
Pia R. Siljander
Carolina Soekmadji
Amit Srivastava
Marca Wauben
Joshua A. Welsh
Kenneth Witwer
Lei Zheng

OUTLINE

1. Why does reproducibility matter?
2. Confounding factors in blood EV research
3. Impact of pre-analytical variables on blood EV research
4. QC and transparent reporting of plasma/serum preparations: the MIBlood-EV

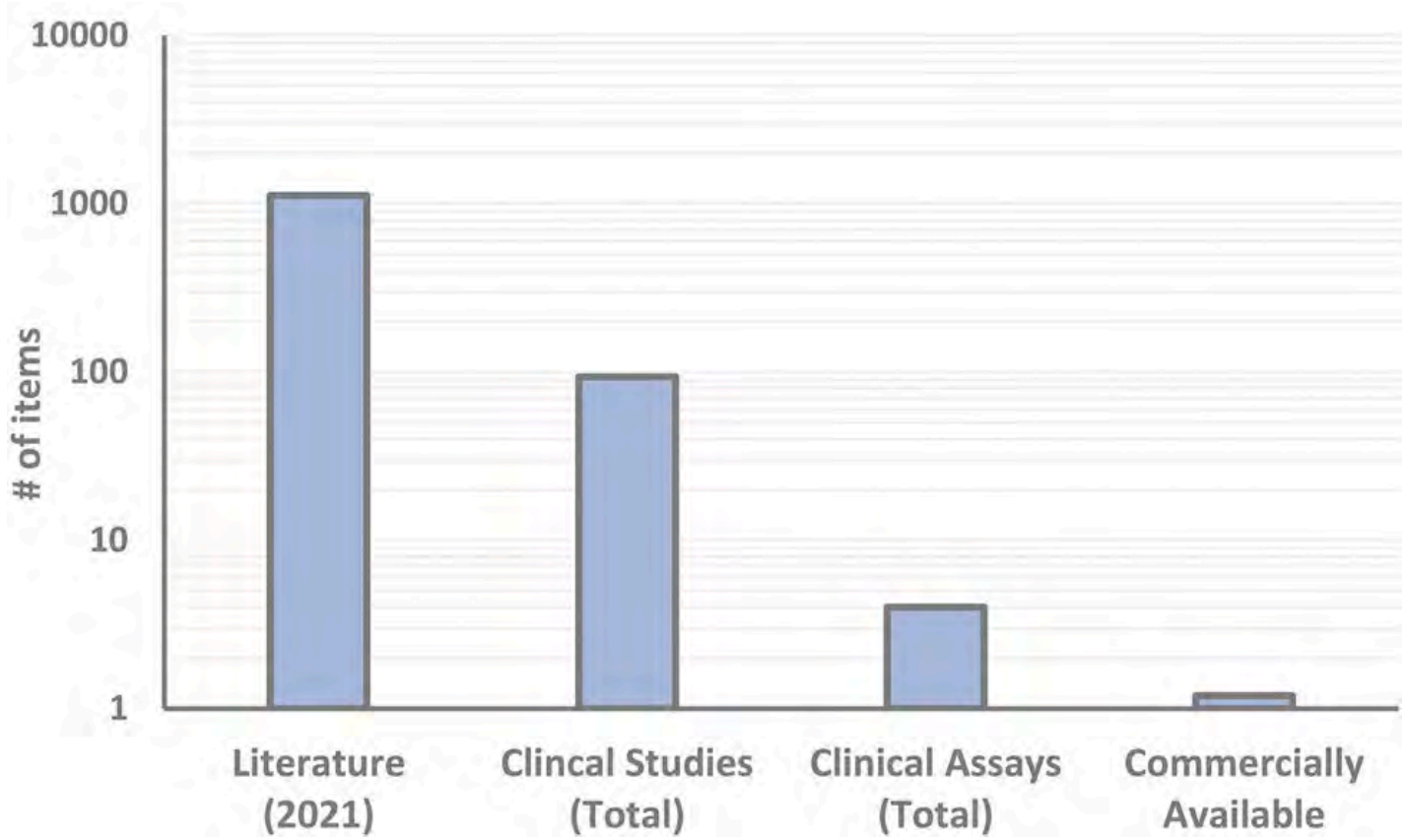
WHAT DOES REPRODUCIBILITY MATTER? COST



Freedman LP et al, PLoS Biology, 2015

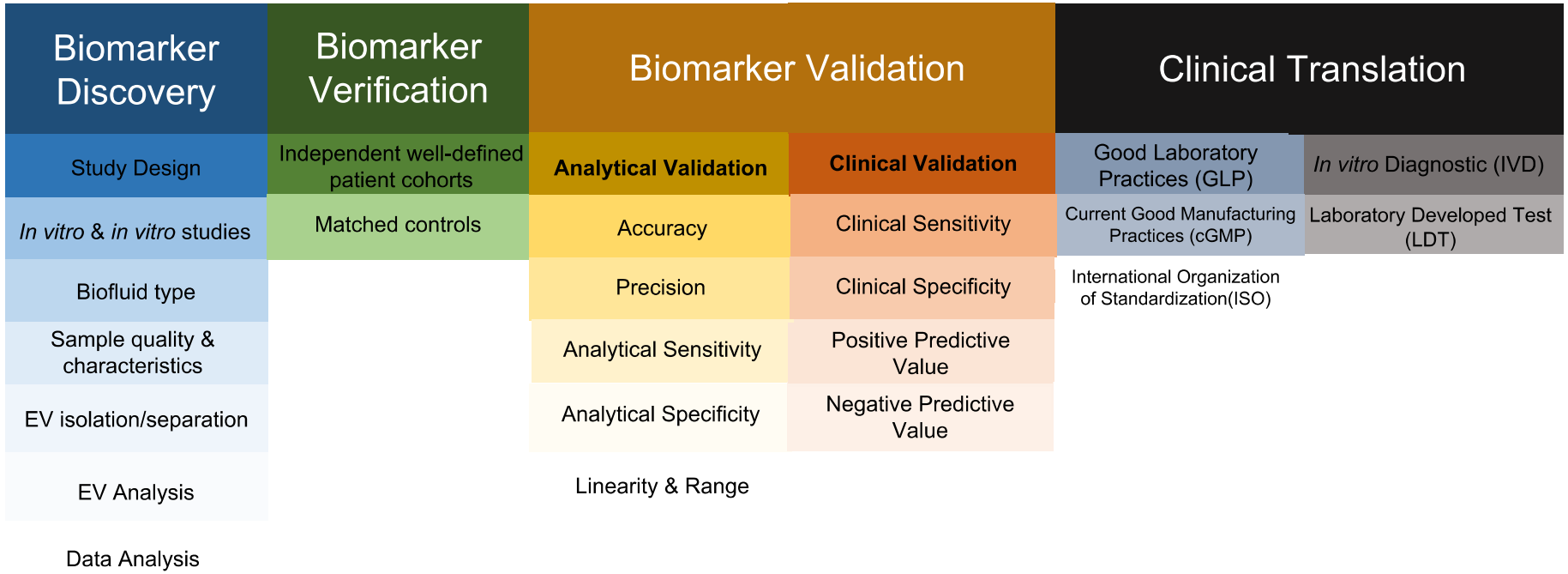
WHAT DOES REPRODUCIBILITY MATTER?
FAILED TRANSLATION TO THE CLINIC

Cumulative number of publications on “extracellular vesicle” and “biomarker” in 2022



Enderle D & Noerholm M, J of Lab Med, 2022

PRE-ANALYTICAL VARIABILITY IS A MAJOR DRIVER OF IRREPRODUCIBLE RESEARCH



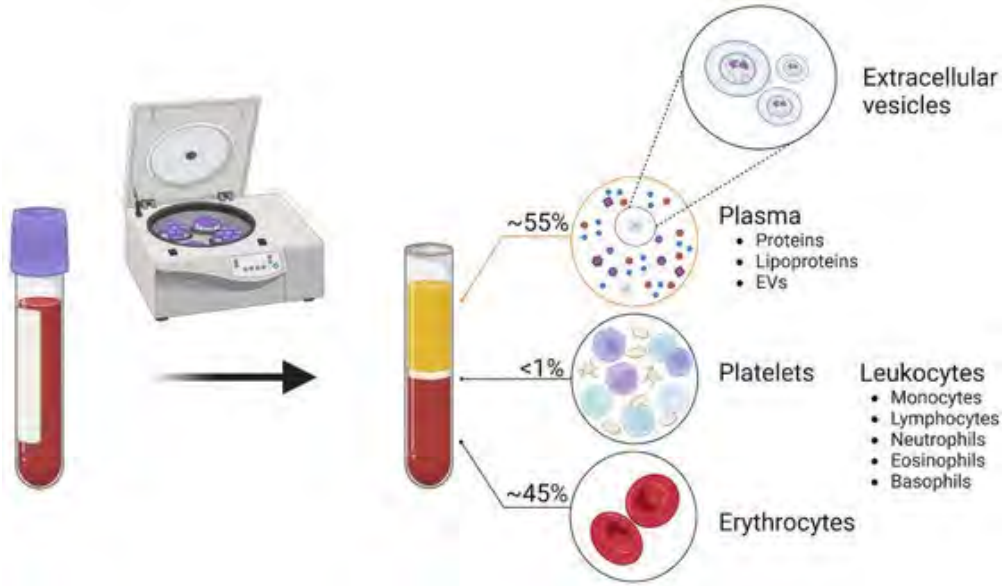
Pre-analytical variables in blood collection may account for >50% of laboratory errors at biomarker discovery stage.

- Hemolysis
- Clotted specimens
- Insufficient sample volume

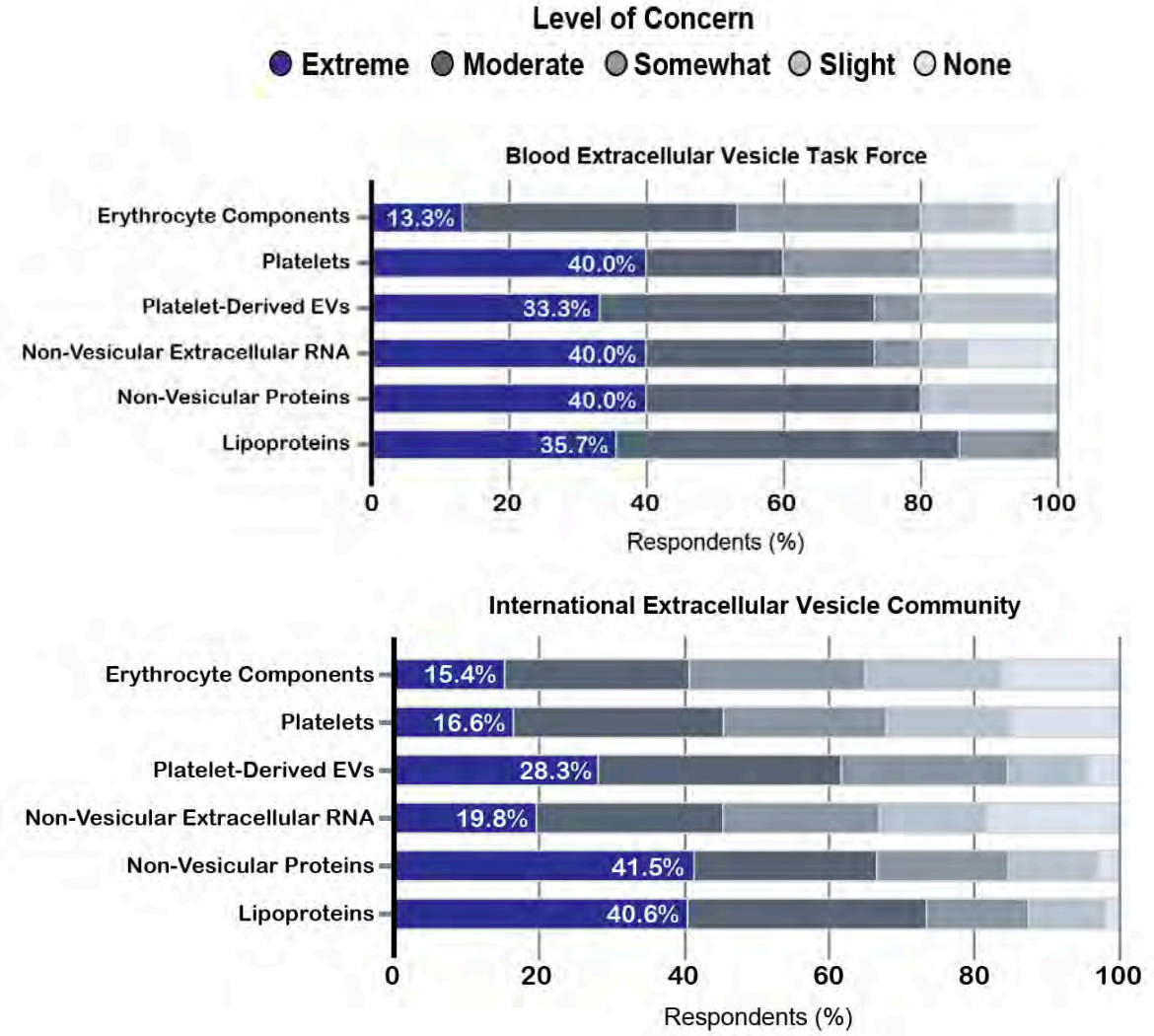
Green SF, Clin Biochem, 2013

Yekula A et al, Methods, 2020

MAJOR CONFOUNDERS IN BLOOD EV RESEARCH



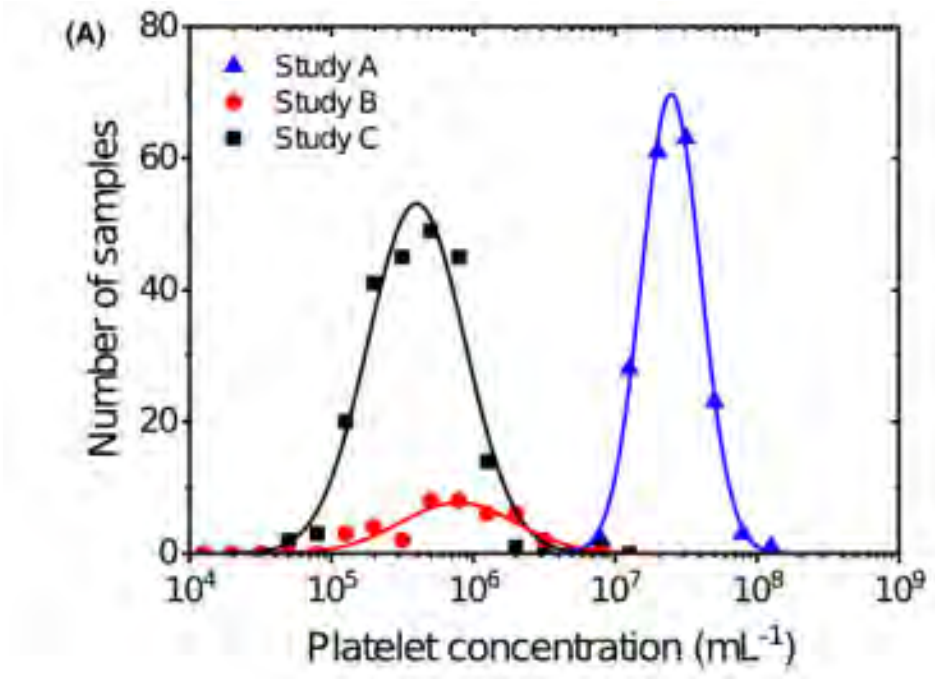
Nieuwland R & Silijander PRM, JEV, 2024



Lucien F, Gustafson D* et al, JEV, 2023*

MAJOR CONFOUNDERS IN BLOOD EV RESEARCH

- Lipoproteins (≈10^{16} lipoproteins/mL) outnumber plasma EVs (≈10^{9} EVs/mL) (Simonsen JB et al, Circulation 2017)
- 0.2% of plasma EVs derived from tissues (Li Y et al, Comp Struct Biotech J, 2020)
  - 0.02-0.05% of plasma EV proteins come from the tumor (Barlin M et al, Mol & Cell Prot 2023)
- Residual platelets is common in prepared plasma (Bettin B et al, JTH, 2022)



CURRENT METHODOLOGICAL GUIDELINES FOR PLASMA/SERUM PREPARATIONS FOR EV RESEARCH

Review

Extracellular Vesicles

Chantal Boulanger, Guest Editor

Methodological Guidelines to Study Extracellular Vesicles

Frank A.W. Coumans, Alain R. Brisson, Edit I. Buzas, Françoise Dignat-George, Esther E.E. Drees, Samir El-Andaloussi, Costanza Emanueli, Aleksandra Gasecka, An Hendrix, Andrew F. Hill, Romaric Lacroix, Yi Lee, Ton G. van Leeuwen, Nigel Mackman, Imre Mäger, John P. Nolan, Edwin van der Pol, D. Michiel Pegtel, Susmita Sahoo, Pia R.M. Siljander, Guus Sturk, Olivier de Wever, Rienk Nieuwland

Circulation 2017

Blood collection

Considerations and Recommendations

- Collect blood from overnight fasting subjects. The choice of anticoagulant depends on downstream analysis.
- Avoid prolonged use of a tourniquet[43] and use a large diameter, 21-gauge needle.[44–46]
- Discard the first 2 to 3 mL of collected blood[47,48] and collect blood in plastic collection tubes at room temperature (see also Coagulation section of this article).
- Properly fill the tubes to obtain the appropriate blood to anticoagulant ratio and mix gently.[49]
- Keep the blood collection tubes in a vertical position during transport.
- The time interval between blood collection and the first centrifugation step to prepare plasma should be minimized or at least be kept constant between samples, to limit effects on the concentration and functional activity of EVs.[39,50–52]
- Preferably, no measurements of EVs in hemolyzed samples should be done. If hemolyzed samples are included, the obtained results should be interpreted with care[28] and the degree of hemolysis should be measured.[53]

Blood processing

Considerations and Recommendations

- Centrifuge blood at room temperature.
- Remove platelets by using 2 subsequent centrifugations steps of 2500*g* for 15 minutes as recommended by International Society on Thrombosis and Haemostasis,[25] and use a clean plastic tube for the second centrifugation step.
- To reduce the risk of platelet and leukocyte contamination do not collect the last 0.5 cm of plasma above the buffy coat and set the lowest deceleration on the centrifuge.
- Quantify residual platelets in platelet-free plasma.
- Removal of platelets may also remove large EVs such as apoptotic bodies and oncosomes.
- Apply identical centrifugations conditions, including speed, deceleration, rotor, and temperature, to each sample within a study.
- Plasma is recommended for most applications because serum contains additional vesicles which are released during in vitro clot formation.

CURRENT METHODOLOGICAL GUIDELINES FOR PLASMA/SERUM PREPARATIONS FOR EV RESEARCH

Received: 22 August 2023 | Accepted: 4 December 2023

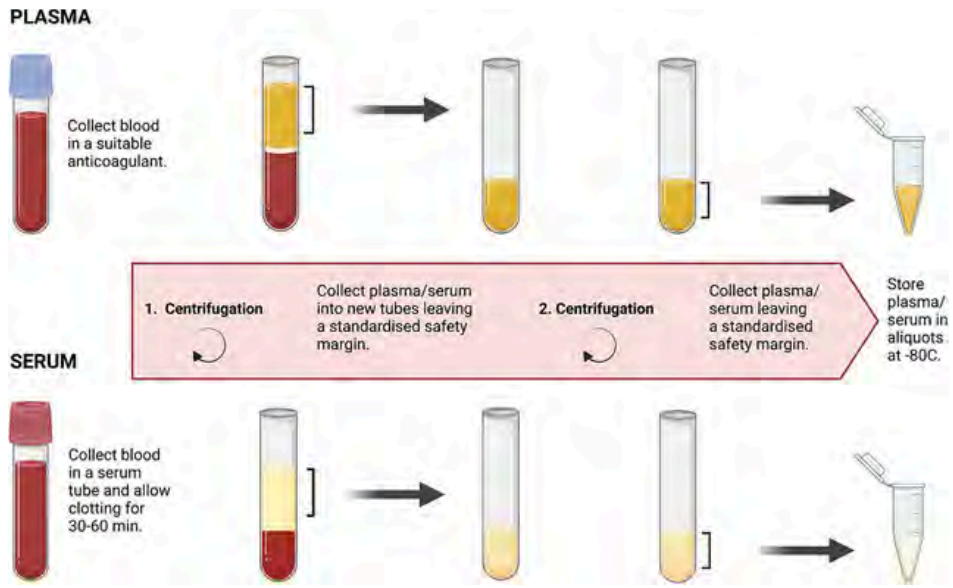
DOI: 10.1002/jev2.12400

ISEV

REVIEW ARTICLE

A beginner's guide to study extracellular vesicles in human blood plasma and serum

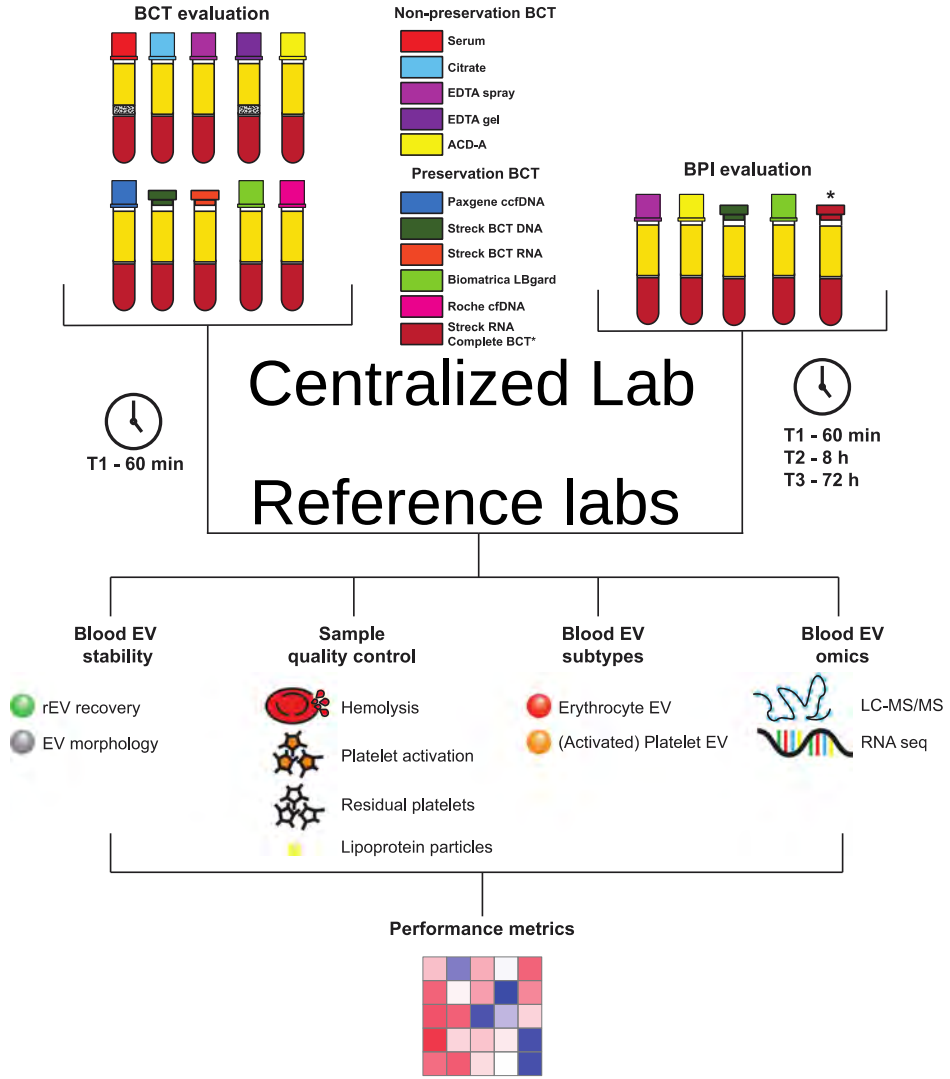
Rienk Nieuwland[1,2] | Pia R-M Siljander[3,4]

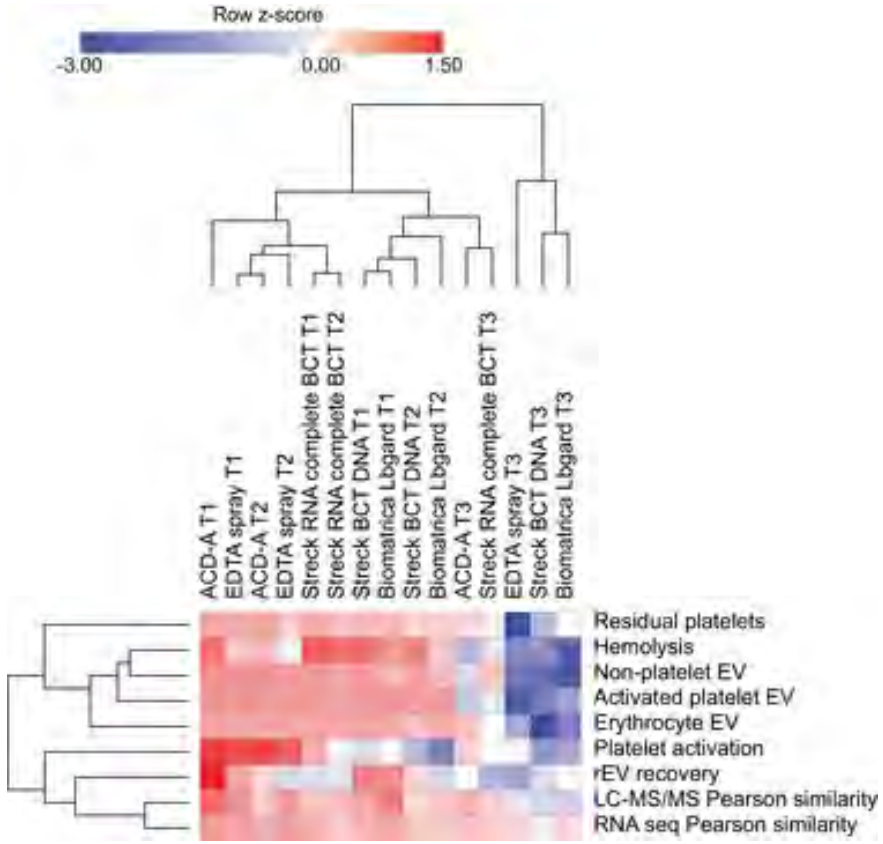


Centrifugation: 2,500 x g – 15 min, no brake

Nieuwland R & Siljander PRM, JEV, 2023

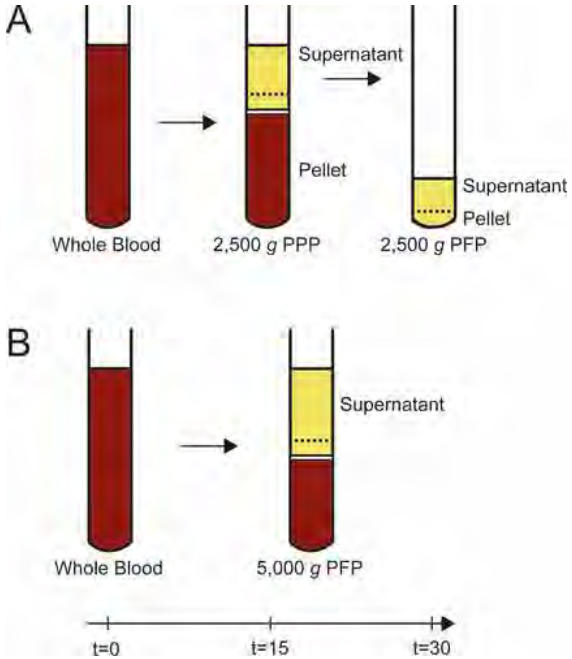
IMPACT OF BLOOD COLLECTION TUBES IN QUALITY OF PLASMA AND SERUM SAMPLES

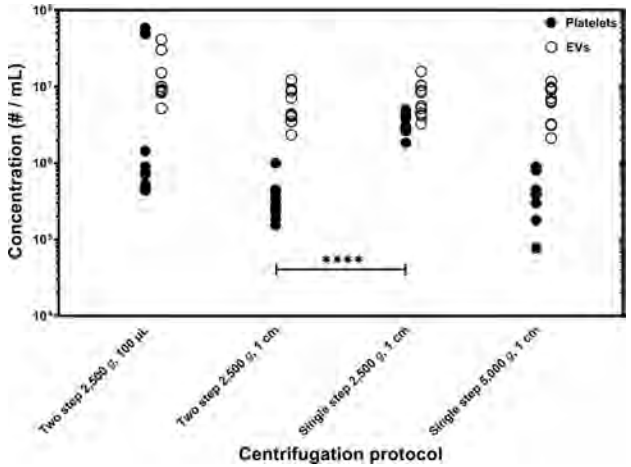




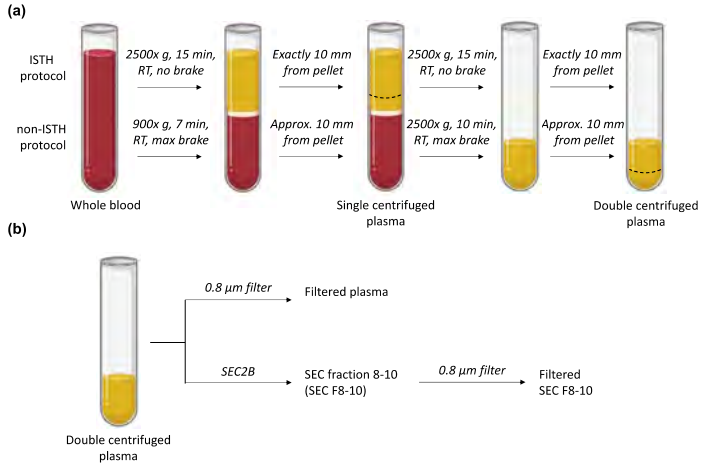
Dhondt B et al, JEV, 2023

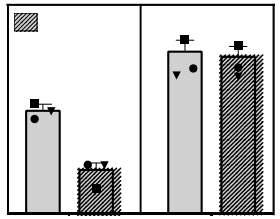
IMPACT OF CENTRIFUGATION ANF FILTRATION ON RESIDUAL PLATELETS AND ERY-GHOSTS





Rikkert LG et al, Platelets, 2021

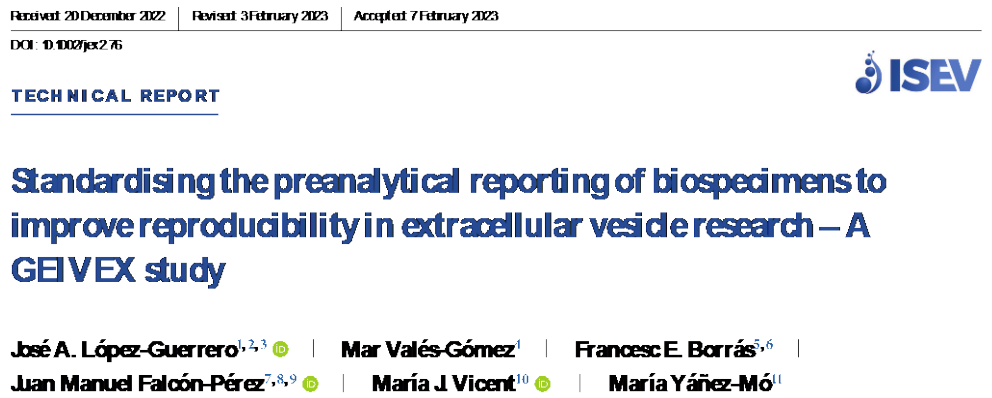




Bracht J et al, JEV, 2023

HETEROGENEITY IN PRE-ANALYTICAL PROCEDURES – A SURVEY OF RESEARCH LABORATORIES

- Survey sent to GEIVEX members, 70 responses, 43 laboratories
- Codification of preanalytical conditions using the SPREC (Standardized PREanalytical Code v3.0)
- 66 out 70 (94%) protocols displayed differences in biofluid handling.
- For blood, there was no lab with similar protocol
  - 13 protocols prepared single-spun plasma. 14 protocols prepared twice-spun plasma
  - 8 different anticoagulants (EDTA #1: 32%).
- Some labs do not have access to pre-analytical conditions employed for sample preparation


Received 20 December 2022 | Revised 3 February 2023 | Accepted 7 February 2023
DOI: 10.1002/jex2.76

TECHNICAL REPORT

ISEV

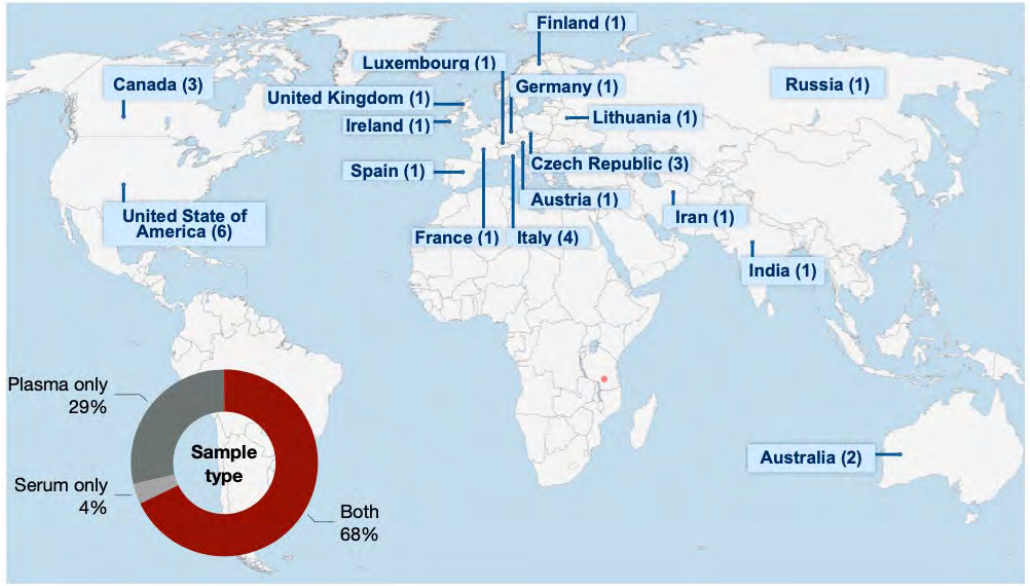
Standardising the preanalytical reporting of biospecimens to improve reproducibility in extracellular vesicle research – A GEIVEX study

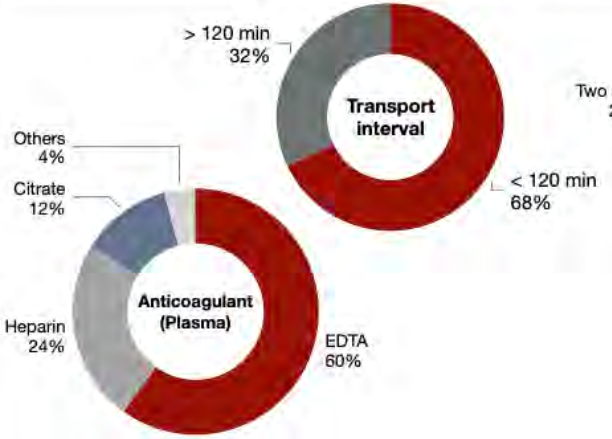
José A. López-Guerrero[1,2,3] | Mar Valés-Gómez[4] | Francesc E. Borrás[5,6] | Juan Manuel Falcón-Pérez[7,8,9] | María J. Vicent[10] | María Yáñez-Mó[11]

- **High variability in protocols used**
- **No adherence to methodological guidelines**
- **Limited information available on sample preparation procedures**

Lopez-Guerrero JA et al, J Extracellular Bio, 2023

HETEROGENEITY IN PRE-ANALYTICAL PROCEDURES – A SURVEY OF BIOBANKS





- Hemolysis assessment : 70%
- Platelete counting: 7%
- Lipoprotein assessment: 3%

- **High variability in protocols used**
- **Most biobanks use single-step centrifugation (<3,000 x g)**
- **No QC of blood and plasma/serum**

CURRENT STATUS QUO IN BLOOD EV RESEARCH

- Lack of standardized protocols across research labs and biobanks
- No evidence-based guidelines
  - Using the best available evidence, expert panels identify and develop SOP recommendations: what to do or **what not to do?**
  - Conduct multi-center benchmarking studies
  - Publication and promotion in society journals
- Does an “universal” SOP for all liquid biopsy analytes feasible?
- Meanwhile, transparent reporting of the pre-analytical variables and QC of plasma/serum is essential to enhance reproducibility

MIBLOOD-EV: REPORTING TOOL FOR BLOOD EV RESEARCH

Received: 23 August 2023 | Revised: 31 October 2023 | Accepted: 10 November 2023

DOI: 10.1002/jev2.12385

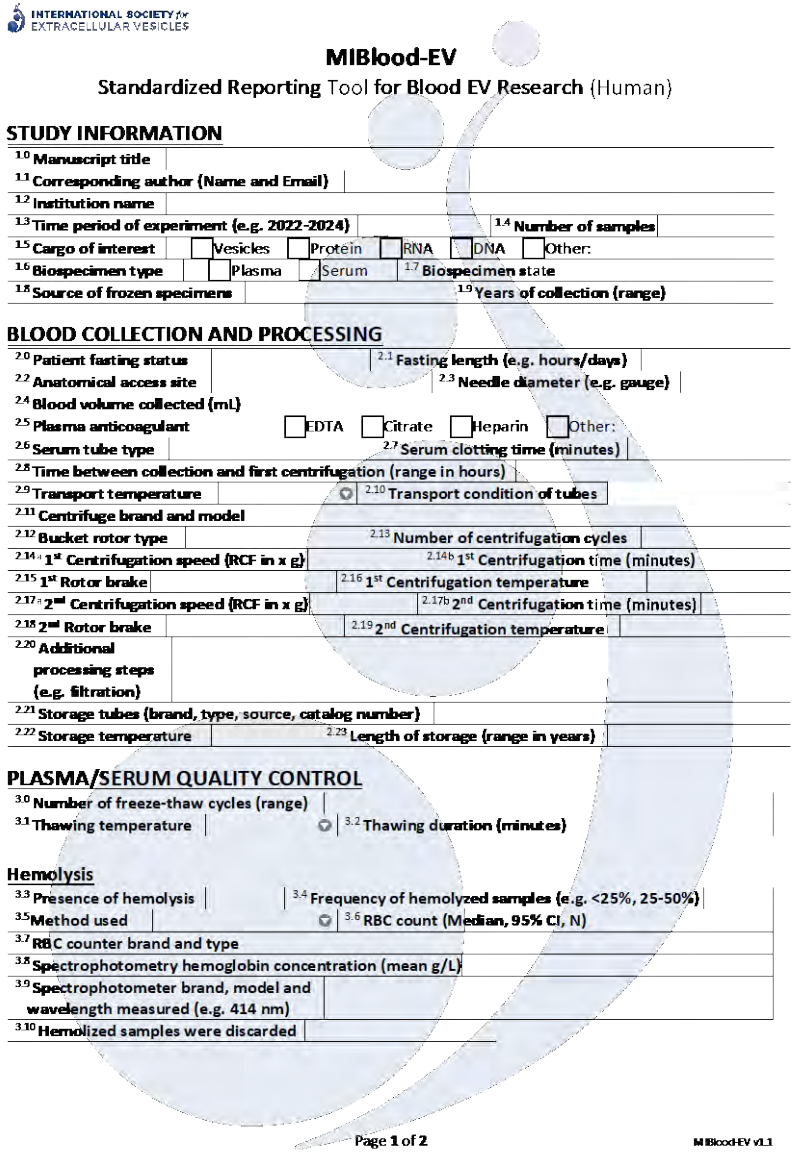
TECHNICAL NOTE

ISEV

MIBlood-EV: Minimal information to enhance the quality and reproducibility of blood extracellular vesicle research

Fabrice Lucien[1,2] | Dakota Gustafson[3,4,5] | Metka Lenassi[6] | Bo Li[7,8] | Jacob J. Teske[1] | Eric Boilard[9] | Katharina Clemm von Hohenberg[10] | Juan Manual Falcón-Perez[11,12] | Alice Gualerzi[13] | Antonia Reale[14] | Jennifer C. Jones[15] | Cecilia Lässer[16] | Charlotte Lawson[17] | Irina Nazarenko[18,19] | Lorraine O'Driscoll[20] | Ryan Pink[21] | Pia R-M Siljander[22] | Carolina Soekmadji[23] | An Hendrix[24] | Joshua A Welsh[25] | Kenneth W. Witwer[25] | Rienk Nieuwland[8]

- Report pre-analytical conditions for blood collection, handling and storage
- Report quality of plasma/serum for 3 parameters (hemolysis, platelets and lipoproteins)
- Available with the original manuscript and in the ISEV R&S website
- Commentaries under review in BIO (ISBER journal) and JLB (ISLB journal)
- Adherence from research groups and biobanks



INTERNATIONAL SOCIETY for EXTRACELLULAR VESICLES

MIBlood-EV

Standardized Reporting Tool for Blood EV Research (Human)

STUDY INFORMATION

1.0 Manuscript title
1.1 Corresponding author (Name and Email)
1.2 Institution name
1.3 Time period of experiment (e.g. 2022-2024) | 1.4 Number of samples
1.5 Cargo of interest: Vesicles, Protein, RNA, DNA, Other:
1.6 Biospecimen type: Plasma, Serum | 1.7 Biospecimen state
1.8 Source of frozen specimens | 1.9 Years of collection (range)

BLOOD COLLECTION AND PROCESSING

2.0 Patient fasting status | 2.1 Fasting length (e.g. hours/days)
2.2 Anatomical access site | 2.3 Needle diameter (e.g. gauge)
2.4 Blood volume collected (mL)
2.5 Plasma anticoagulant: EDTA, Citrate, Heparin, Other:
2.6 Serum tube type | 2.7 Serum clotting time (minutes)
2.8 Time between collection and first centrifugation (range in hours)
2.9 Transport temperature | 2.10 Transport condition of tubes
2.11 Centrifuge brand and model
2.12 Bucket rotor type | 2.13 Number of centrifugation cycles
2.14a 1st Centrifugation speed (RCF in x g) | 2.14b 1st Centrifugation time (minutes)
2.15 1st Rotor brake | 2.16 1st Centrifugation temperature
2.17a 2nd Centrifugation speed (RCF in x g) | 2.17b 2nd Centrifugation time (minutes)
2.18 2nd Rotor brake | 2.19 2nd Centrifugation temperature
2.20 Additional processing steps (e.g. filtration)
2.21 Storage tubes (brand, type, source, catalog number)
2.22 Storage temperature | 2.23 Length of storage (range in years)

PLASMA/SERUM QUALITY CONTROL

3.0 Number of freeze-thaw cycles (range)
3.1 Thawing temperature | 3.2 Thawing duration (minutes)

Hemolysis

3.3 Presence of hemolysis | 3.4 Frequency of hemolyzed samples (e.g. <25%, 25-50%)
3.5 Method used | 3.6 RBC count (Median, 95% CI, N)
3.7 RBC counter brand and type
3.8 Spectrophotometry hemoglobin concentration (mean g/L)
3.9 Spectrophotometer brand, model and wavelength measured (e.g. 414 nm)
3.10 Hemolized samples were discarded

Page 1 of 2

MIBlood-EV v1.1

THANK YOU !

Erasmus MC
University Medical Center Rotterdam

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1st EV Technology Meeting of 2024

Quality collection and biobanking for urinary EVs

Dr. Elena S. Martens-Uzunova

Department of Urology, Erasmus MC

Erasmus MC UROLOGY RESEARCH
www.erasmusurologyresearch.nl

GUSEV
ISEV SPECIAL INTEREST GROUP

Join us ☺ !!!

INTERNATIONAL SOCIETY for EXTRACELLULAR VESICLES

GUSEV

an ISEV Special Interest Group on Genitourinary System EVs

Register here

https://isev.memberclicks.net/SIGGUSEV

...or contact the GUSEV board

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ue2u@uvahealth.org

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e.martens@erasmusmc.nl

Section Chair Americas
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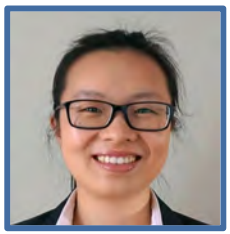
dburger@uottawa.ca



Section Chair Asia Pacific
Monica Ng, PhD MBBS(hons)
Monica.Ng@health.qld.gov.au

Content

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1st EV Technology Meeting of 2024

- **General considerations**
- A practical example
- What is up(coming)
- *Some art….*

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A simple PubMed search …

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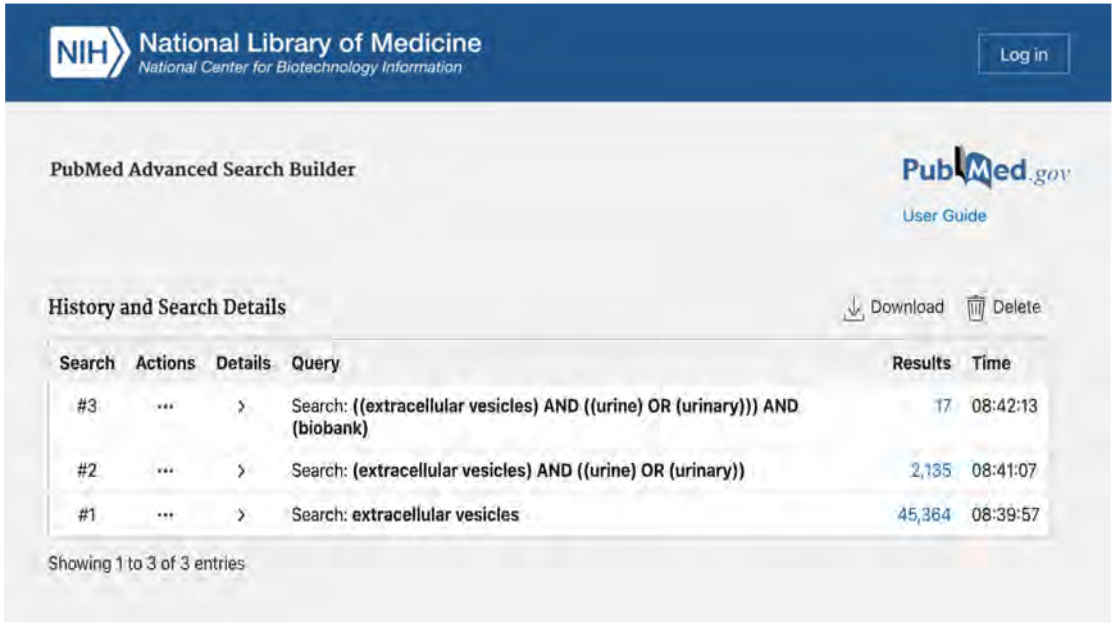
- How many publications in PubMed focus on the biobanking of urine for the analysis of uEVs?



NIH National Library of Medicine
National Center for Biotechnology Information

Log in

PubMed Advanced Search Builder

PubMed.gov

User Guide

History and Search Details

Download Delete

Search	Actions	Details	Query	Results	Time
#3	···	›	Search: **((extracellular vesicles) AND ((urine) OR (urinary))) AND (biobank)**	17	08:42:13
#2	···	›	Search: **(extracellular vesicles) AND ((urine) OR (urinary))**	2,135	08:41:07
#1	···	›	Search: **extracellular vesicles**	45,364	08:39:57

Showing 1 to 3 of 3 entries

2024

- 17 from which:
  - 1 review
  - 1 position paper
  - 4 Methodology
  - 11 research articles with a different research focus (on cargo)

2023

- 12 from which:
  - 1 review
  - 1 position paper
  - 10 research articles with a different research focus (on cargo)

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Methodological gaps in the current uEVs work flow

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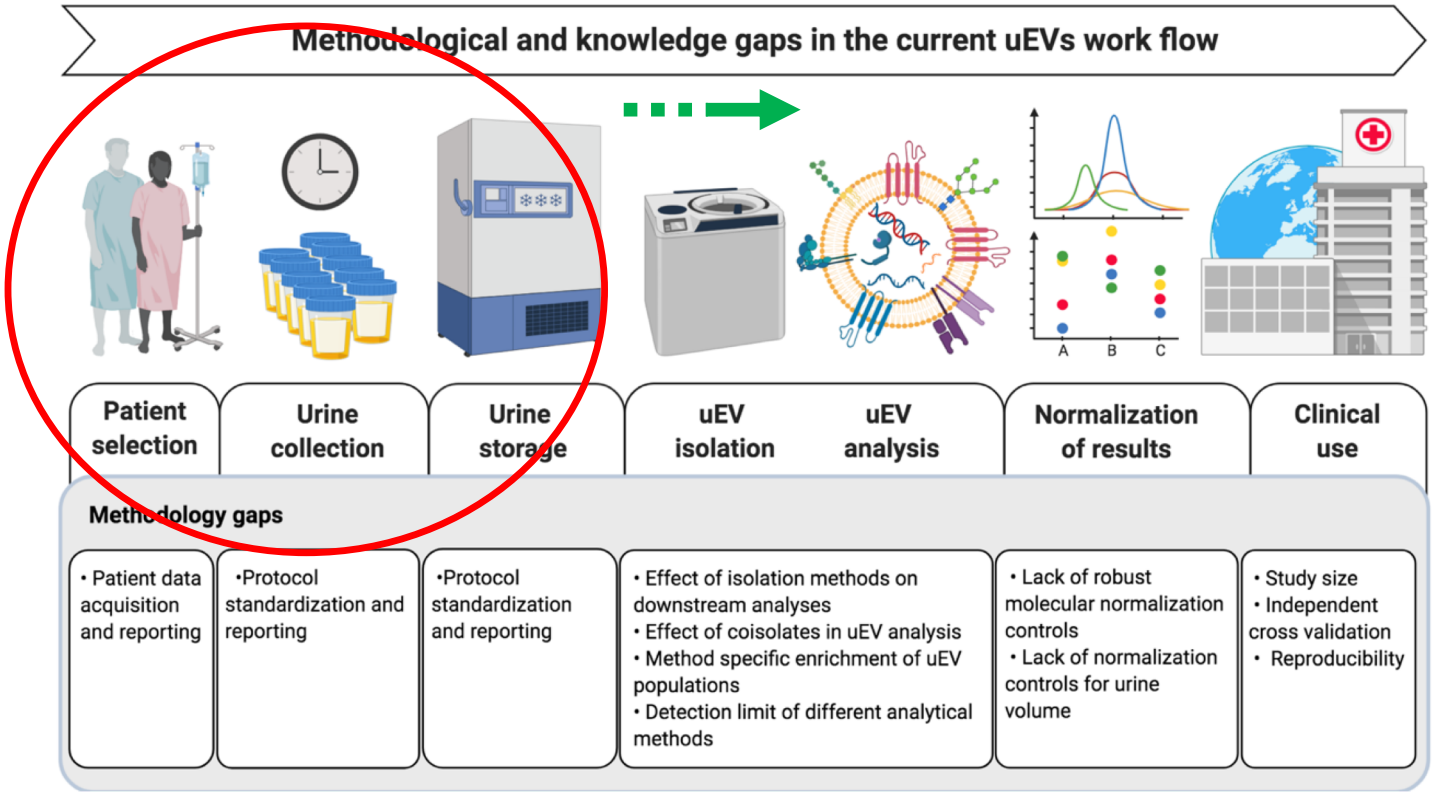
Preanalytical steps



Erdbrügger et al., **Urinary EVs: A position paper.** *(2021) J Extracell Vesicles*

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Preanalytical steps and critical factors that affect them and the final results

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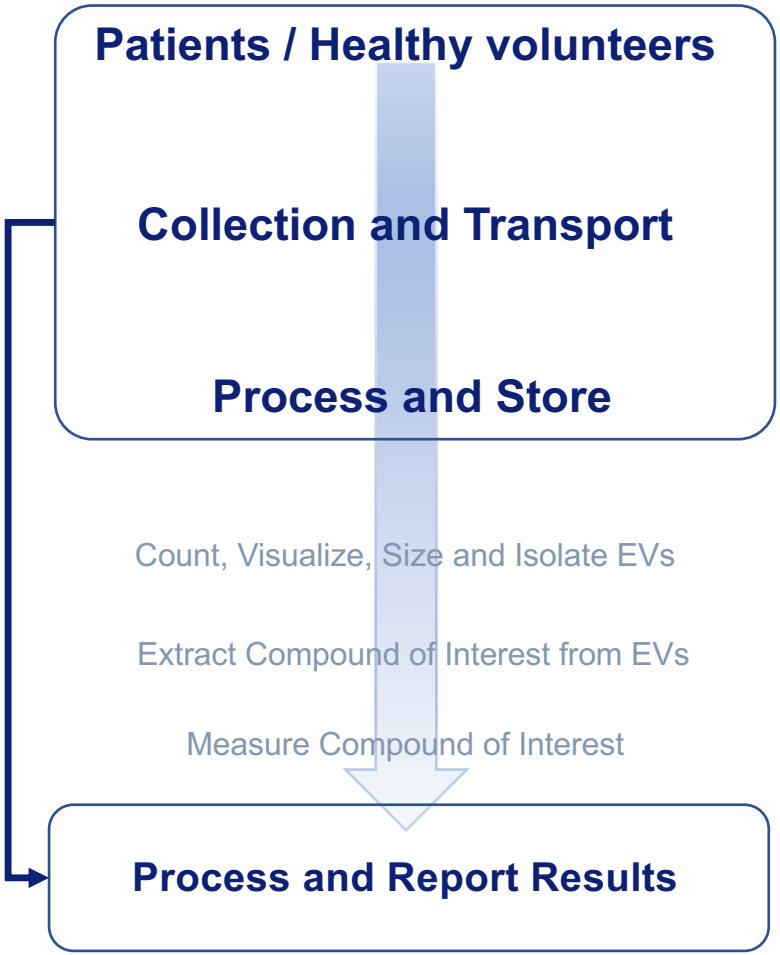

Known factors

- Treatment: Radiotherapy, Chemotherapy, *etc.*
- Comorbidities (Known)
- Multicenter variations in patient population

- Time to collect large enough cohort -> insufficient statistical power
- Physical examination prior collection, *e.g.* DRE -> composition, concentration
- Time of collection, **random (spot)**, morning, 24h urine
- Dedicated collection devise / certified containers
- Sample type, e.g. first void / midstream urine

- Sample centrifugation / freezing cells - Clinical lab (yes/no; capacity)
- Aliquots
- Costs of centralized biobanking ($$$, €€€)

Unknown factors

- Individual's medical history, non-compliance, etc.
- Logistics (insufficient/incomplete database or storage facilities)
- Human error, inconsistent or incomplete documentation/reporting

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Preanalytical steps and critical factors that affect them and the final results

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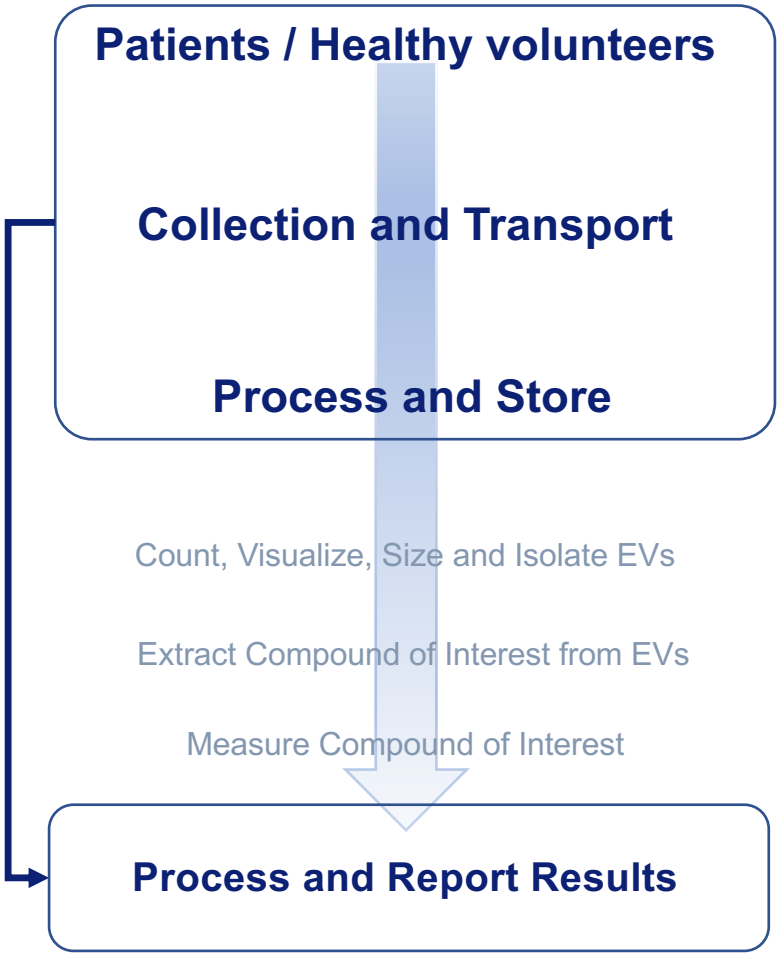

Position papers:

- Welsh et al. **Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches** *(2024) J Extraell Vesicles*
- Erdbrügger et al., **Urinary EVs: A position paper.** *(2021) J Extracell Vesicles*

SOPs for collecting and storage of urine (and blood)

- brd.nci.nih.gov/brd/sop-compendium/show/1583
- ➢ van Royen et al. **The Quick Reference Card: Storage of urinary EVs**. (*2023) J Extracell Vesicles*
- Fabrice Lucien **MIBlood-EV**: **Minimal information to enhance the quality and reproducibility of blood extracellular vesicle research** *(2023) J Extracell Vesicles*

Guidelines, Reproducibility and Reporting Standards

- Nieuwland et al**., Reproducibility of EV research.** *(2022) Eur J Cell Biol.*
- EV-TRACK Consortium: **EV reporting**. (2017) Nat Methods.

Open Access and Repositories

- Deposit data in Vesiclepedia, EVpedia, ExoCarta, exRNA, miRandola, Zenodo, etc.
- Biospecimen Research Database (Biobank details & SOPs)

  E.g. H.M Moore. **The NCI Biospecimen Research Network,** *(2011) BiotechHistochem*

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The IMMPROVE consortium project

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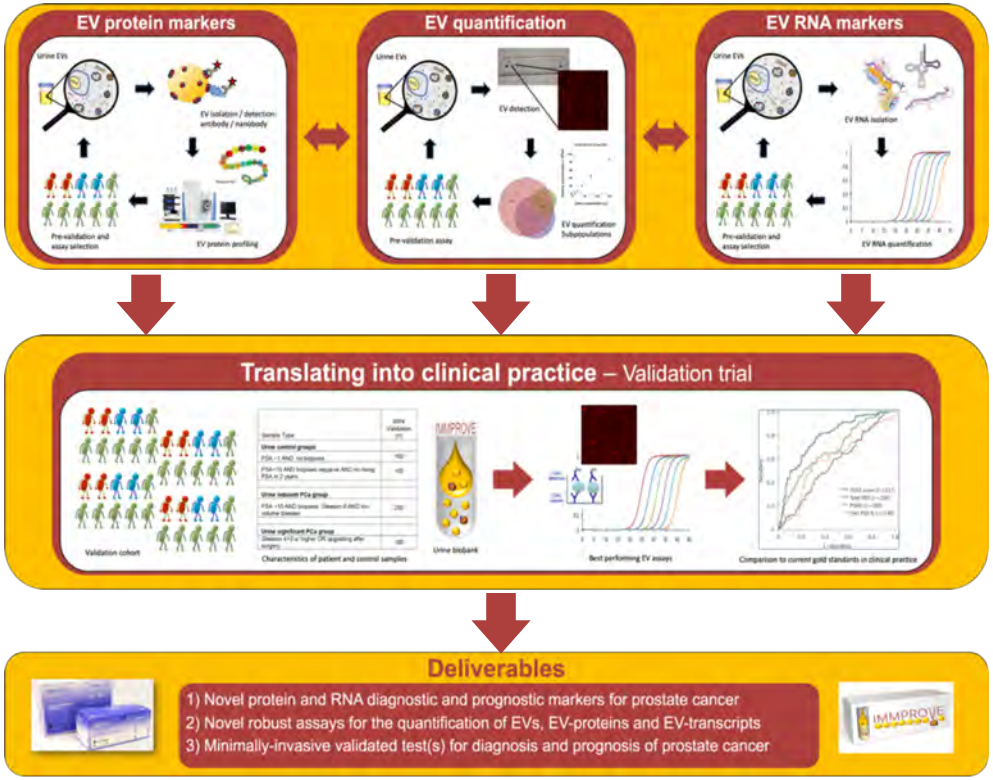
2016

2019

2022



- Innovative Measurements and Markers for PROstate cancer diagnosis and prognosis using Extracellular Vesicles

Alpe d'HuZes opgevenisgeenoptie.nl

Een actie voor KWF KANKER BESTRIJDING

Erasmus MC University Medical Center Rotterdam

Radboudumc university medical center

Amsterdam UMC Universitair Medische Centra

TU Delft

Erasmus MC Cancer Institute

Biobanking

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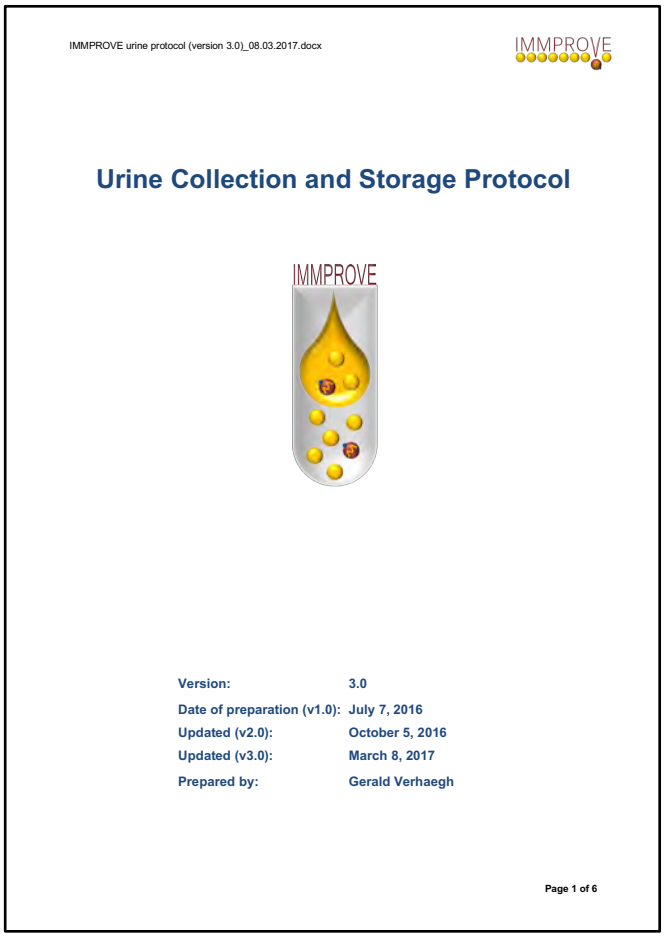
IMMPROVE urine protocol (version 3.0)_08.03.2017.docx

IMMPROVE

Urine Collection and Storage Protocol

IMMPROVE

Version:	3.0
Date of preparation (v1.0):	July 7, 2016
Updated (v2.0):	October 5, 2016
Updated (v3.0):	March 8, 2017
Prepared by:	Gerald Verhaegh

Page 1 of 6

- **Collection parameters**
  - 3 medical centers, EMC, AUMC, RDBUMC
  - Uniform collection protocol
    - Prior to 1st biopsy
    - Post-DRE
    - 24-48 ml
    - First void urine
    - EV-containing supernatant processed within 4 hours (500 g)
    - 4 ml aliquots stored at -80°C

- **Clinical data and experimental measurements**
  - CASTOR EDC database
    - Serum PSA (sPSA)
    - Biopsy Gleason Score
    - Creatinine
    - Urinary PSA
    - …..
    - …..
  - Over 50 different sample parameters

Including clinicopathological characteristics

Biobanking - The Collection protocol

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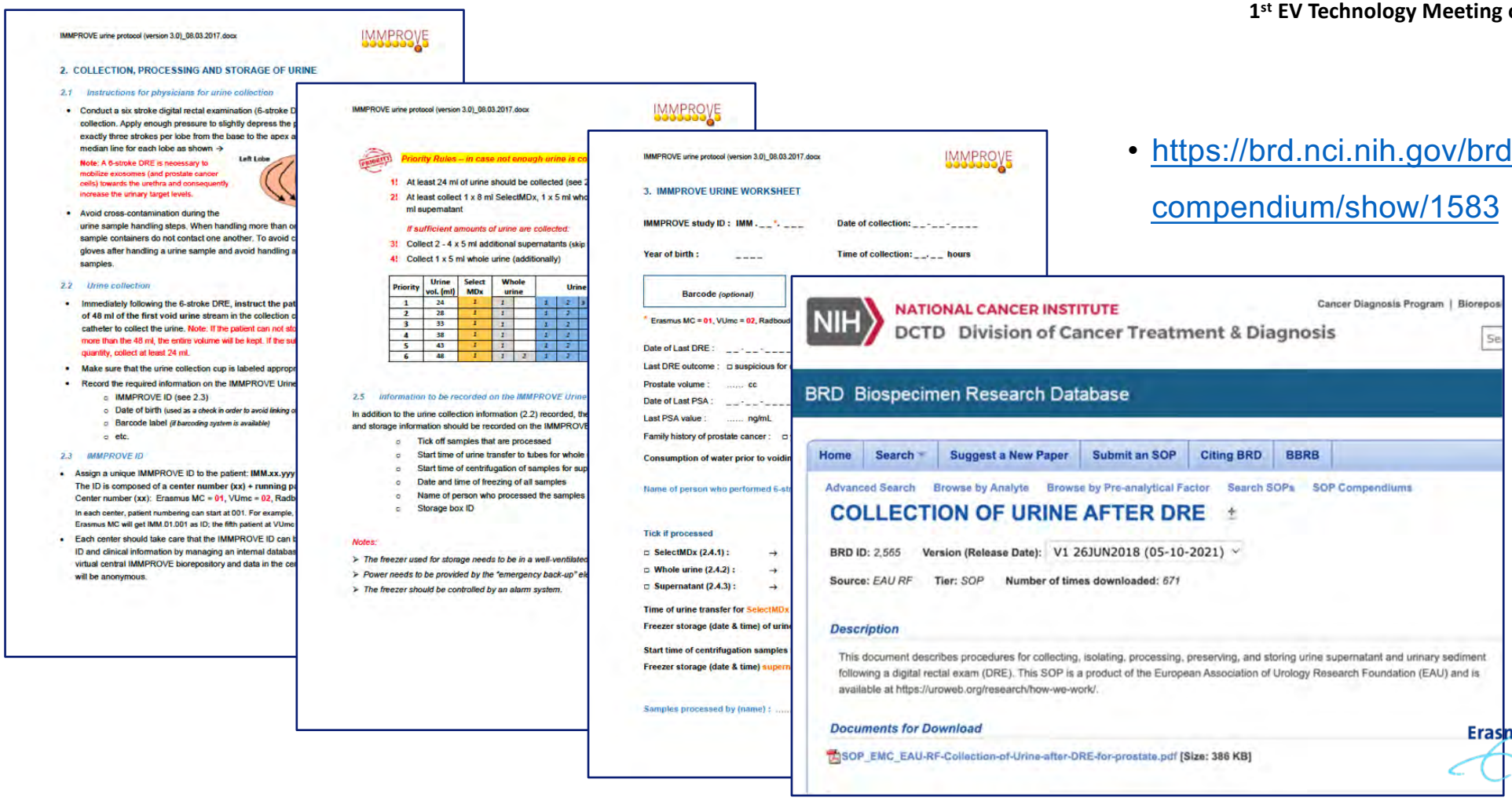

- https://brd.nci.nih.gov/brd/sop-compendium/show/1583

Biobanking: The Database

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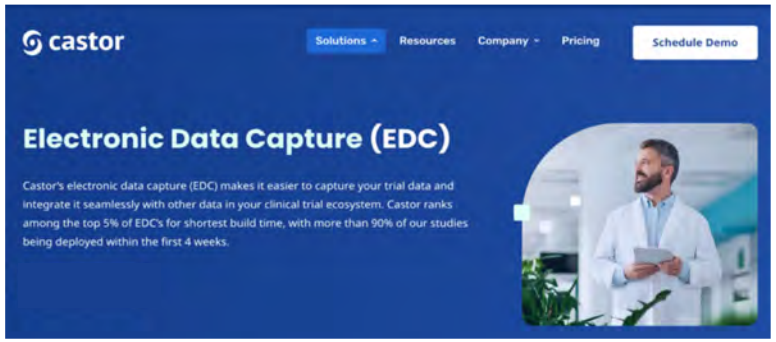
1st EV Technology Meeting of 2024



- **Clinical data and measurements**
  - CASTOR EDC database
  - Over 50 different sample parameters including clinicopathological characteristics

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Biobanking: The Database

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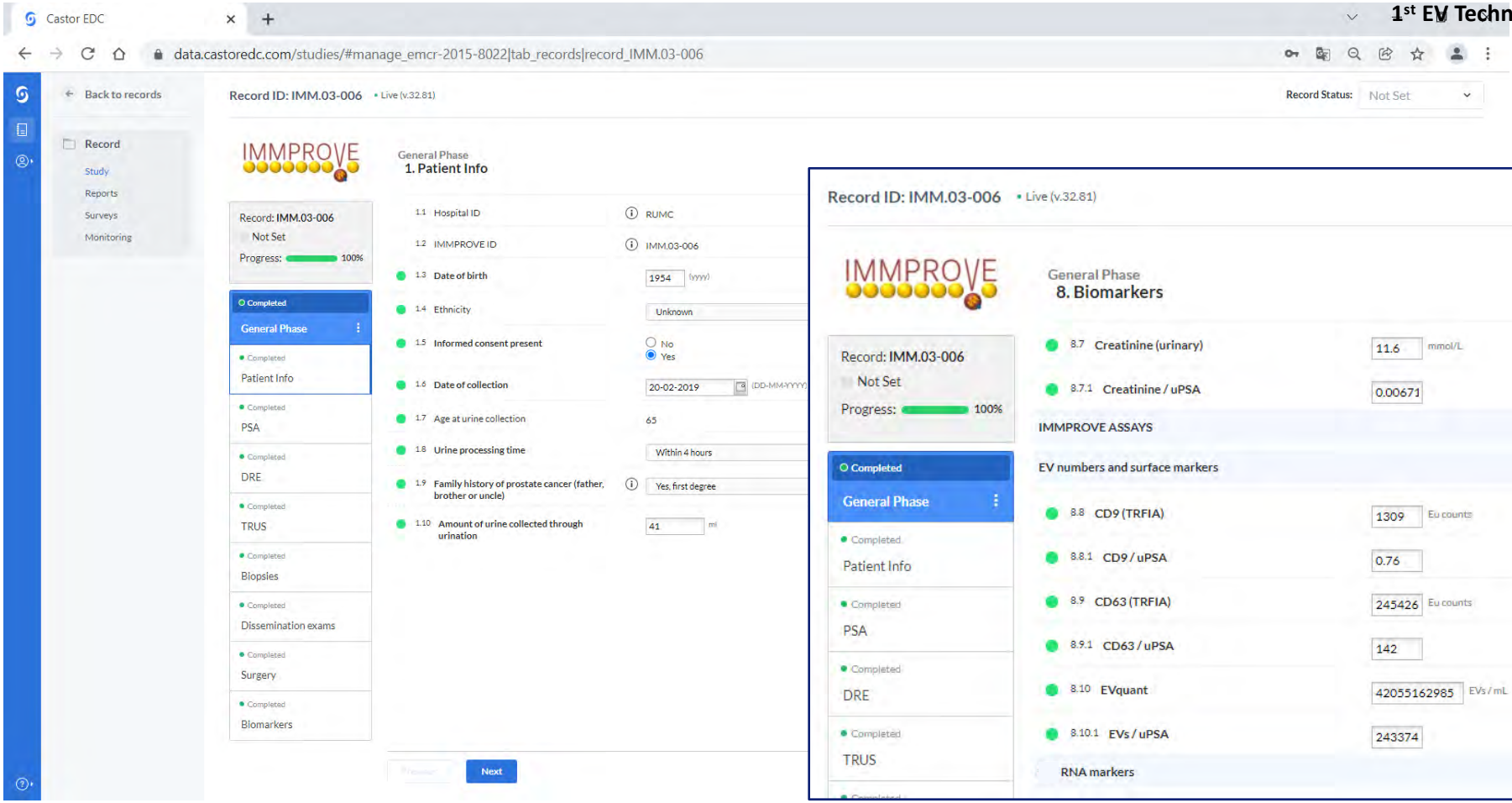

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Quality control: Multicenter comparison

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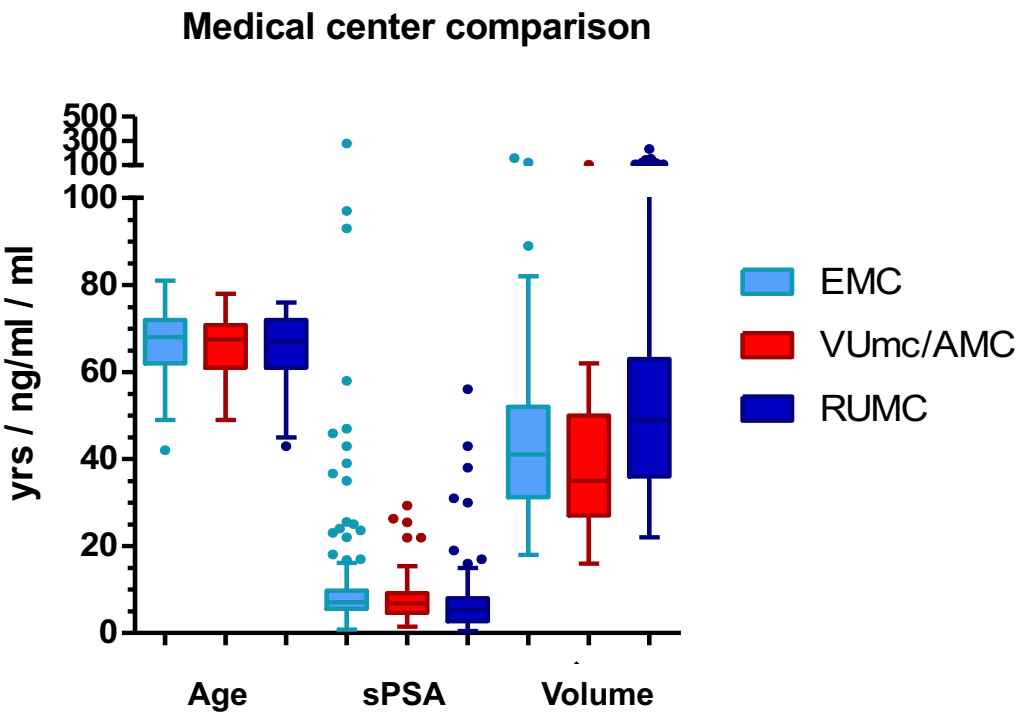
- **Collected samples** **525**
- **Biopsied samples** **328**
  - **PCa negative (N=111)** **111**
    - *sPSA <3, no biopsy* 107
    - *negative biopsies* 4
  - **PCa positive** **221**
    - *GS = 6* 64
    - *GS = 7* 98
    - *GS = 8* 24
    - *GS = 9* 29
    - *GS = 10* 6



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Quality control

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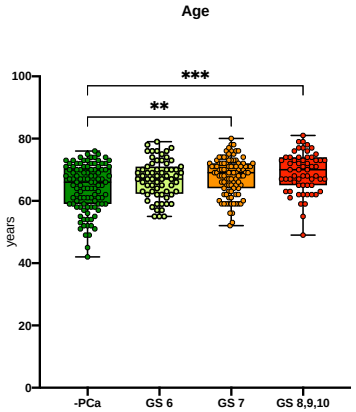
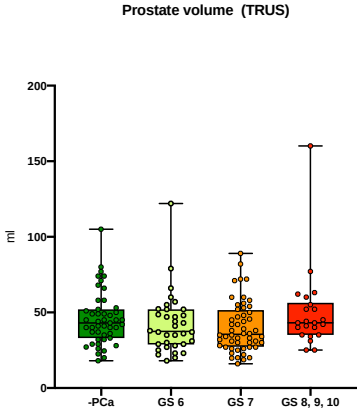
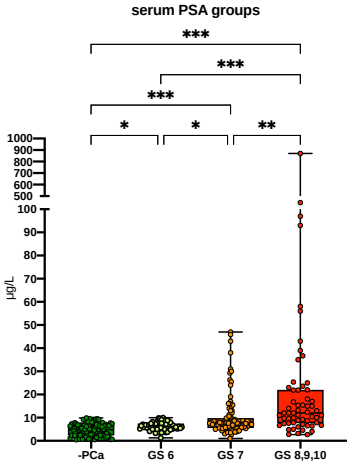
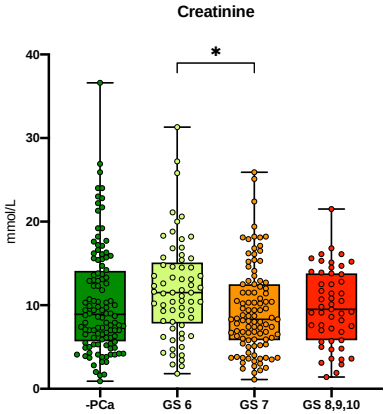
Clinicopathological characteristics

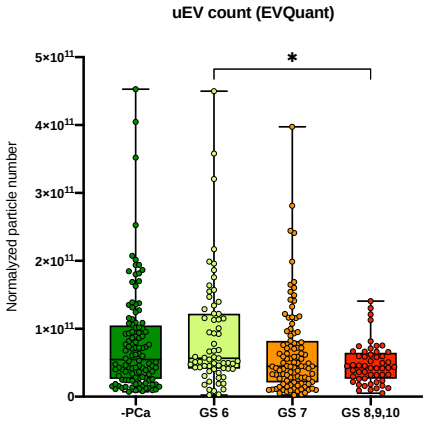


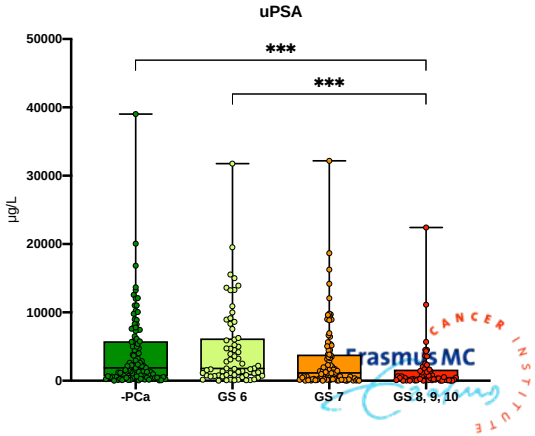




Biofluid cohort characteristics







Methodology for downstream processing and analysis

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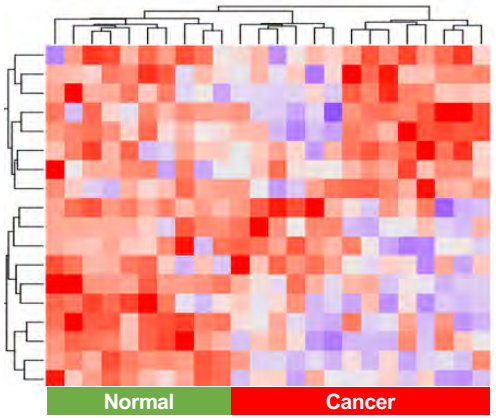
Define targets

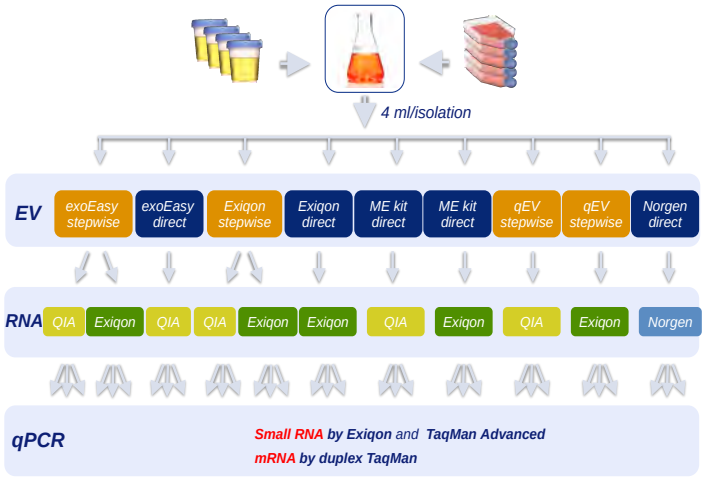
1. 11 candidate-biomarker small RNAs from previous tissue-based studies



- microRNAs
- isoMIRs
- tRNA fragments
- snoRNAs
- snoRNA fragments

Define & optimize methodology

2. Selection of uEVs isolation method and small RNA quantitation



- Norgen Direct uEV capture and RNA isolation kit
- Duplex TaqMan advanced qPCR

Reiterate

3. Evaluation in clinical samples

Cohort 1 *(n=10,Detection in uEVs)*

Small RNAs: 11
Samples: 5 benign, 5 cancer

Cohort 2 *(n=91, Diagnostic performance)*

Small RNAs: 4
Samples: 22 benign, 69 cancer

Cohort 3 *(n=329,Validation)*

Small RNAs: 2
Samples: 107 benign, 221 cancer

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A Quick Reference Card for urine and uEVs

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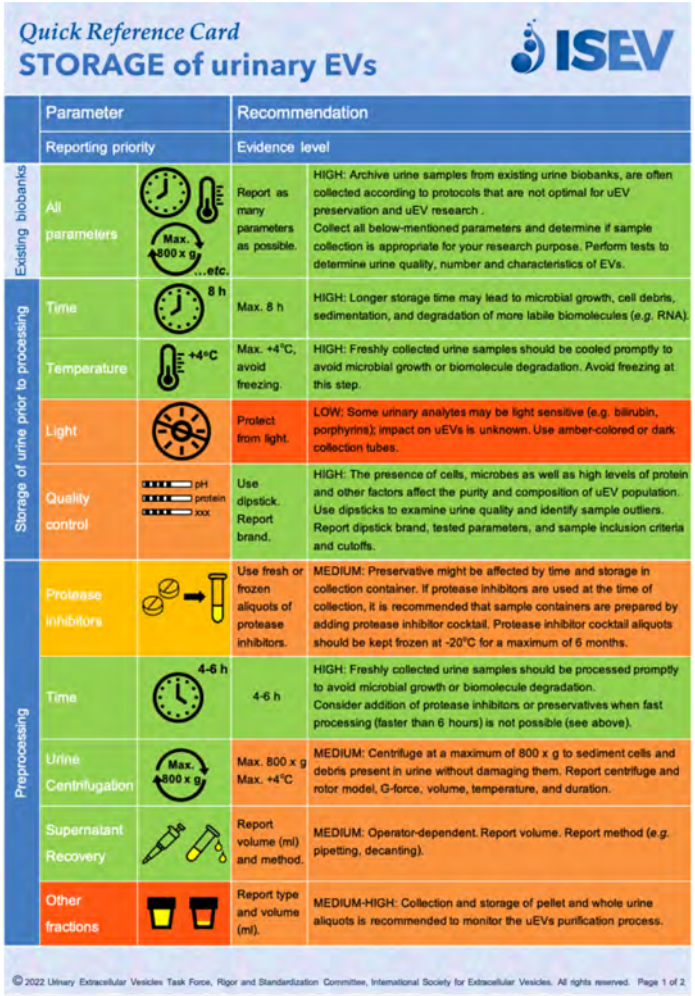
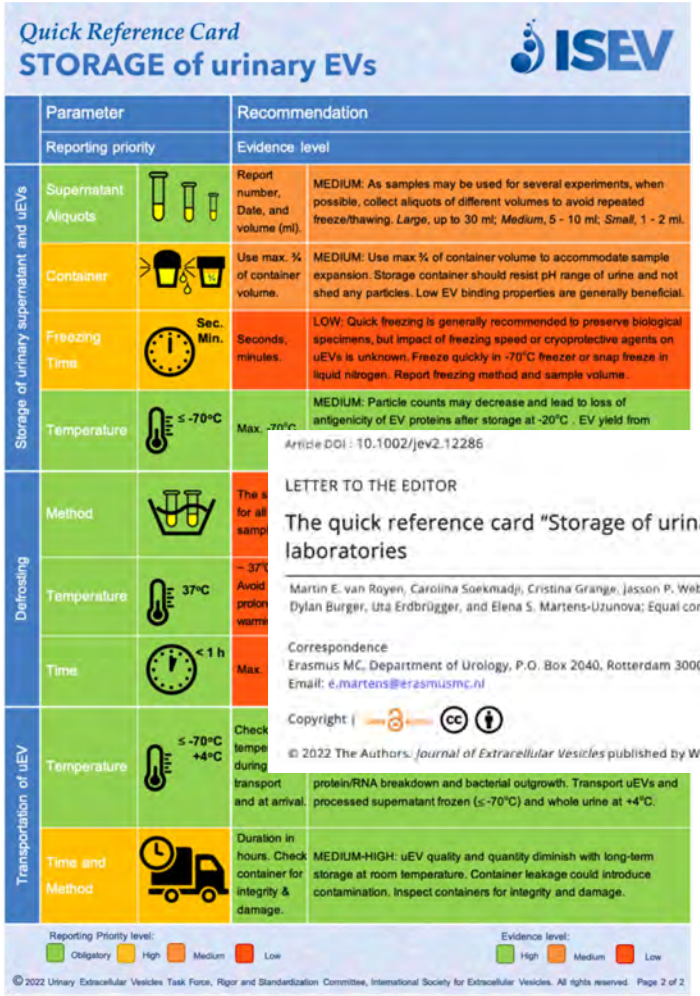


Quick Reference Card
STORAGE of urinary EVs

ISEV

	Parameter		Recommendation	
	Reporting priority		Evidence level	
Existing biobanks	All parameters	Max. 800 x g ...etc.	Report as many parameters as possible.	HIGH: Archive urine samples from existing urine biobanks, are often collected according to protocols that are not optimal for uEV preservation and uEV research . Collect all below-mentioned parameters and determine if sample collection is appropriate for your research purpose. Perform tests to determine urine quality, number and characteristics of EVs.
Storage of urine prior to processing	Time	8 h	Max. 8 h	HIGH: Longer storage time may lead to microbial growth, cell debris, sedimentation, and degradation of more labile biomolecules (*e.g.* RNA).
	Temperature	+4°C	Max. +4°C, avoid freezing.	HIGH: Freshly collected urine samples should be cooled promptly to avoid microbial growth or biomolecule degradation. Avoid freezing at this step.
	Light		Protect from light.	LOW: Some urinary analytes may be light sensitive (e.g. bilirubin, porphyrins); impact on uEVs is unknown. Use amber-colored or dark collection tubes.
	Quality control	pH protein xxx	Use dipstick. Report brand.	HIGH: The presence of cells, microbes as well as high levels of protein and other factors affect the purity and composition of uEV population. Use dipsticks to examine urine quality and identify sample outliers. Report dipstick brand, tested parameters, and sample inclusion criteria and cutoffs.
Preprocessing	Protease inhibitors		Use fresh or frozen aliquots of protease inhibitors.	MEDIUM: Preservative might be affected by time and storage in collection container. If protease inhibitors are used at the time of collection, it is recommended that sample containers are prepared by adding protease inhibitor cocktail. Protease inhibitor cocktail aliquots should be kept frozen at -20°C for a maximum of 6 months.
	Time	4-6 h	4-6 h	HIGH: Freshly collected urine samples should be processed promptly to avoid microbial growth or biomolecule degradation. Consider addition of protease inhibitors or preservatives when fast processing (faster than 6 hours) is not possible (see above).
	Urine Centrifugation	Max. 800 x g	Max. 800 x g Max. +4°C	MEDIUM: Centrifuge at a maximum of 800 x g to sediment cells and debris present in urine without damaging them. Report centrifuge and rotor model, G-force, volume, temperature, and duration.
	Supernatant Recovery		Report volume (ml) and method.	MEDIUM: Operator-dependent. Report volume. Report method (*e.g.* pipetting, decanting).
	Other fractions		Report type and volume (ml).	MEDIUM-HIGH: Collection and storage of pellet and whole urine aliquots is recommended to monitor the uEVs purification process.



Quick Reference Card
STORAGE of urinary EVs

ISEV

	Parameter		Recommendation	
	Reporting priority		Evidence level	
Storage of urinary supernatant and uEVs	Supernatant Aliquots		Report number, Date, and volume (ml).	MEDIUM: As samples may be used for several experiments, when possible, collect aliquots of different volumes to avoid repeated freeze/thawing. *Large*, up to 30 ml; *Medium*, 5 - 10 ml; *Small*, 1 - 2 ml.
	Container		Use max. ¾ of container volume.	MEDIUM: Use max ¾ of container volume to accommodate sample expansion. Storage container should resist pH range of urine and not shed any particles. Low EV binding properties are generally beneficial.
	Freezing Time	Sec. Min.	Seconds, minutes.	LOW: Quick freezing is generally recommended to preserve biological specimens, but impact of freezing speed or cryoprotective agents on uEVs is unknown. Freeze quickly in -70°C freezer or snap freeze in liquid nitrogen. Report freezing method and sample volume.
	Temperature	≤ -70°C	Max. -70°C	MEDIUM: Particle counts may decrease and lead to loss of antigenicity of EV proteins after storage at -20°C . EV yield from
Defrosting	Method		The s for all samp	
	Temperature	37°C	– 37° Avoid prolon warmi	
	Time	< 1 h	Max.	
Transportation of uEV	Temperature	≤ -70°C +4°C	Check tempe during transport and at arrival.	protein/RNA breakdown and bacterial outgrowth. Transport uEVs and processed supernatant frozen (≤-70°C) and whole urine at +4°C.
	Time and Method		Duration in hours. Check container for integrity & damage.	MEDIUM-HIGH: uEV quality and quantity diminish with long-term storage at room temperature. Container leakage could introduce contamination. Inspect containers for integrity and damage.

Reporting Priority level: Obligatory, High, Medium, Low

Evidence level: High, Medium, Low

Article DOI : 10.1002/jev2.12286

LETTER TO THE EDITOR

The quick reference card "Storage of urinary EVs" – A practical guideline tool for research and clinical laboratories

Martin E. van Royen, Carolina Soekmadji, Cristina Grange, Jasson P. Webber, Tobias Tertel, Marvin Droste, Anja Bluescher, Bernd Giebel, Guido Jenster, Alicia Llorente, Charles J. Blijdorp, Dylan Burger, Uta Erdbrügger, and Elena S. Martens-Uzunova; Equal contributions

Correspondence
Erasmus MC, Department of Urology, P.O. Box 2040, Rotterdam 3000 CA, The Netherlands.
Email: e.martens@erasmusmc.nl

A product of
the **Urine EV Task Force**
Working group **STORAGE**

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Storage effects on EV content including main points for of urine and uEVs

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STORAGE FRAMEWORK

Frozen Urine from Biobank
- Quality Control parameter
- Pre-processing method prior to storage
- Storage duration (XX years)?
- Storage temperature
- Minimum volume

Fresh Urine
- Quality control parameter
- Methodology
- Minimum volume

Freezing
- Temperature
- Maximum storage duration

Fresh
- Physical condition during sample prep
- Maximum time allowed before isolation

Influence of processing parameters
- Enzyme treatment / inhibitors
- (DNase/RNase/protease)
- Buffer pH,
- Buffer composition
- Addition of carriers and/or blocking agents (e.g. albumin)
- Processing temperature?
- Centrifugation and/or filtration prior to storage

Frozen urine samples
- Defrosting methods
- Frequency freeze and thaw
- Quality Control parameter
- Removal / dispersion of sediment

Sample treatment prior to EV isolation
- Pre-processing methods

EV isolation
- Minimum volume
- Quality control parameter
- Recommended isolation methods
- Characterisation (markers etc)
- Quality Control methodology

Urine EV Task Force & GUSEV
Work group STORAGE
Cristina Grande, Martin Van Roojen

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Clinical Translation Roadblocks in urinary EV biomarker research (*manuscript in preparation*)

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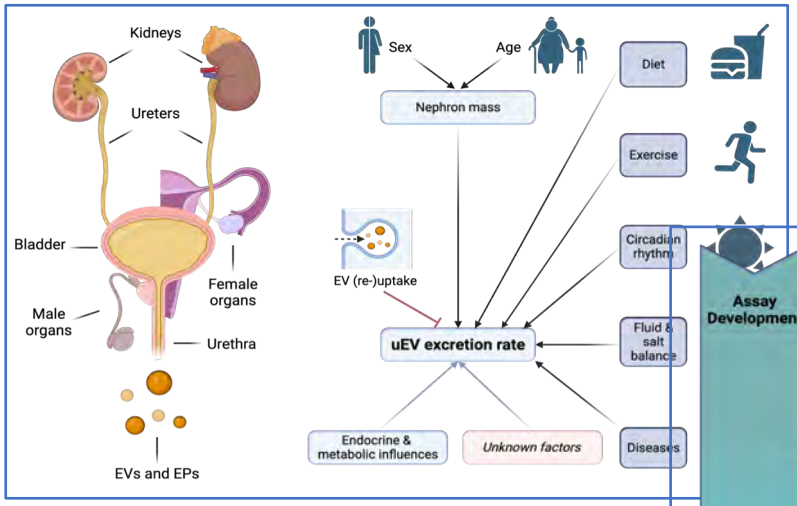
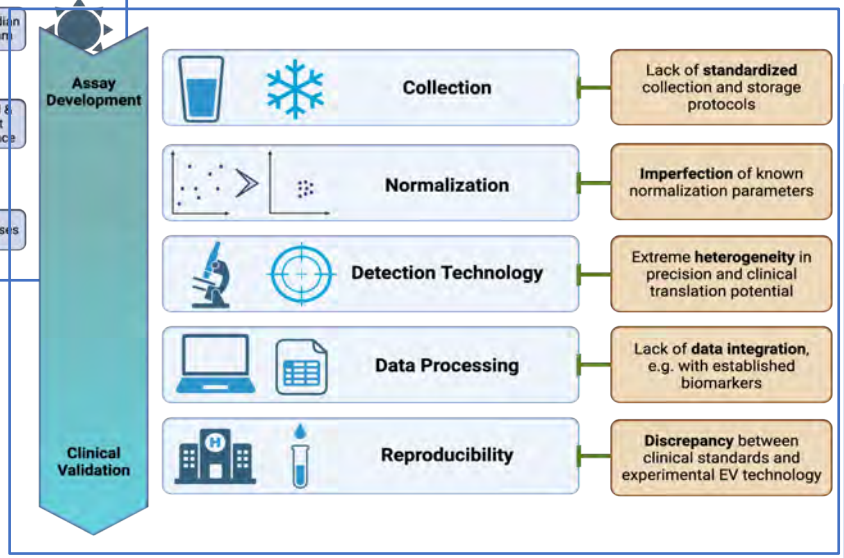
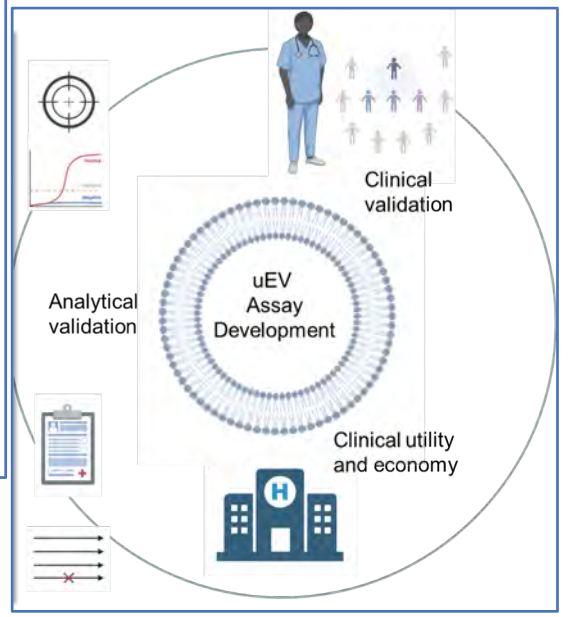
Factors determining uEV excretion



Technical roadblocks



Factors affecting uEV assay development



A product of

GUSEV

ISEV SPECIAL INTEREST GROUP

How to deal with known and unknown variables?

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• Make an **Inventory** of known variables and order them on expected impact	Literature / Colleagues / Join a community
• **Test** different variables for their effect	Conclusion / Publish
• **Standardize** as many (high impact) variables as possible	SOPs
• **Keep a Record** of methods and SOP deviations	Data management protocols
• **Re-interpret**, Learn and Adapt	Reiterate

• **Accept** that you will never be able to control:
  • all known variables
  • certainly not the unknown ones
  • EV technology advancement (that might change impact of variables)
	Be (at) the ***State-of-the-Art***

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In conclusion

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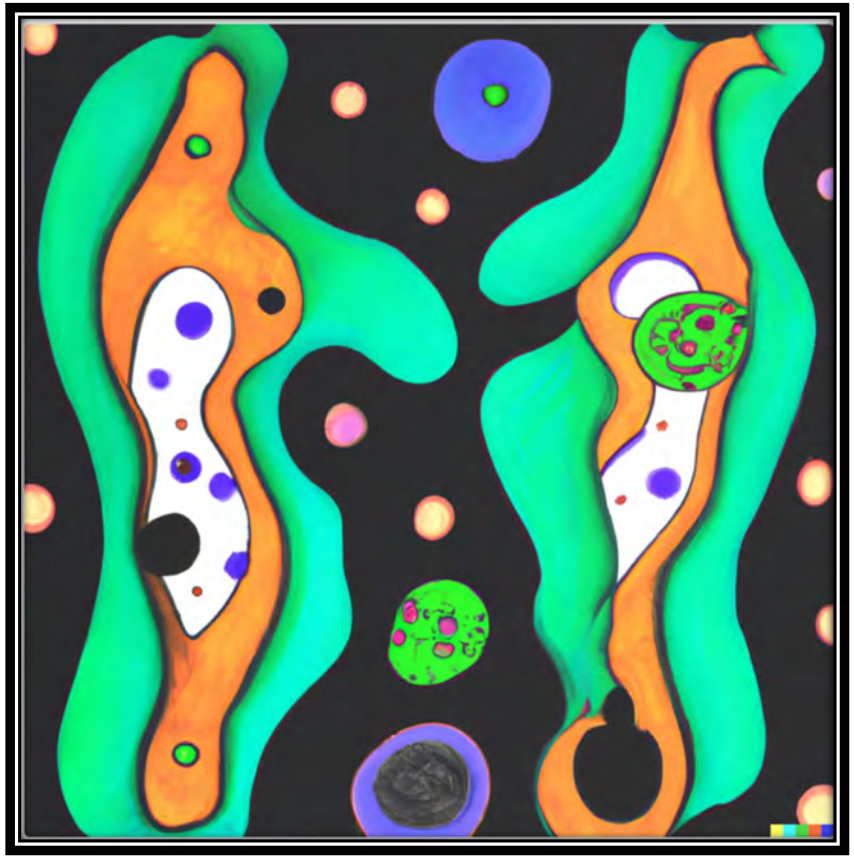
BEST care starts with BEST research!!!

…. But while you are on the journey of science discovery, do not forget to have fun and explore new paths ….



Extracellular Vesicles

Elena × DALL-E
Human & AI

Erasmus MC Cancer Institute

Thank you!

ISEV

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Erasmus MC

Experimental Urology Lab

Guido Jenster
Chris Bangma
Natasja Dits
Mirella van den Berg

Erasmus MC UROLOGY RESEARCH
www.erasmusurologyresearch.nl

Pathology Lab

Martin van Royen
Thomas Hartjes

IMMPROVE

Radboudumc university medical center

Jack Schalken
Gerald Verhaegh

Amsterdam UMC Universitair Medische Centra

Connie Jimenez
Irene Bijnsdorp

TU Delft Delft University of Technology

Michiel Kreutzer
Volkert van Steijn
Serhii Mytnyk

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Fellowship

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ISEV SPECIAL INTEREST GROUP

The Urine EV Task Force

Uta Erdbruegger
Alicia Llorente
Charles Blijdorp
Dylan Burger




uEVs STORAGE Work group

Martin van Royen
Carolina Soekmadji
Cristina Grange
Jasson Webber
Tobias Tertel
Marvin Droste
Anja Buescher
Bernd Giebel
Lei Zeng

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