Biomarkers for Precision Oncology

- **Alain R. Thierry**
- Director of Research, INSERM - Institute of Research on Oncology de Montpellier
- Director of the “Biomarkers for Precision Oncology” team
- Inserm U1194 – IRCM, Montpellier

A focus: Circulating DNA in oncology

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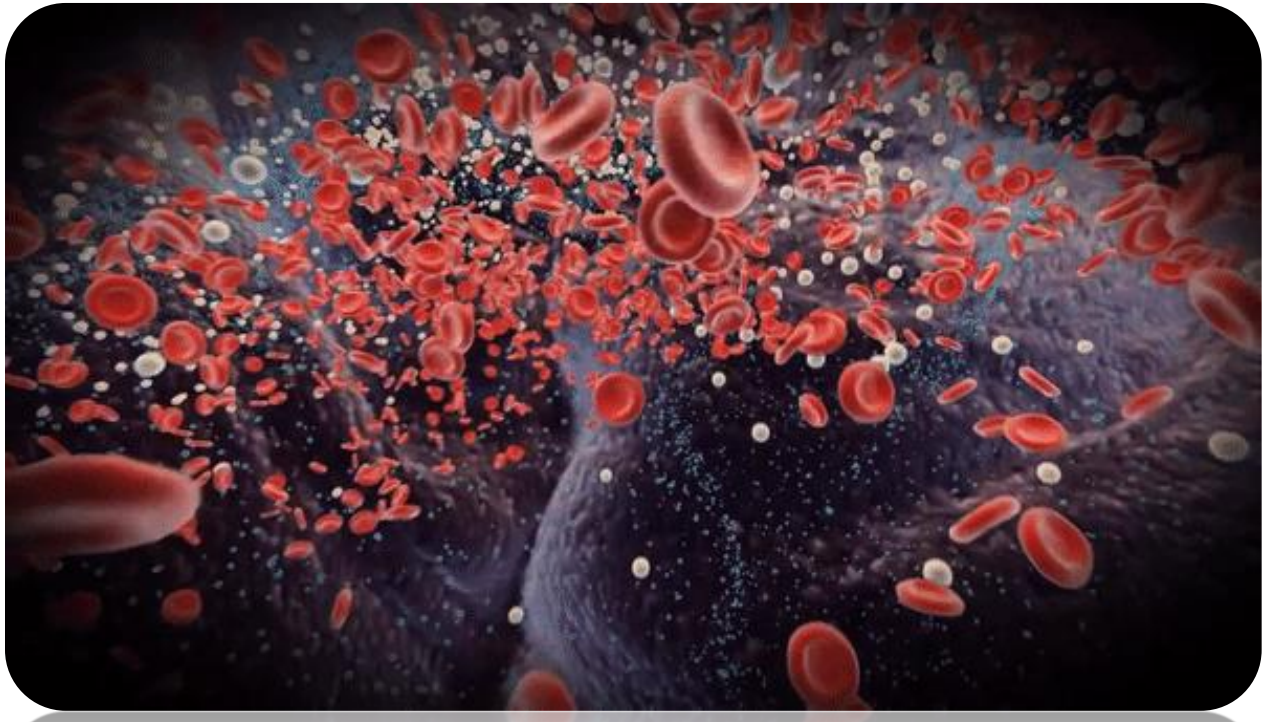
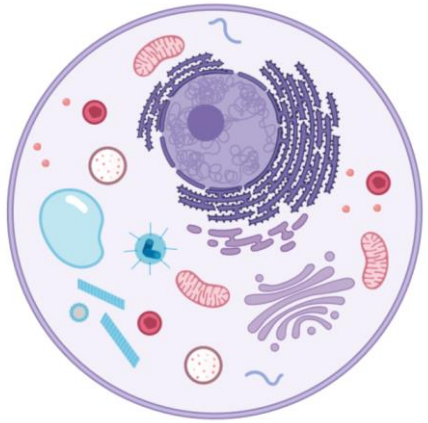




Nuclear and mitochondrial DNA release

Milk
Urine
saliva
mucous suspension
spinal fluid / and amniotic fluid

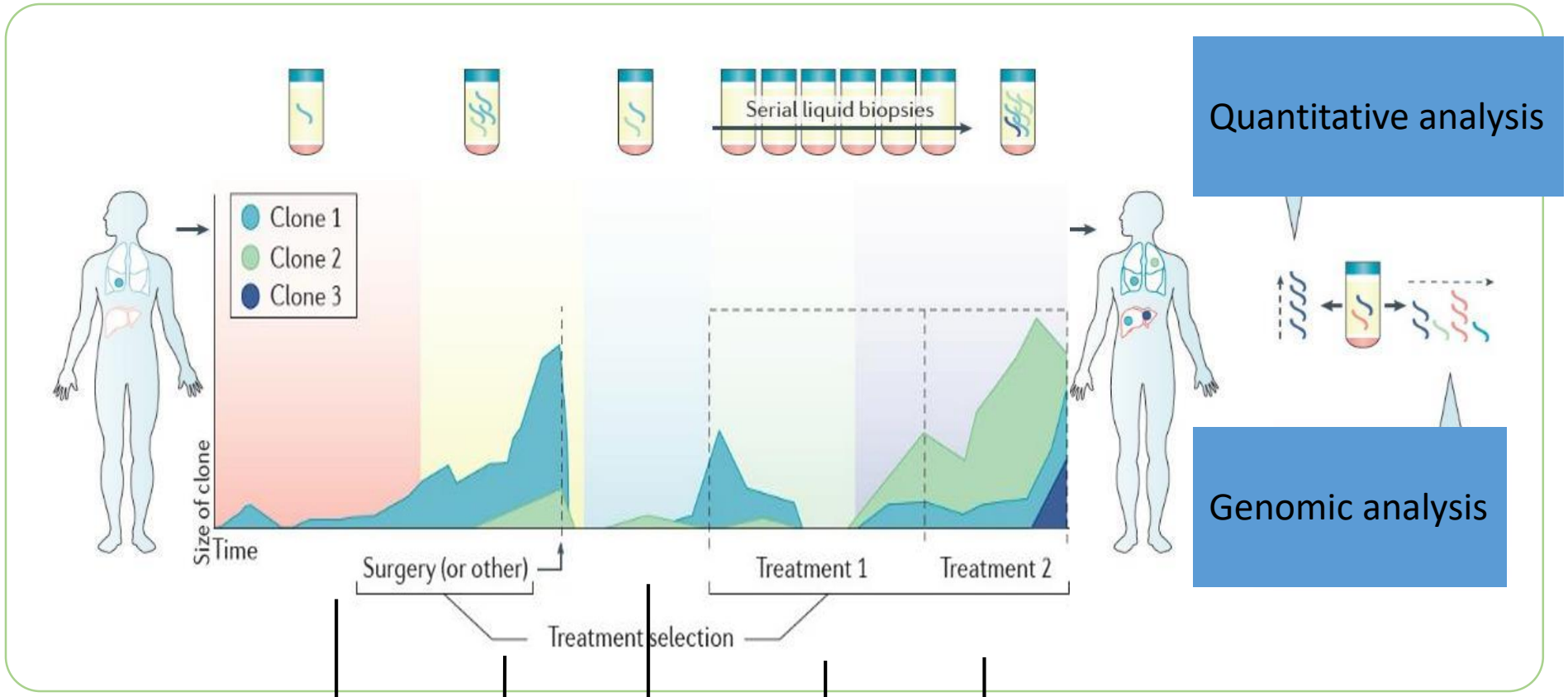
Cell culture supernatants from :
cell lines
primary cells
organoids
embryo cultures

Physiological
circulating fluids
« Blood »

Extracellular circulating DNA

Extracellular DNA

Potential clinical applications of circulating DNA in the course of cancer management care



6. Screening test

2. Deciding on the use of adjuvante chemotherapy

4. Control of Treatment efficacy

1. Guiding on the use of targeted thérapy

5.Surveillance of the recurrence

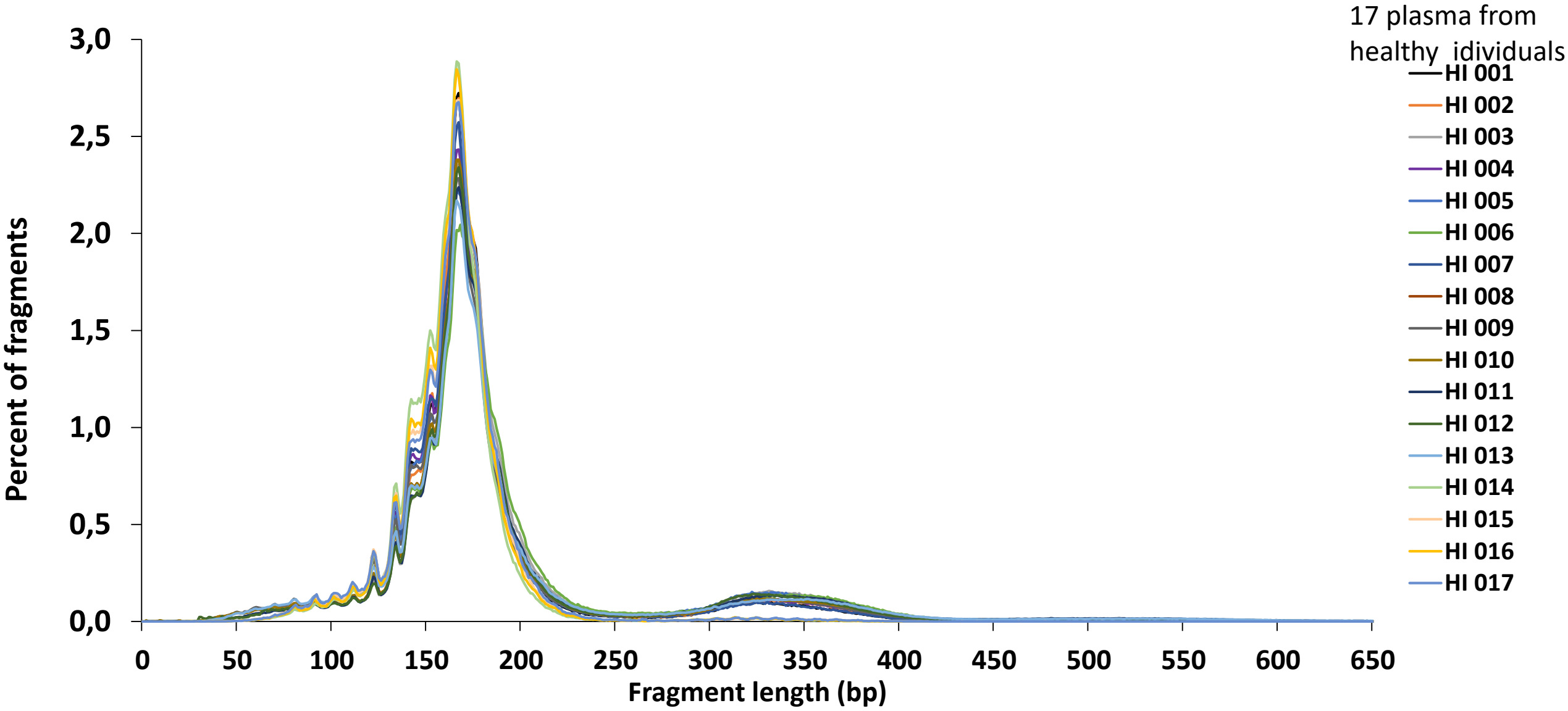
3. Early detection of emerging résistance

Wan et al, Nat.Rev.Cancer, 2017)

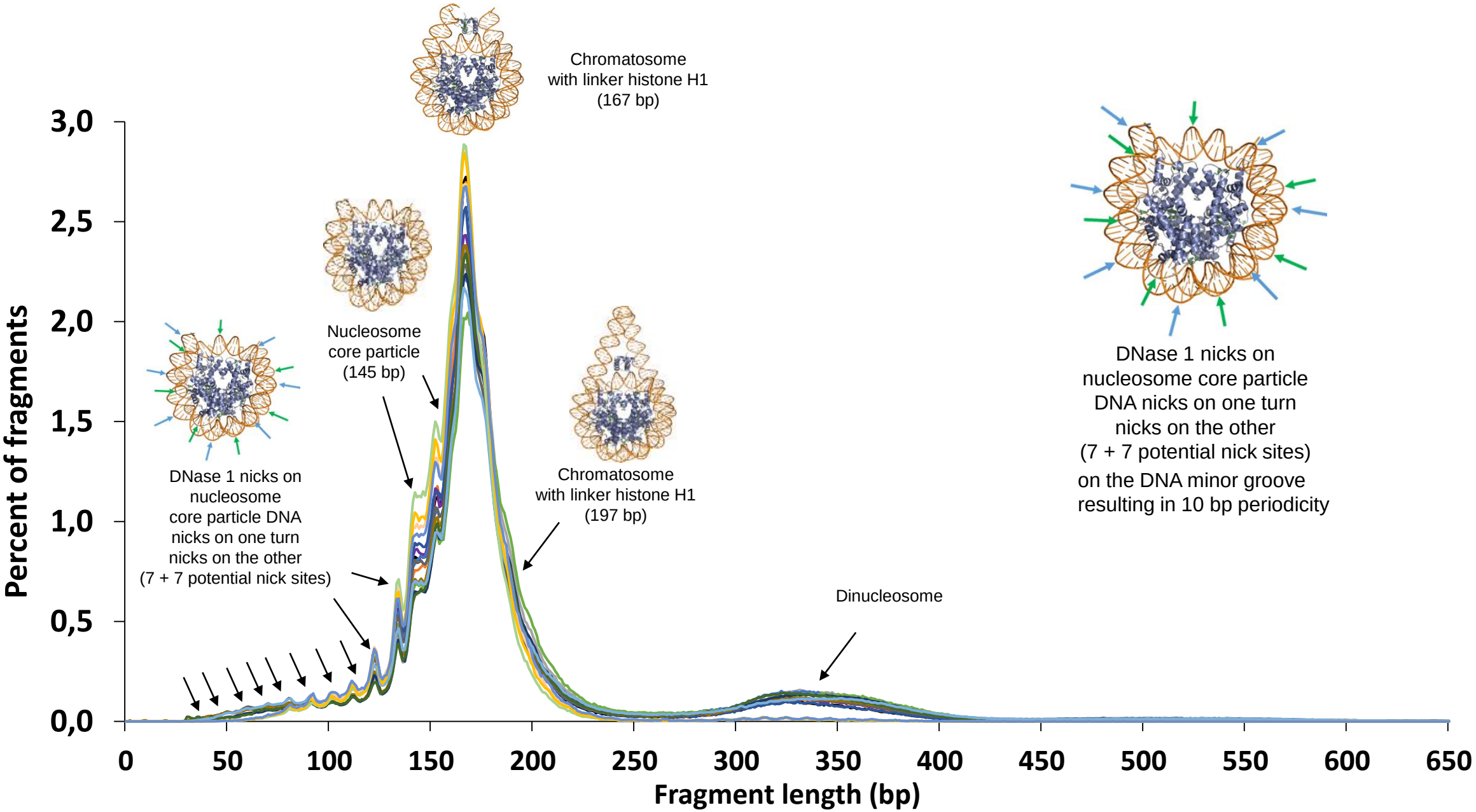
Study of the Topology of circulating nuclear DNA

Analysis of the size fragment

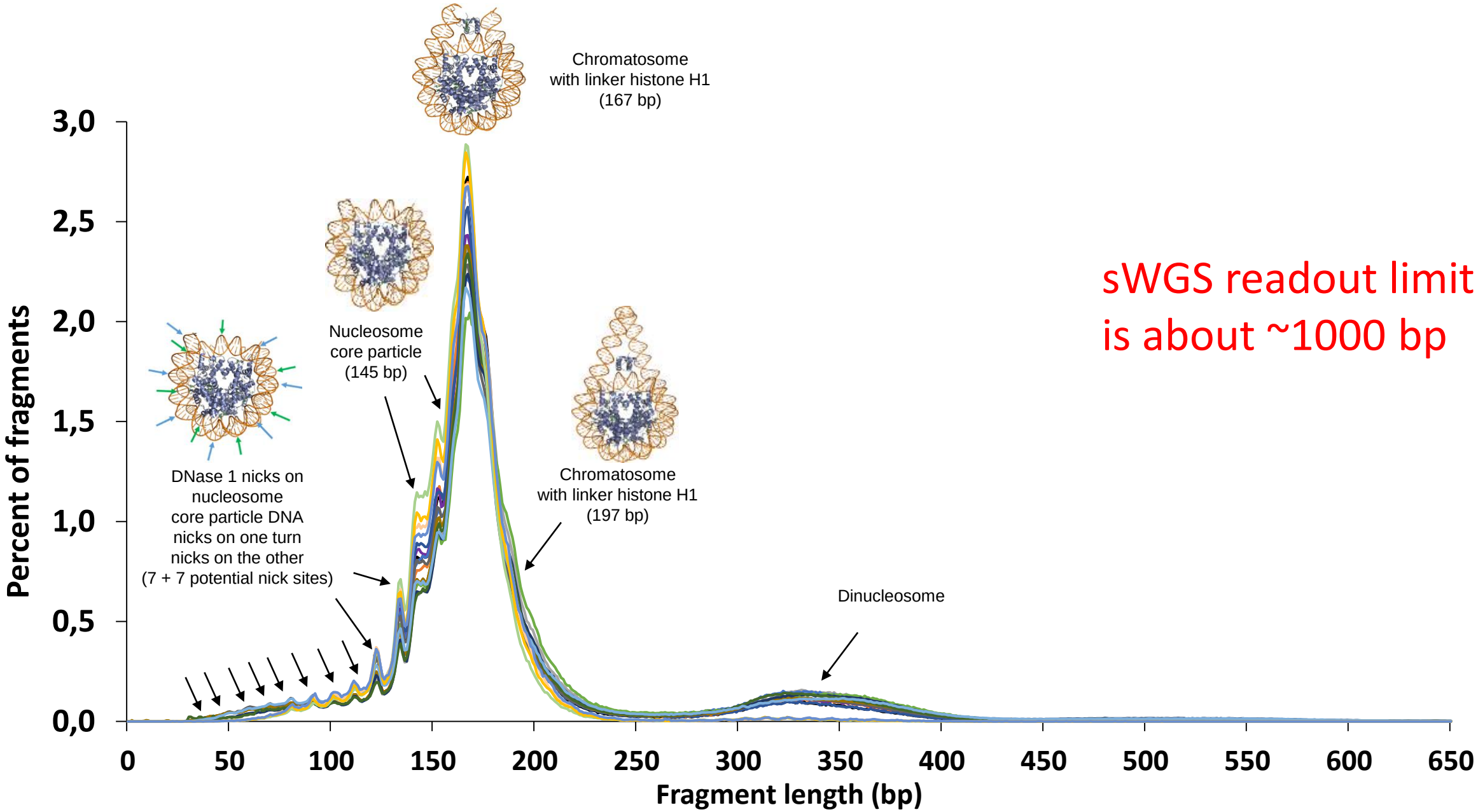
cfDNA fragmentomics study by shallow Whole Genome Sequencing



cfDNA fragmentomics study by shallow Whole Genome Sequencing

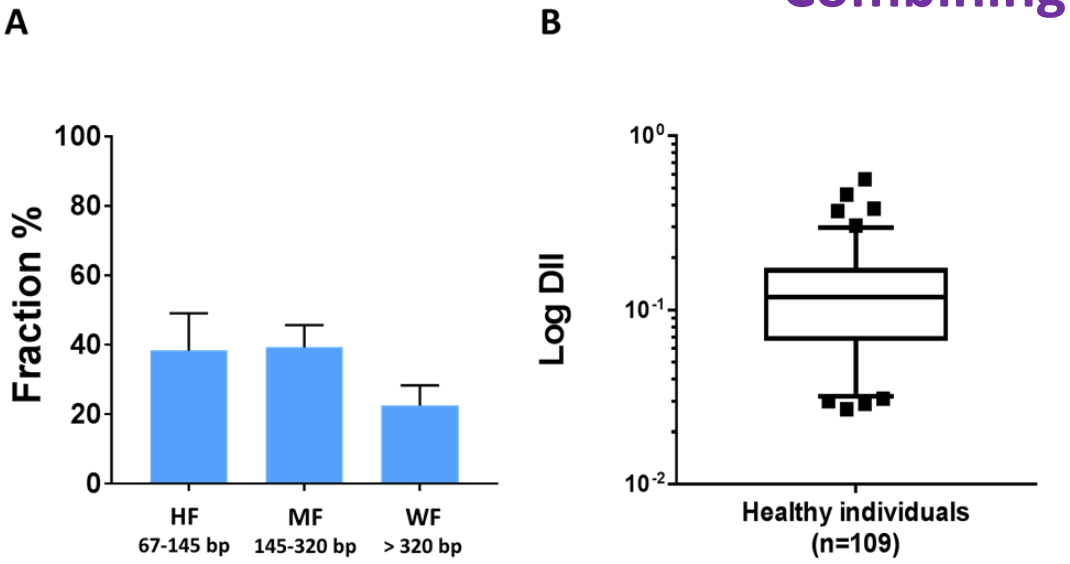


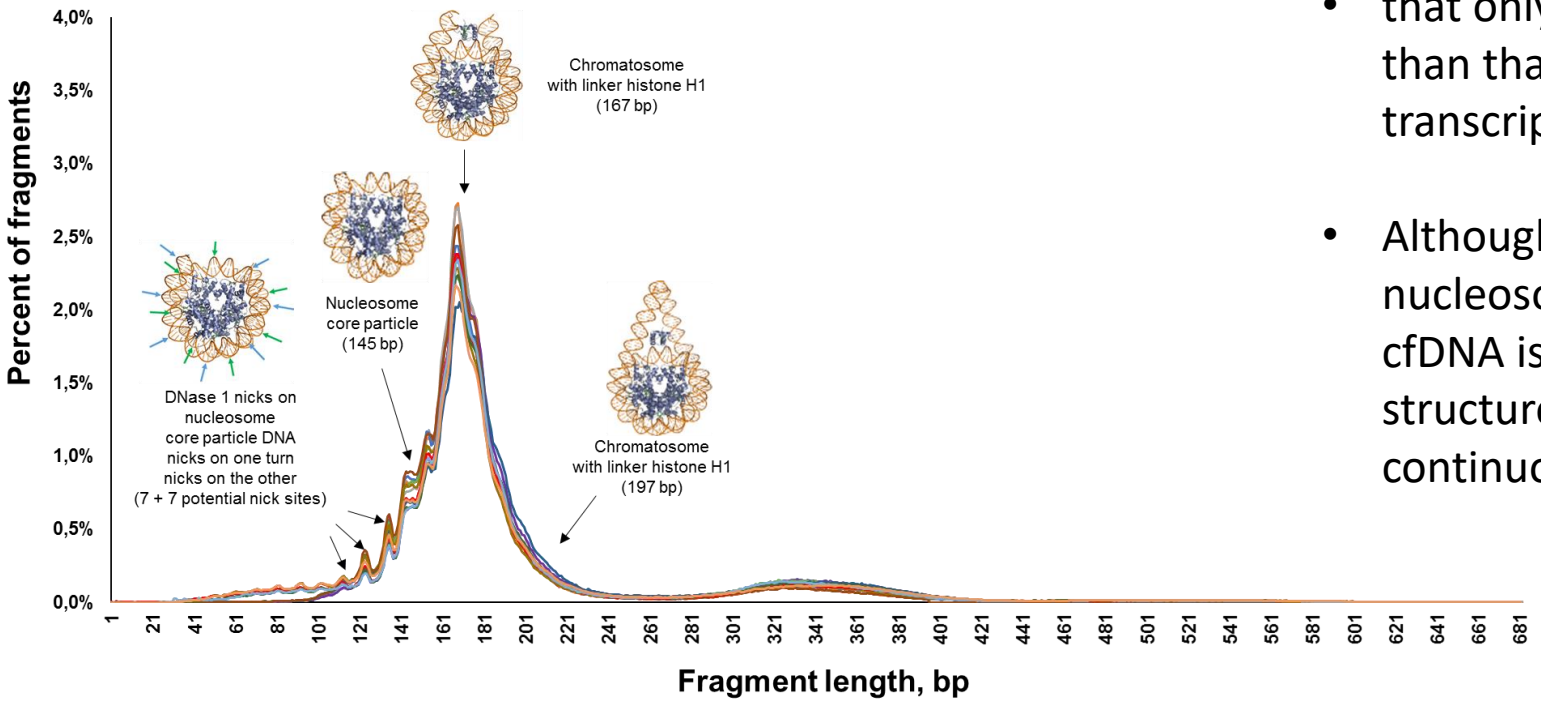
cfDNA fragmentomics study by shallow Whole Genome Sequencing



sWGS readout limit
is about ~1000 bp

Combining sWGS and Q-PCR assays





Taken together our data showed:

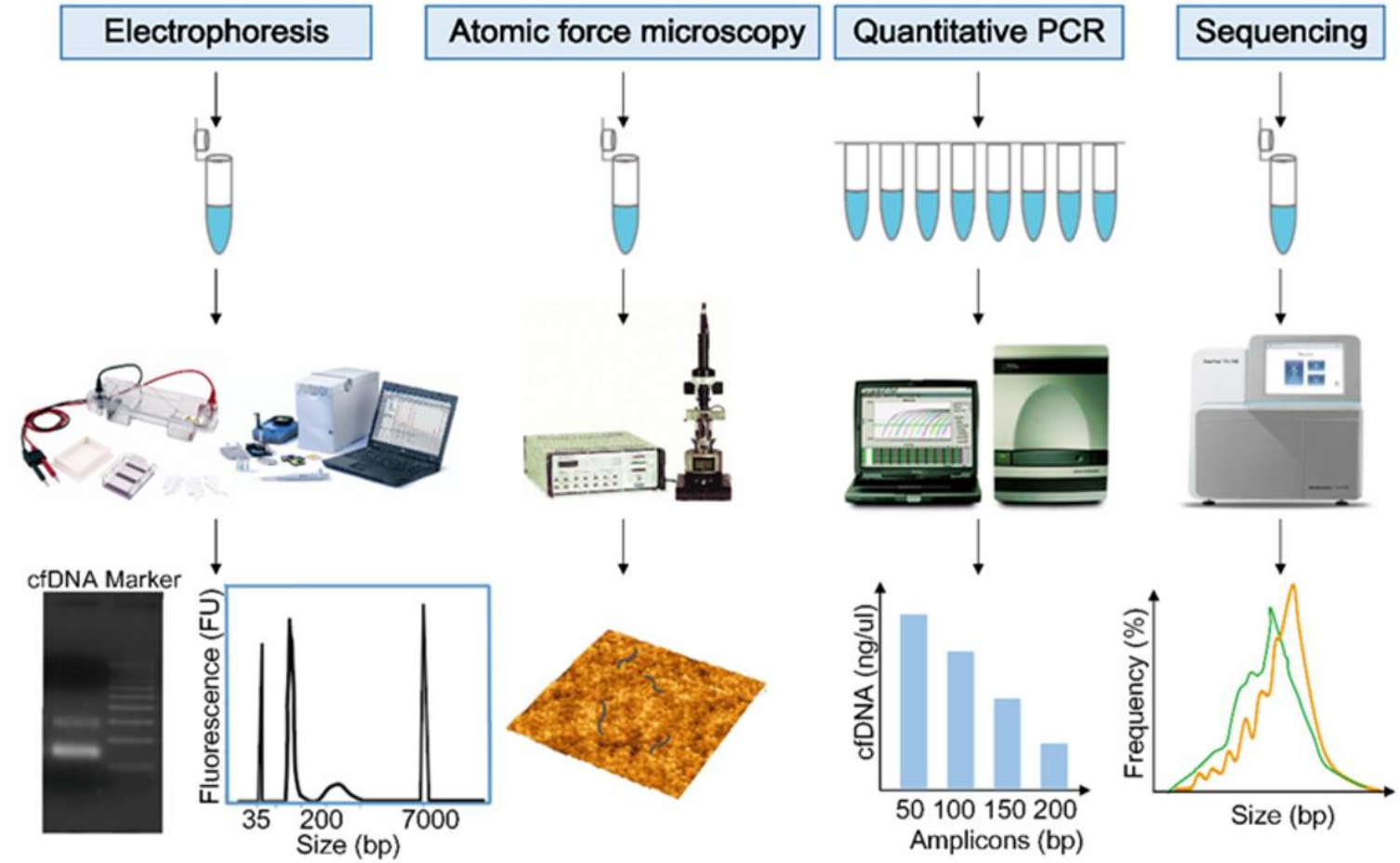
- We could estimate the proportion of cfDNA inserted in
  -mono-nucleosomes, 67.5-80%,
  -di-nucleosomes, 9.4-11.5%
  and chromatin (>1,000bp) as 8.5-21.0%.

- that only a minor proportion of cfDNA is bigger than that existing in mono-nucleosome or transcription factor complexes circulating in blood.

- Although DNA on single chromatosomes or mono-nucleosomes is detectable, our data revealed that cfDNA is highly nicked (97-98%) on those structures, which appear to be subjected to continuous nuclease activity in the bloodstream.

Sanchez et al. JCI Insight, 2021

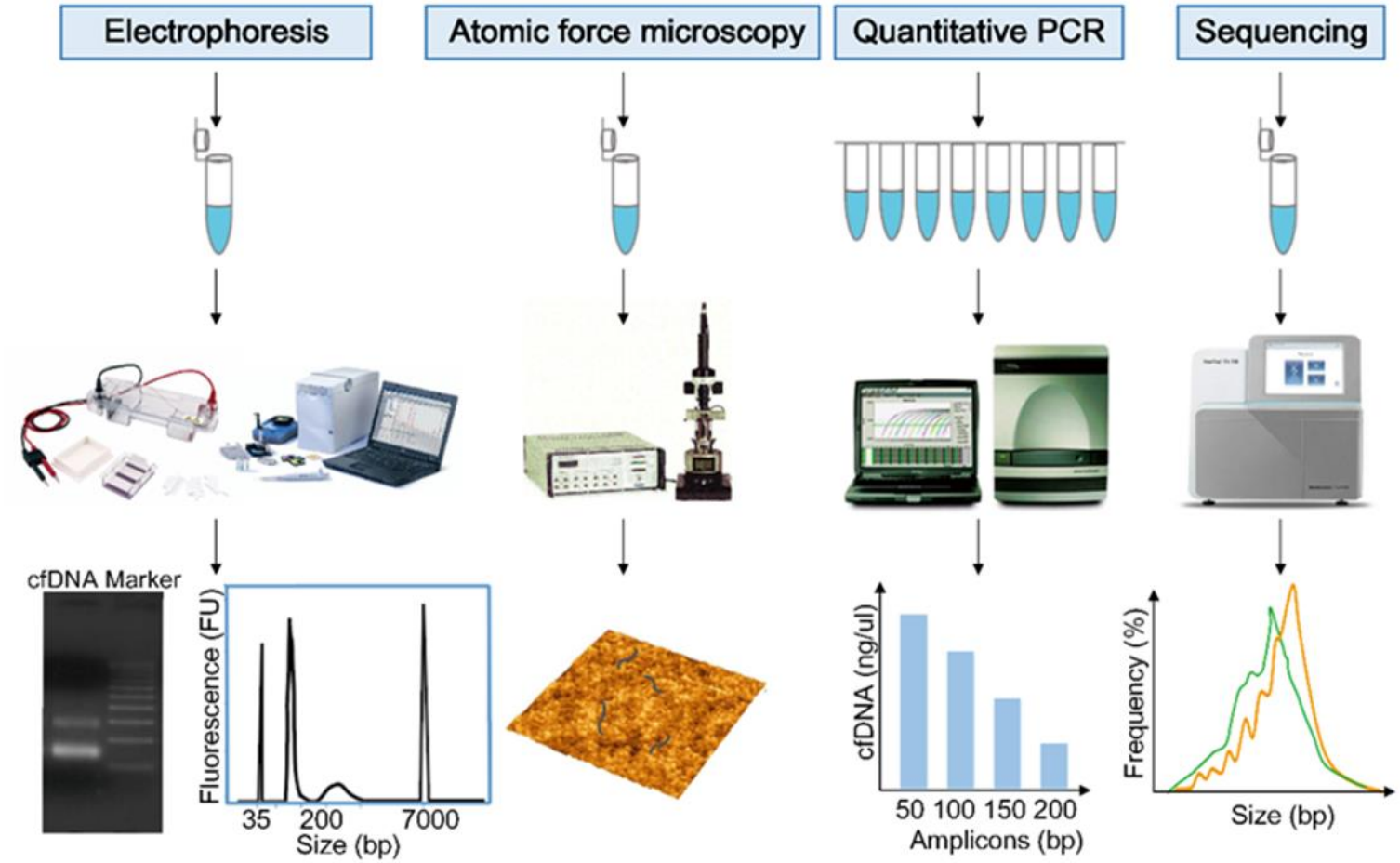
cfDNA fragmentomics study



HIGHLIGHTS:

- We first demonstrated the cfDNA high fragmentation
- Tumor derived cfDNA are more fragmented than non-tumor cell derived cfDNA
- Size distribution as determined by Q-PCR analysis alike SSP- Sequencing
- SSP-Sequencing as novel way for analyzing cfDNA

cfDNA fragmentomics study



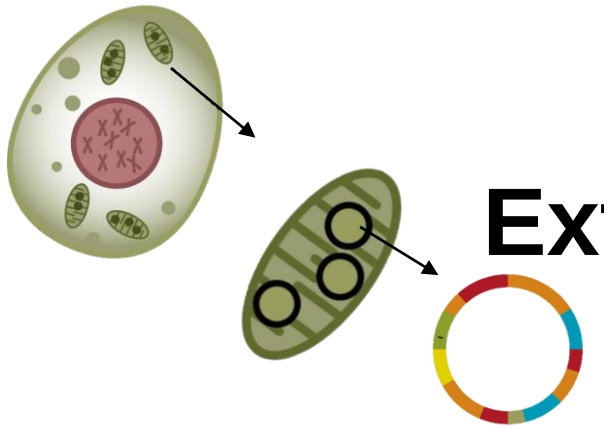
Our strategy is to combined the available methods to

- provide the harmonization of the cfDNA size profile
- elucidate the structures associated with cfDNA in the circulation
- fragmentomics as a possible way toward diagnostics

Identify the **structural characterization** of

extracellular DNA

Specifically



Extracellular mitochondrial DNA

Circulating mitochondrial DNA structure:

Fragmentation of McfDNA:

I. Highly fragmented:

Sci Rep. 2016; 6: 36097. PMCID: PMC5095883
Published online 2016 Nov 4. doi: 10.1038/srep36097 PMID: 27811968

Very Short Mitochondrial DNA Fragments and Heteroplasmy in Human Plasma

Ruoyu Zhang,[1,*] Kiichi Nakahira,[2,*] Xiaoxian Guo,[1] Augustine M.K. Choi,[2] and Zhenglong Gu[a,1]

II. Full length genome:

Mol Genet Metab. 2018 Dec;125(4):332-337. doi: 10.1016/j.ymgme.2018.10.002. Epub 2018 Oct 16.

Plasma-derived cell-free mitochondrial DNA: A novel non-invasive methodology to identify mitochondrial DNA haplogroups in humans.

Newell C[1], Hume S[2], Greenway SC[3], Podemski L[2], Shearer J[4], Khan A[5].

Structure/Forms (Topology) of McfDNA:

I. Both particle-associated and free mitochondrial DNA are present in plasma:

Clin Chem. 2003 May;49(5):719-26.

Quantitative analysis of circulating mitochondrial DNA in plasma.

Chiu RW[1], Chan LY, Lam NY, Tsui NB, Ng EK, Rainer TH, Lo YM.

II. Packed in vesicles:

Proc Natl Acad Sci U S A. 2017 Oct 24;114(43):E9066-E9075. doi: 10.1073/pnas.1704862114. Epub 2017 Oct 11.

Packaging and transfer of mitochondrial DNA via exosomes regulate escape from dormancy in hormonal therapy-resistant breast cancer.

Sansone P[1,2], Savini C[3,4,5], Kurelac I[6], Chang Q[3], Amato LB[6], Strillacci A[3,7], Stepanova A[8], Iommarini L[9], Mastroleo C[3], Daly L[3], Galkin A[8,10], Thakur BK[2,11], Soplop N[12], Uryu K[12], Hoshino A[2], Norton L[3], Bonafé M[4,5], Cricca M[4], Gasparre G[13], Lyden D[2,11], Bromberg J[1,12].

J Neural Transm (Vienna). 2010 Jan;117(1):1-4. doi: 10.1007/s00702-009-0288-8. Epub 2009 Aug 13.

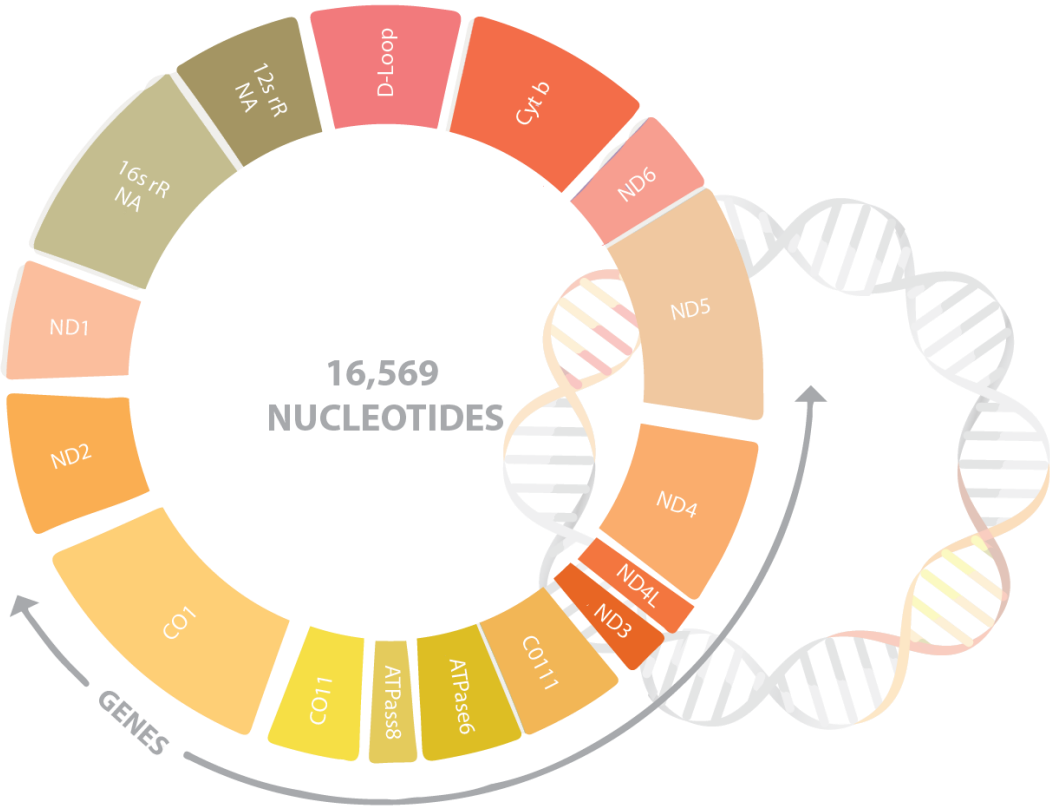
Astrocytes and Glioblastoma cells release exosomes carrying mtDNA

Michele Guescini [1], Susanna Genedani, Vilberto Stocchi, Luigi Francesco Agnati

The circulating mitochondrial DNA structures are poorly known

Mitochondrial DNA :
Important structural differences with nuclear DNA:

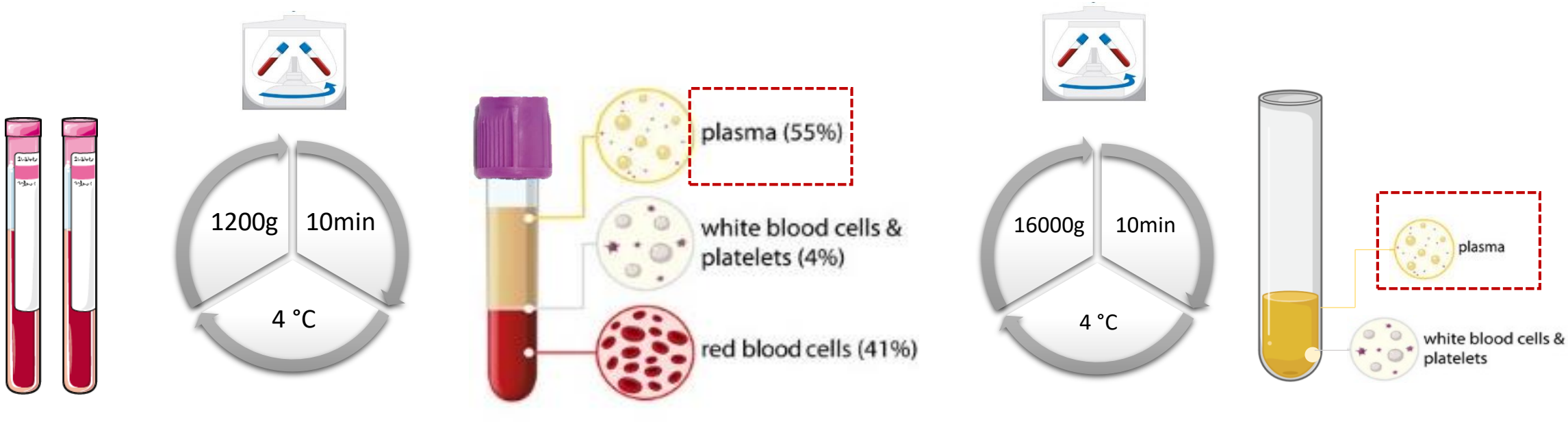
- Mitochondrial DNA is a small DNA
- Circular
- Elevated mitochondrial DNA copy number
- Unprotected by histones



Study of the Topology of circulating mitochondrial DNA

Analysis of the size fragment

Plasma isolation by double centrifugation

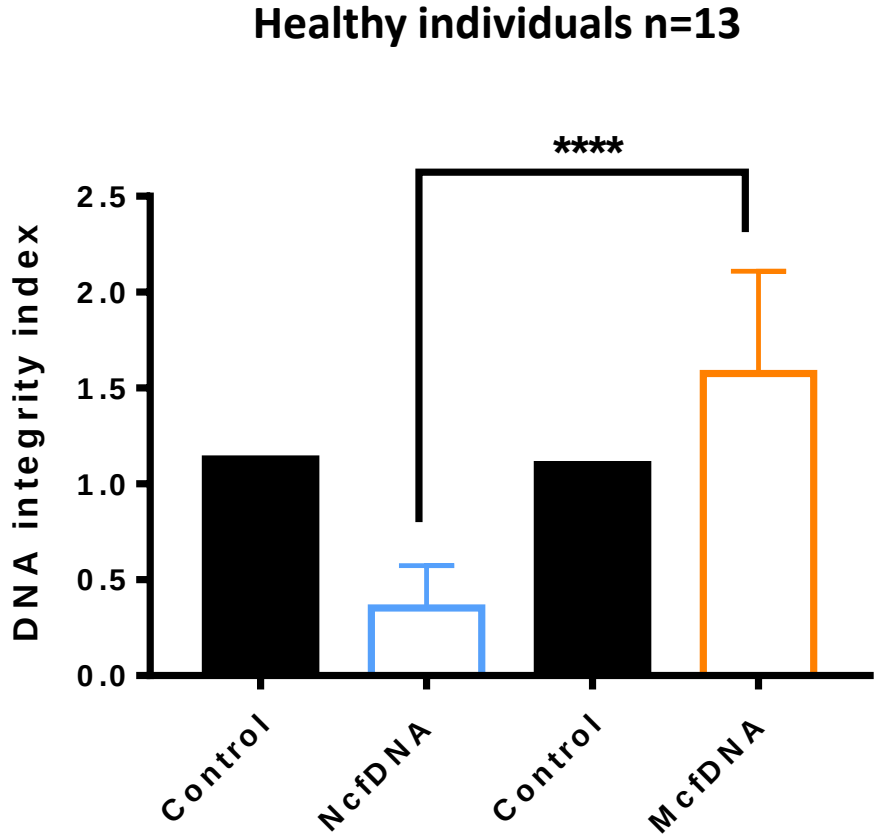


This is the conventional plasma preparation to extract cfDNA

Mitochondrial circulating DNA is less fragmented than nuclear circulating DNA

DNA integrity index= $\frac{\textit{300 bp amplicon Concentration}}{\textit{67 bp amplicon Concentration}}$

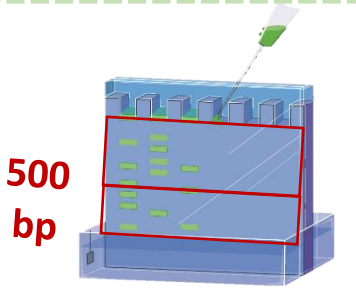
about the proportion of the fragments over 300 bp



▲ Control = Exracted genomic DNA

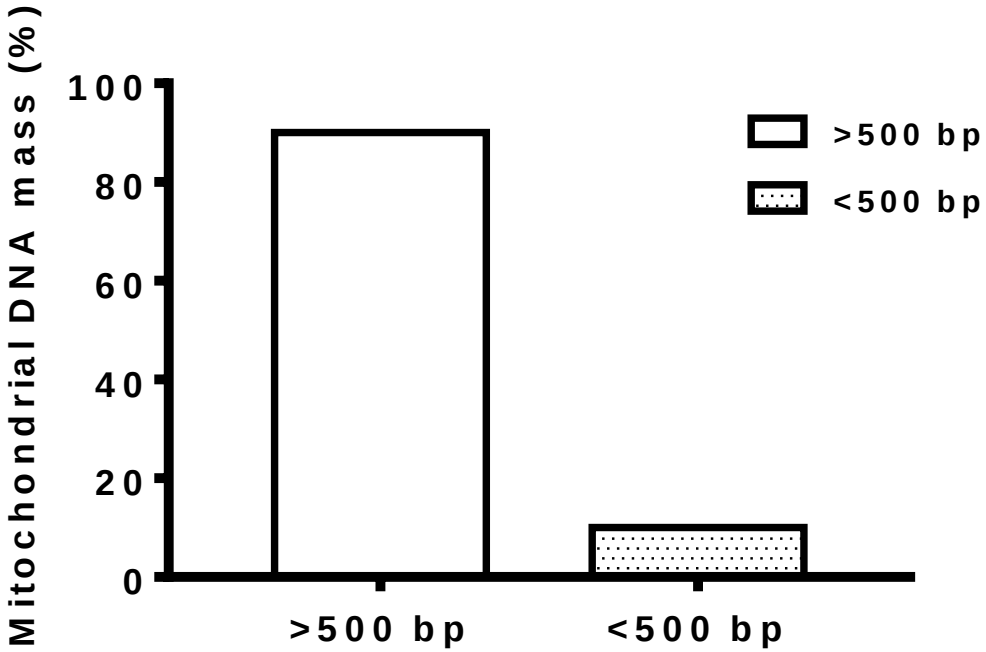
Size distribution of mitochondrial circulating DNA by q-PCR

DNA extract
(Plasma pool from healthy individuals)



Agarose gel electrophoresis

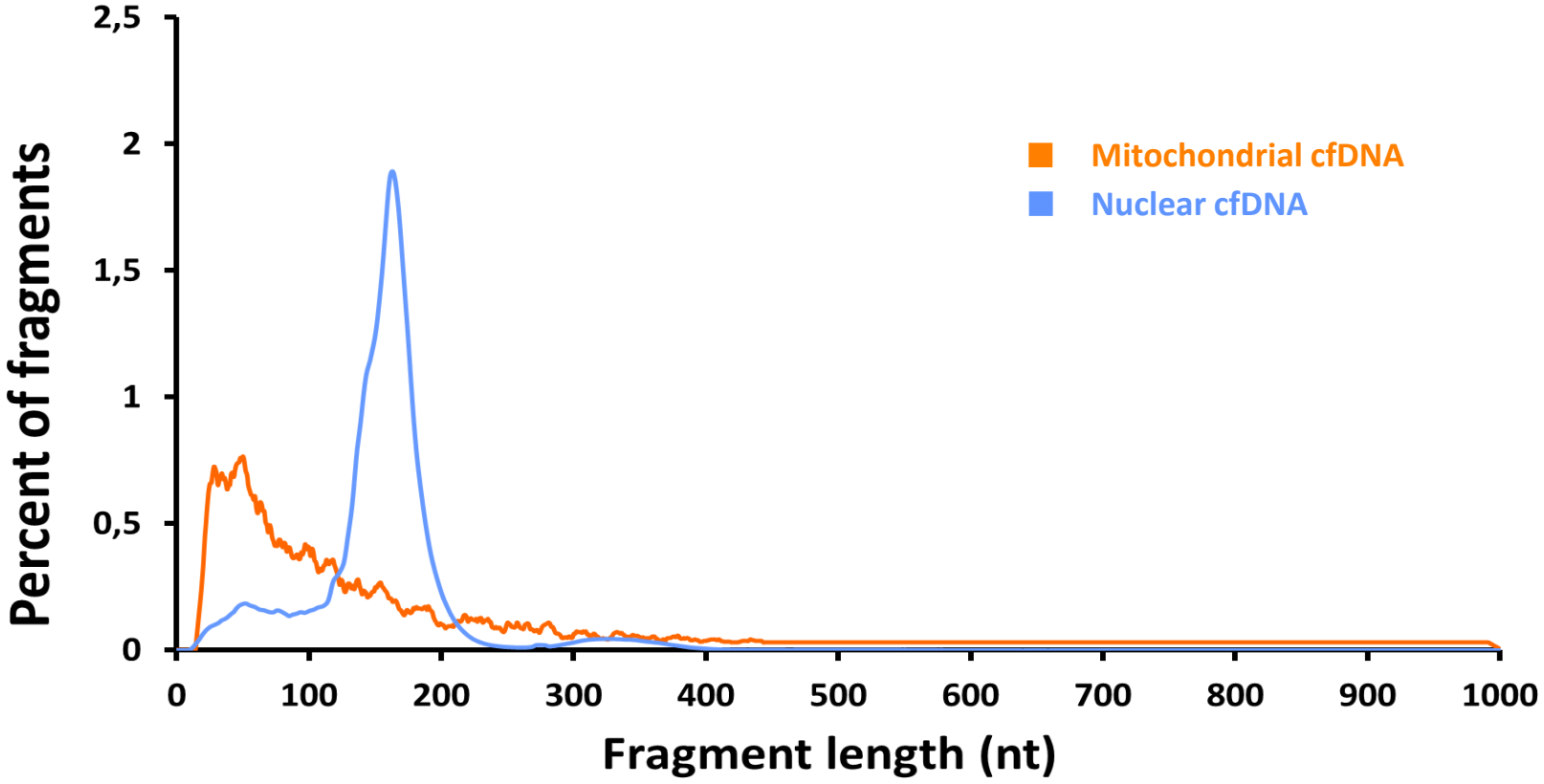
q-PCR quantification



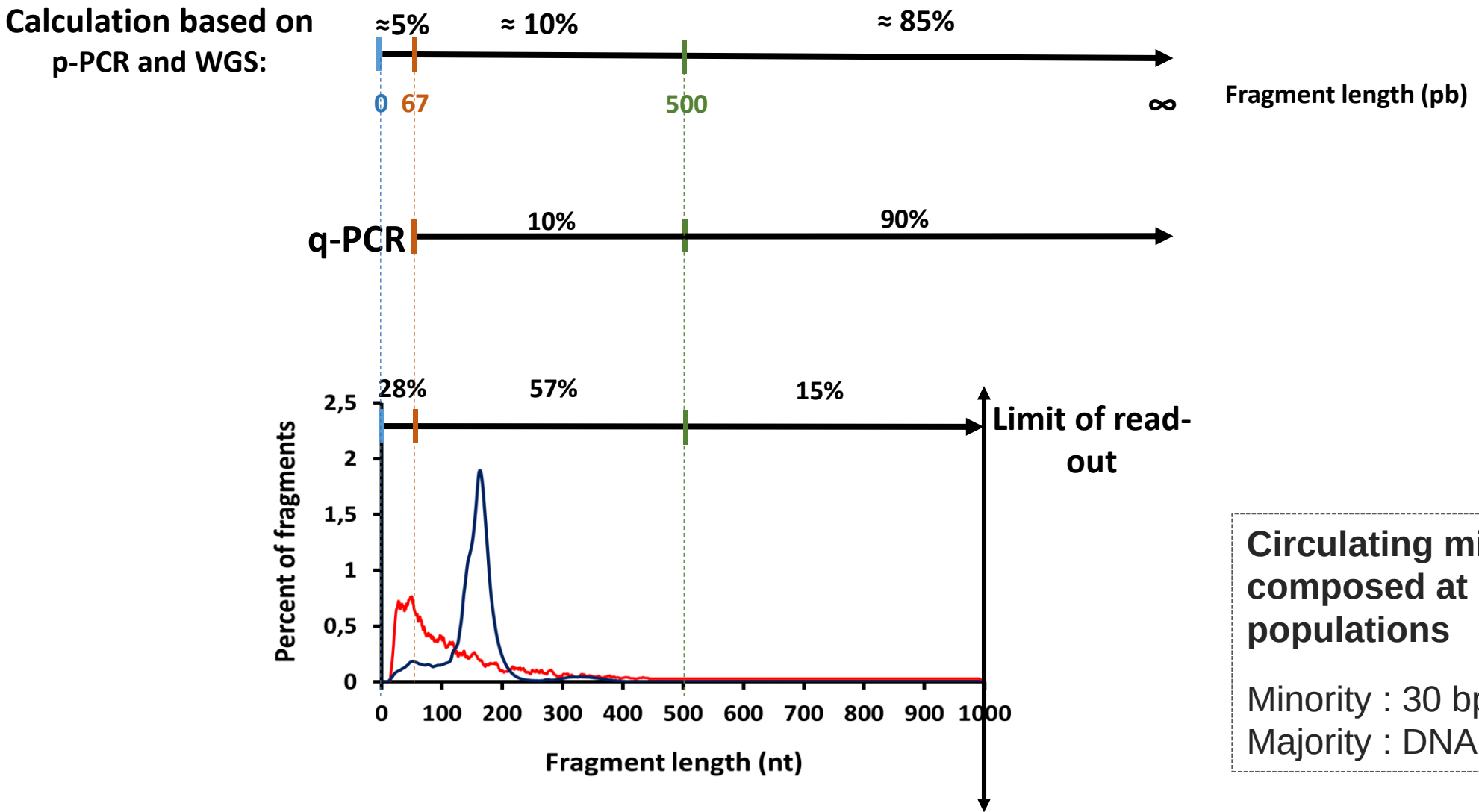
Approximately 10 % of cirDNA mass is composed of fragments below 500 bp

Size distribution of mitochondrial circulating DNA by sWGS

Healthy individuals



Size distribution of mitochondrial circulating DNA



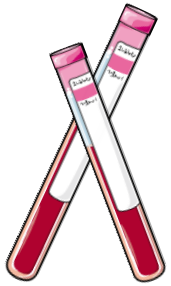
Circulating mitochondrial DNA is composed at least from two populations

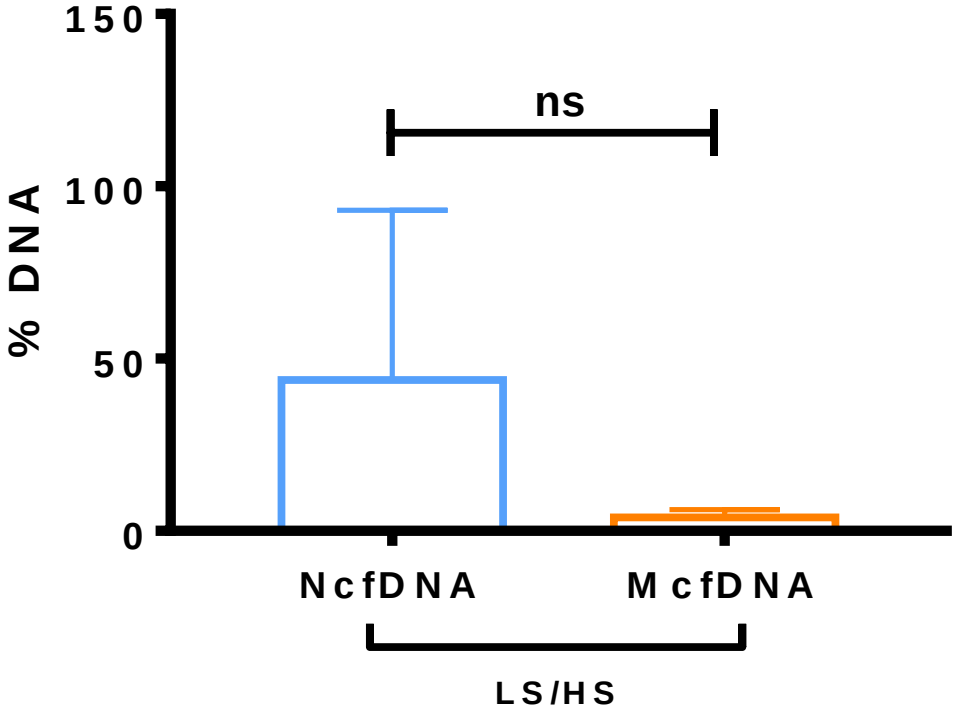
Minority : 30 bp ≤ DNA ≤ 300 bp
Majority : DNA ≥ 1 000 bp

Study of the Topology of circulating mitochondrial DNA

Indirect physical analysis

McfDNA and NcfDNA sedimentation differences as determined by centrifugation

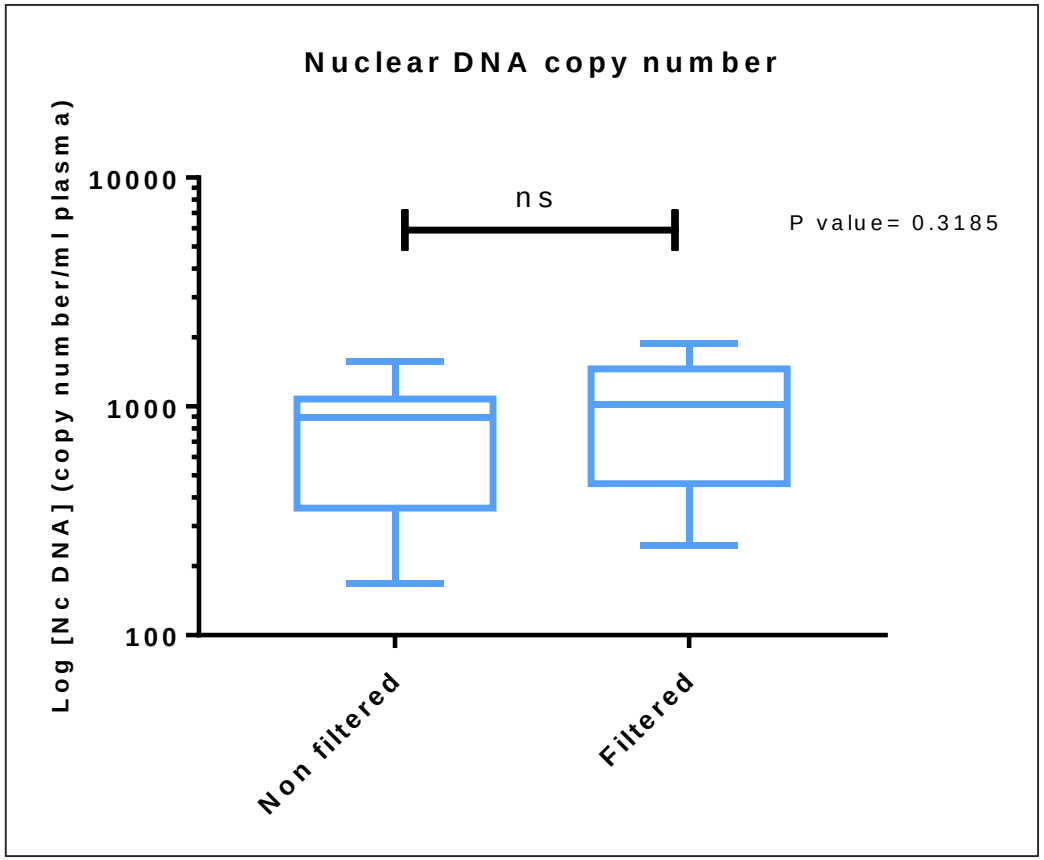


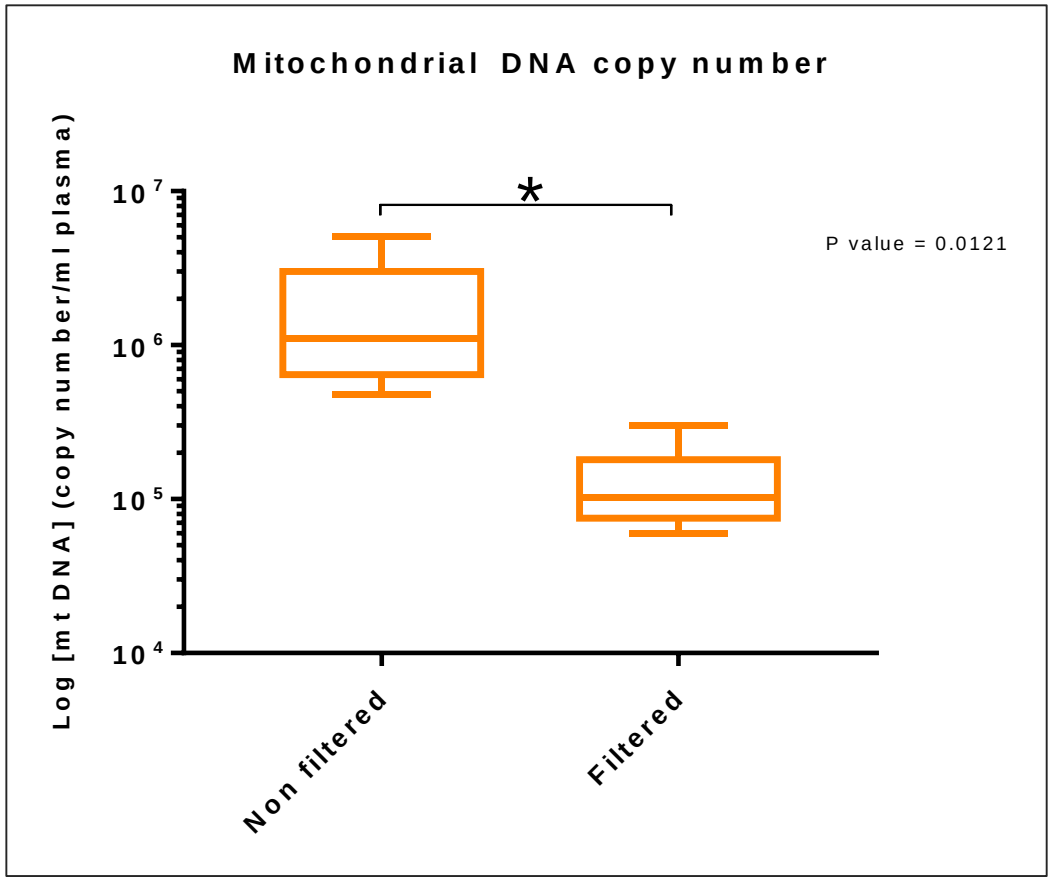


The supernatant DNA concentration variation between 400g (LS) and 16000g (HS) for mitochondrial DNA is **significantly more important** than nuclear DNA in centrifuged plasma

➡ **A significant fraction of particles contains mitochondrial DNA in plasma**

McfDNA and NcfDNA overall size differences as determined by plasma 0.22µM filtration





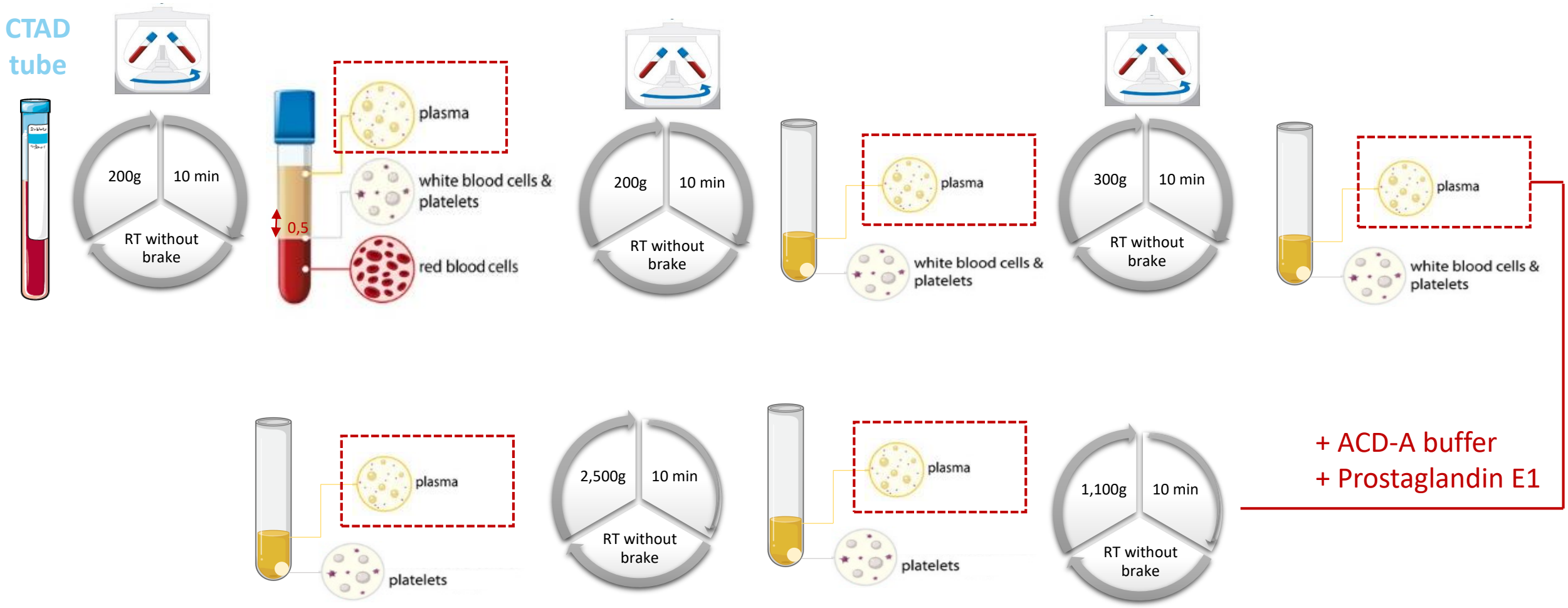
Mitochondrial DNA concentration decreases while nuclear DNA is stable in plasma after filtration

The data support the existence of a significant fraction of particles pelletable at 16,000g and filterable with 0,22µm that contain mitochondrial DNA

Plausible candidates include:

- In vitro mitochondria released by activated platelets
- Platelets
- Macrovesicles containing mitochondrial DNA
- Mitochondria released by cells

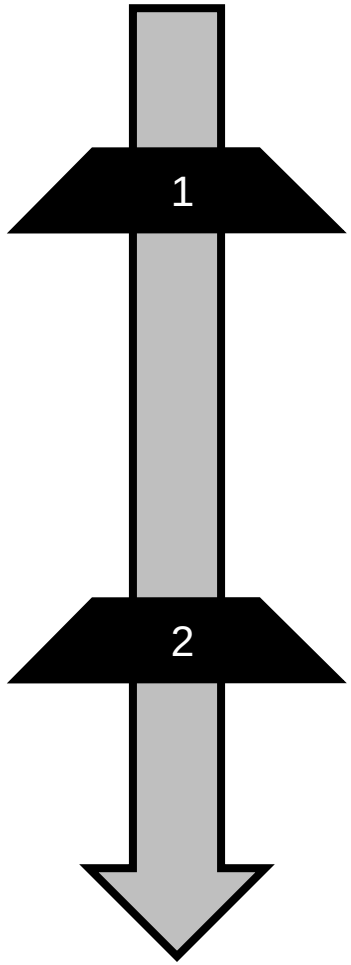
Plasma isolation without platelets activation

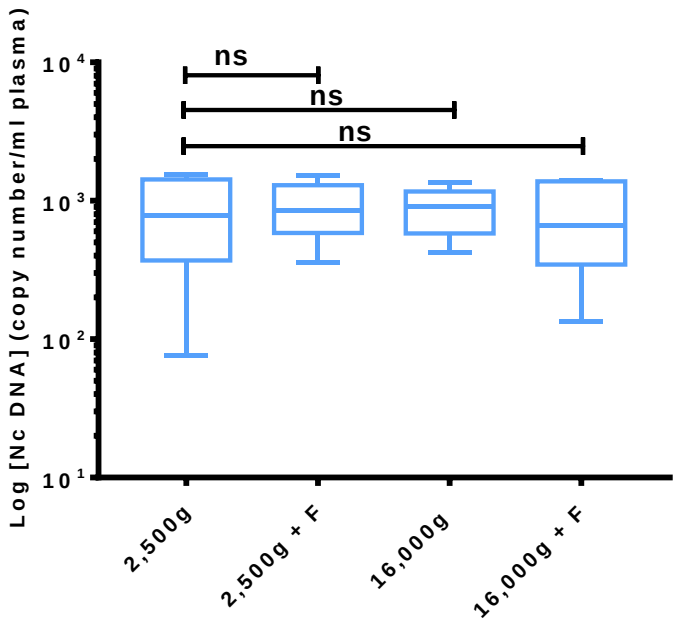


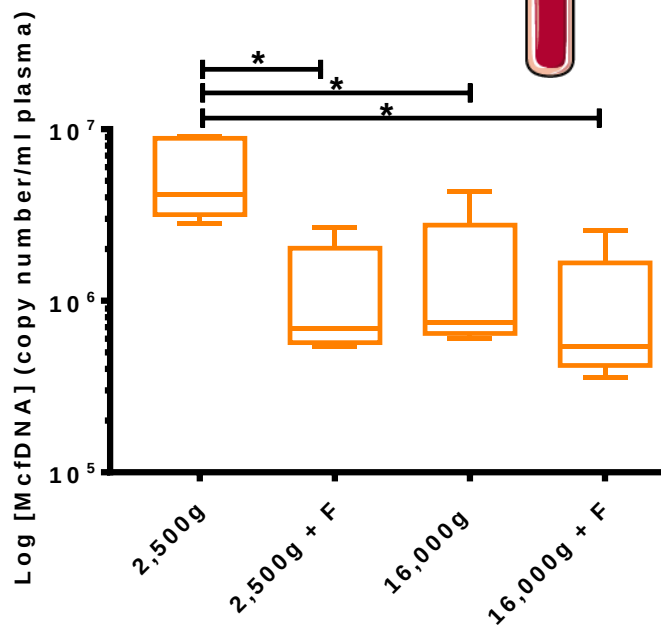
Significant fraction of particles contains mitochondrial DNA in plasma

Differential centrifugation effect on plasma
(2 500g / 16 000g)

Filtration using hydrophilic 0,22µm filters effect on plasma







DNA concentration variation between
2500g and 16000g
for mitochondrial DNA is **significantly more important**
than nuclear DNA in centrifuged plasma

The data support the existence of a significant fraction of particles that contain mitochondrial DNA

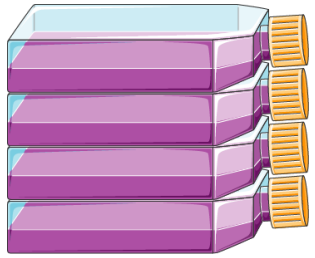
Plausible candidates include:

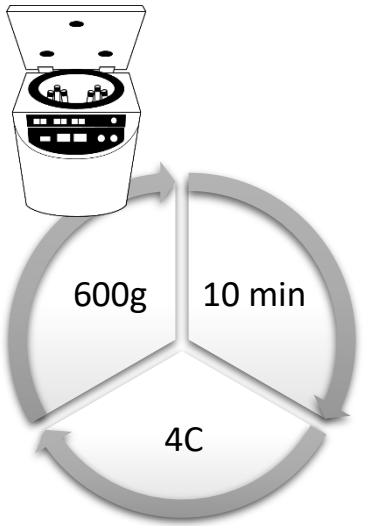
- ~~Mitochondria released by activated platelets~~
- Platelets
- Macrovesicles containing mitochondrial DNA
- Mitochondria released by cells

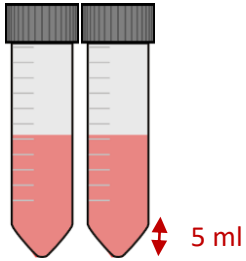
Cell culture supernatant isolation

- **DLD1 (ATCC)**
- **SW620 (ATCC)**
- **CCD-18Co (Turtoi's Lab)**

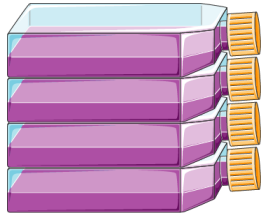
24h or 60h



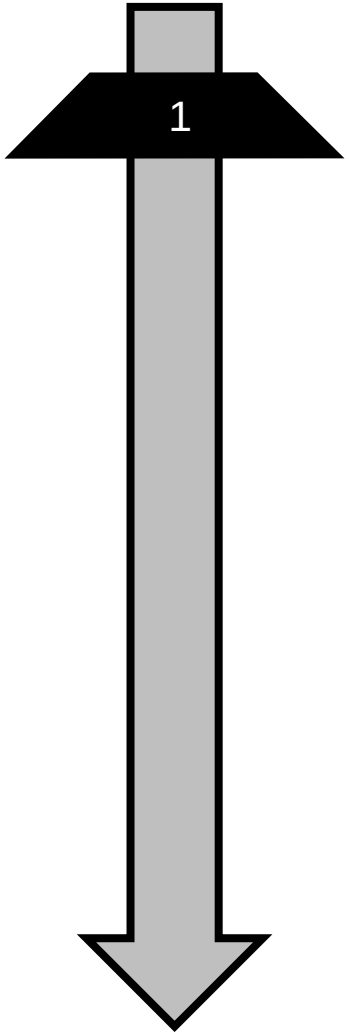


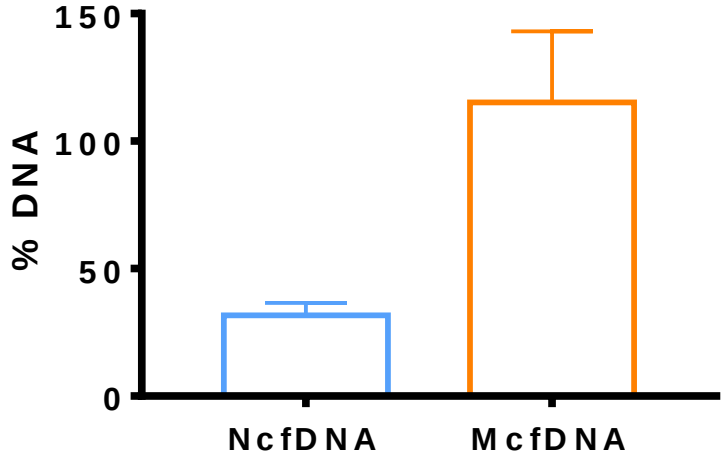


Significant fraction of particles contains mitochondrial DNA in DLD1, SW620, CCD-18Co culture media



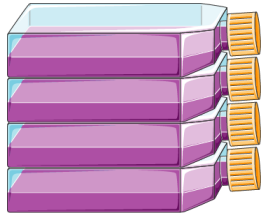
Differential centrifugation effect on plasma
(600g / 16 000g)





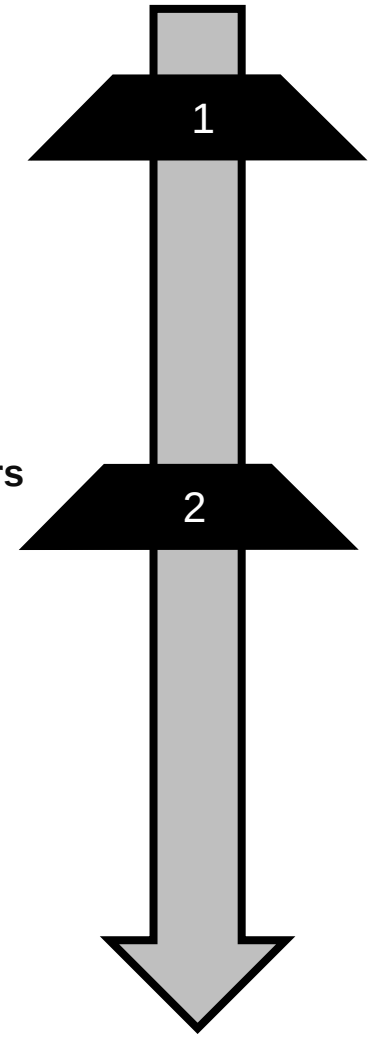
Mitochondrial DNA in 16,000g is not free

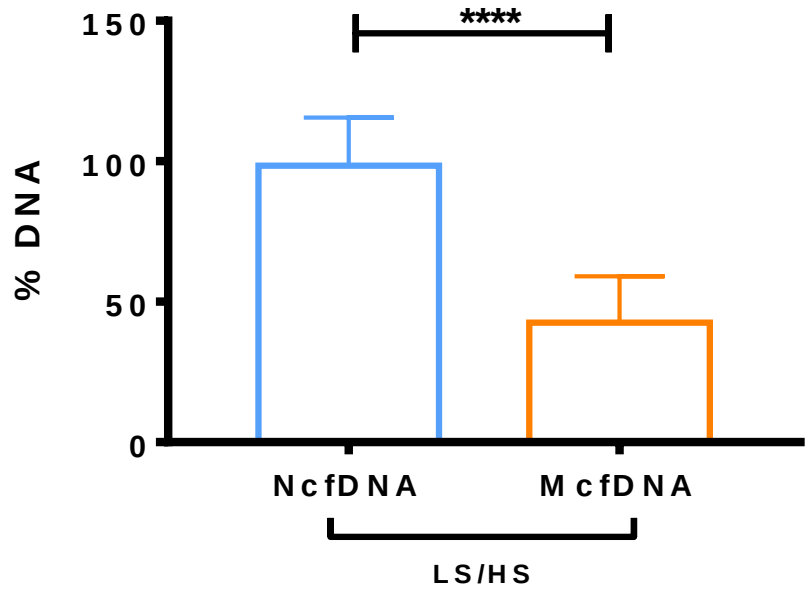
Significant fraction of particles contains mitochondrial DNA in DLD1, SW620, CCD-18Co culture media



Differential centrifugation effect on plasma
(600g / 16 000g)

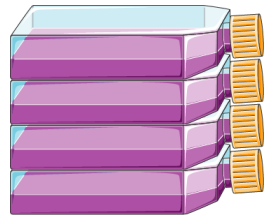
Filtration using hydrophilic 0,22µm filters effect on plasma





DNA concentration variation between control and filtered cell media
for mitochondrial DNA is **significantly more important** than nuclear DNA

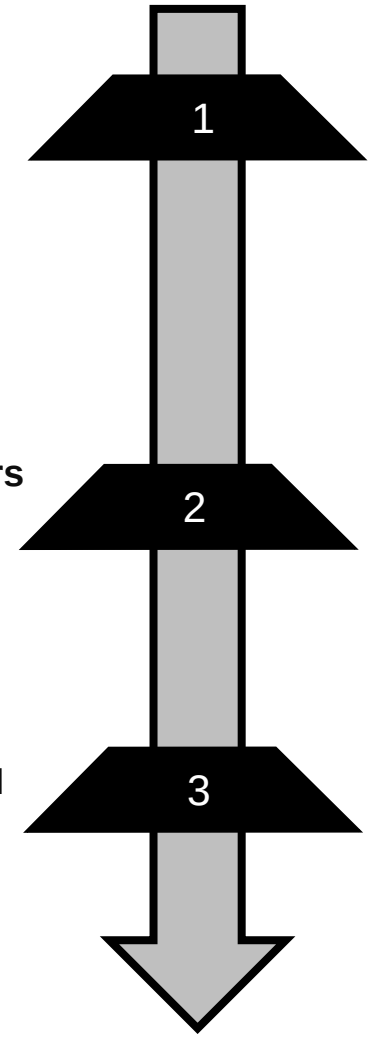
Significant fraction of particles contains mitochondrial DNA in DLD1, SW620, CCD-18Co culture media

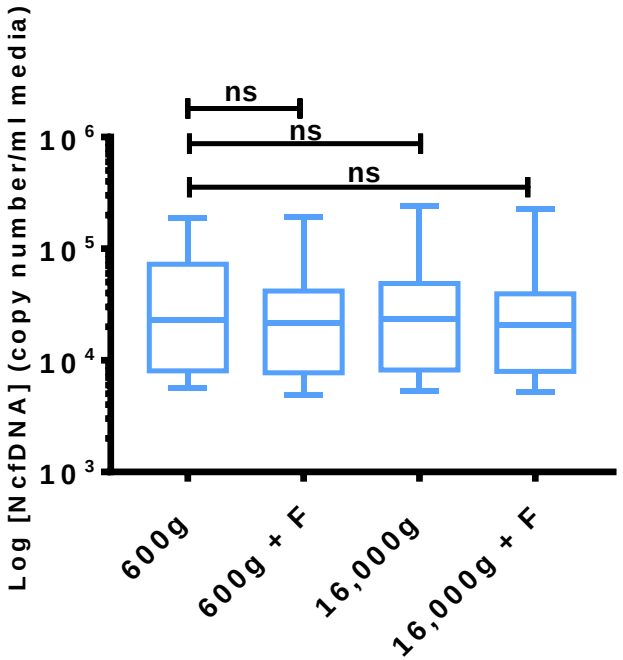


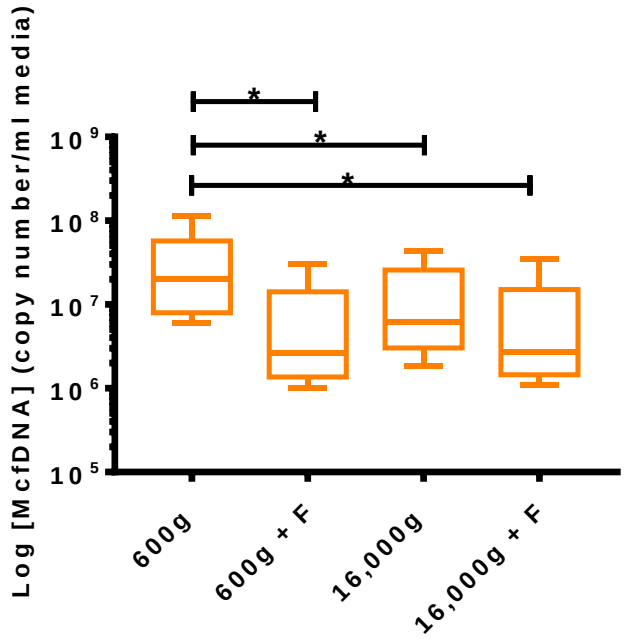
Differential centrifugation effect on plasma
(600g / 16 000g)

Filtration using hydrophilic 0,22µm filters effect on plasma

Treatment of 16,000g pellet with Dnase I







DNA concentration variation between 400g and 16000g for mitochondrial DNA is **significantly more important** than nuclear DNA in centrifuged cell media

Plasma and cell culture media contain McfDNA in dense and stable structure larger than 0,22µm excluding platelets as a source candidates.

The data support the existence of a significant fraction of particles that contain mitochondrial DNA

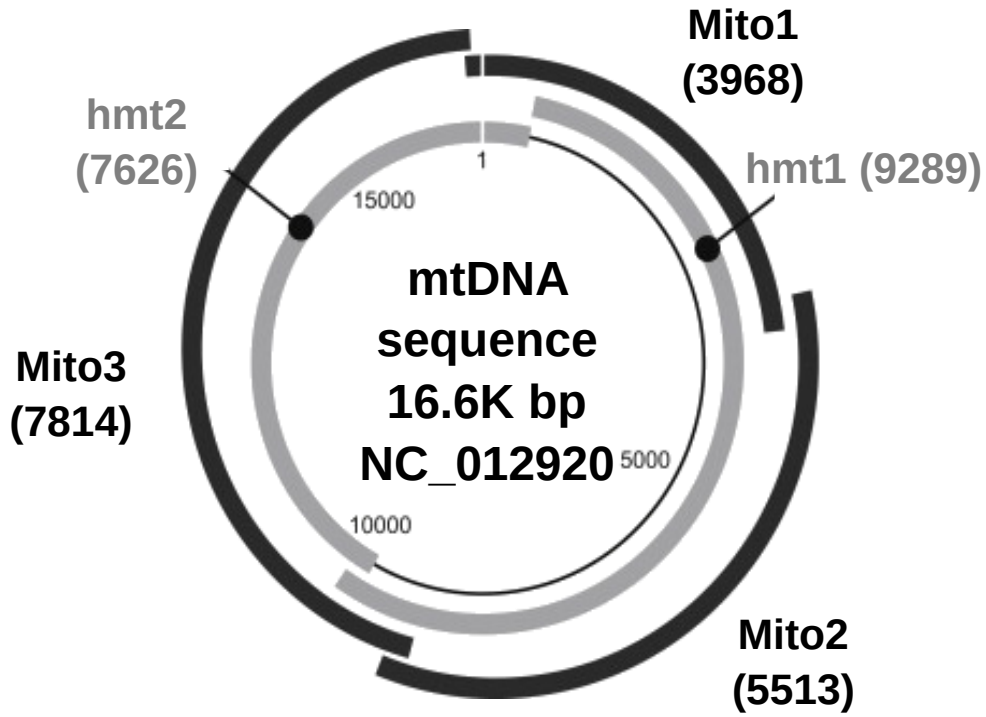
Plausible candidates include:

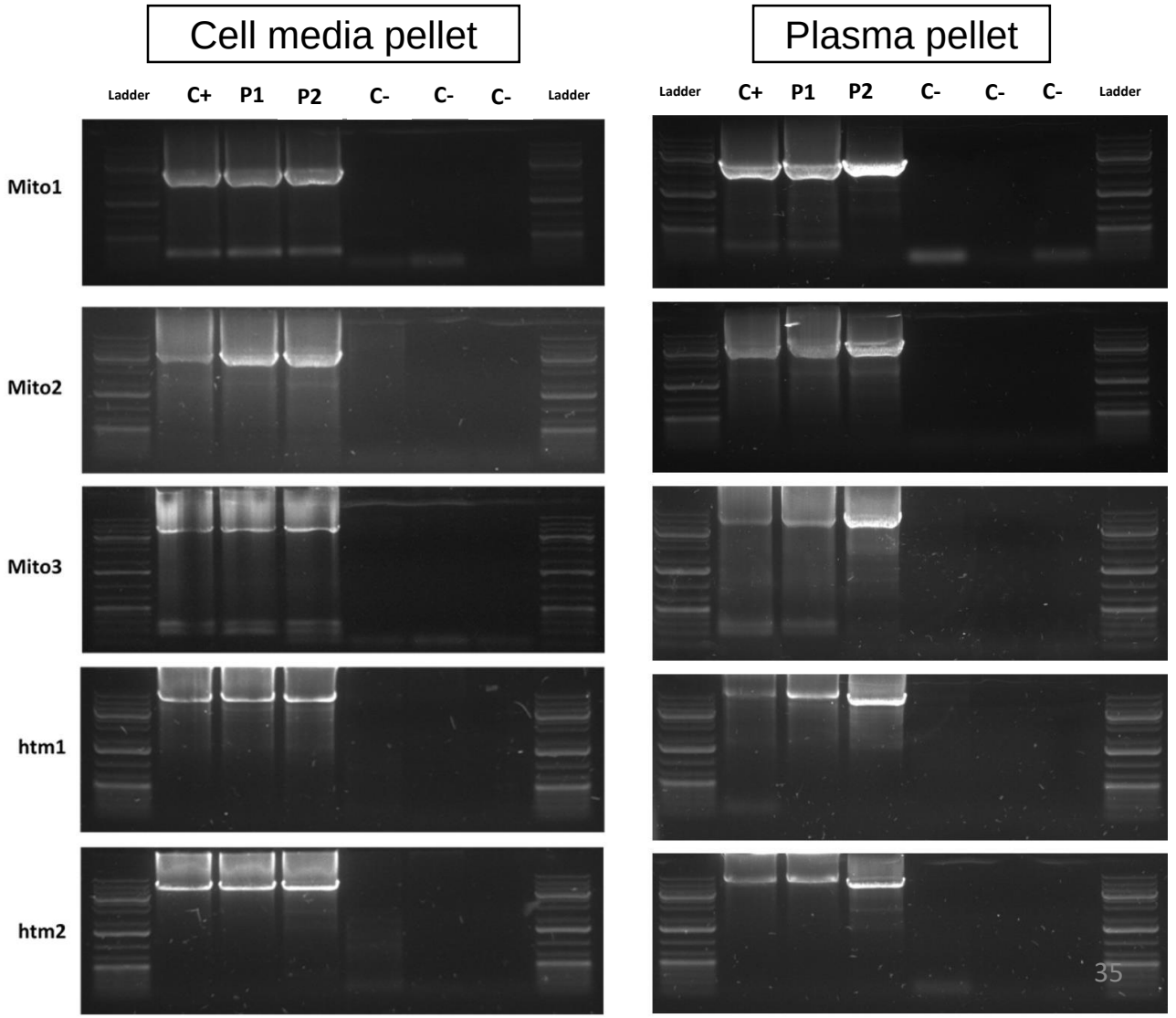
- ~~Mitochondria released by activated platelets~~
- ~~Platelets~~
- Macrovesicles containing mitochondrial DNA
- Mitochondria released by cells

Study of the Topology of circulating mitochondrial DNA

Genome analysis

The presence of full length mitochondrial DNA in the pellet of cell media and plasma





The Development of Next-Generation Sequencing Assays for the Mitochondrial Genome and 108 Nuclear Genes Associated with Mitochondrial Disorders.
Dames et al.

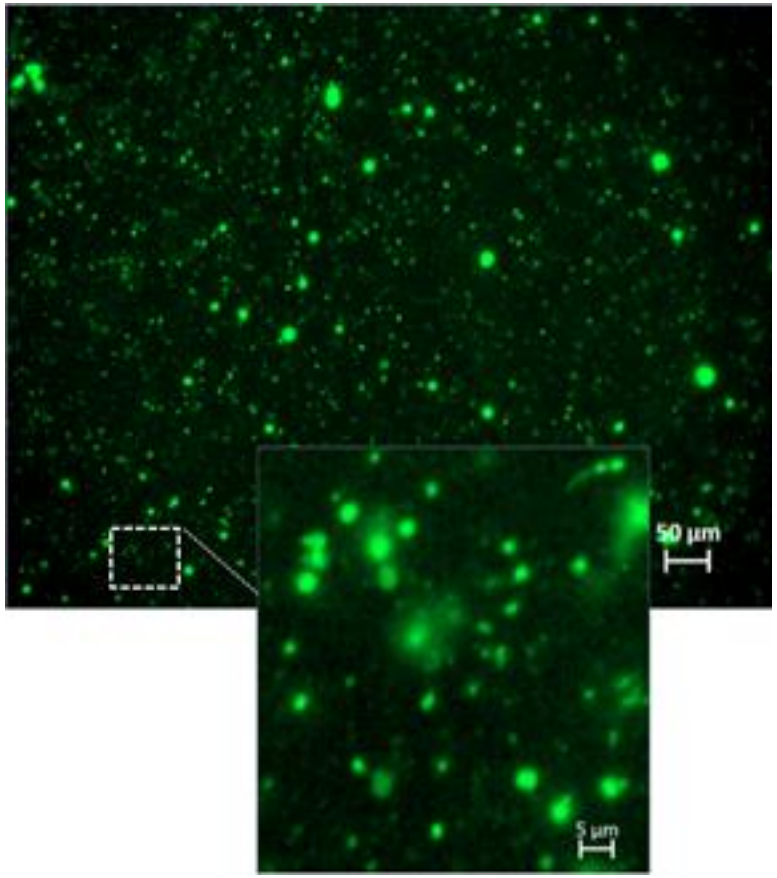
Are these results a signature of the presence of extracellular mitochondria?

Study of the Topology of circulating mitochondrial DNA

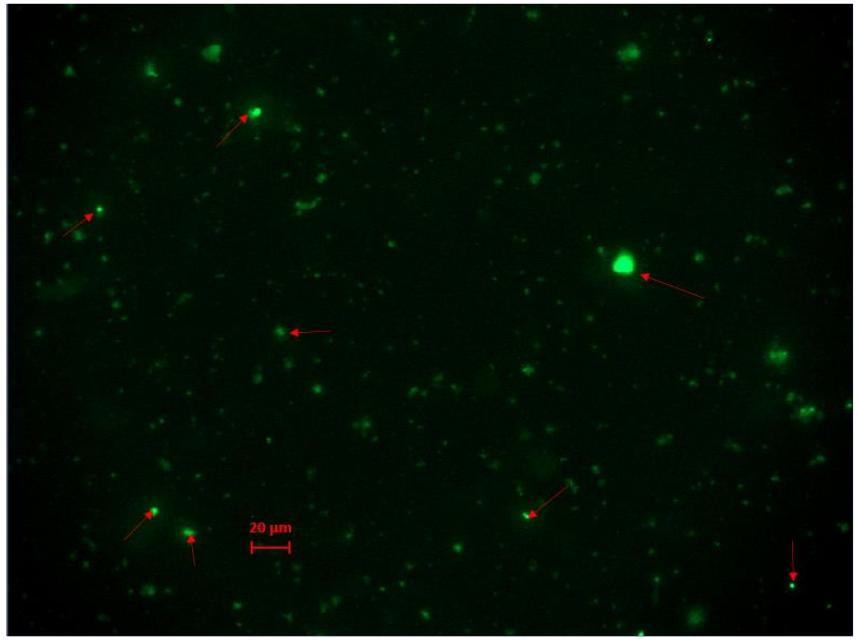
Microscopy analysis

Cell culture media and plasma Pellet stained using Mitotracker green

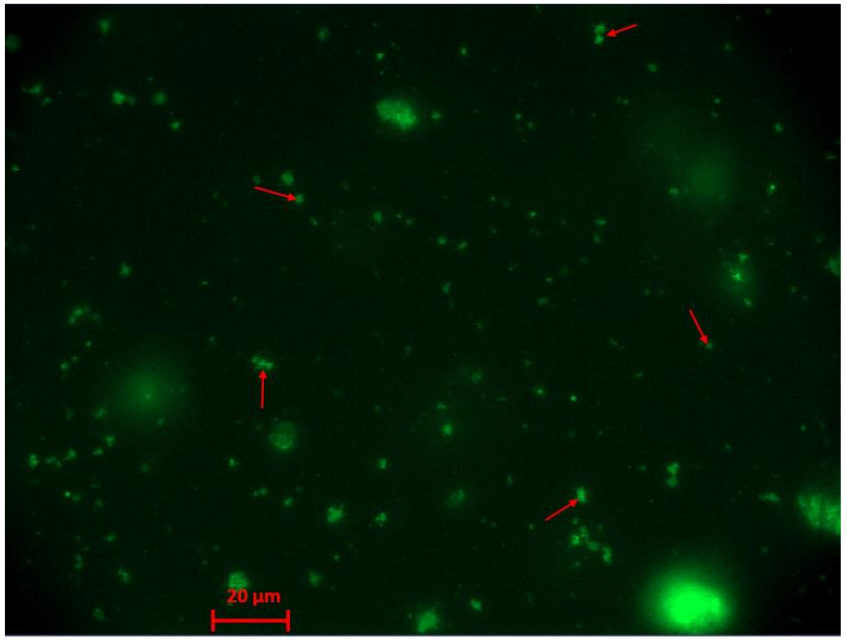
Plasma without activation



SW620



DLD1



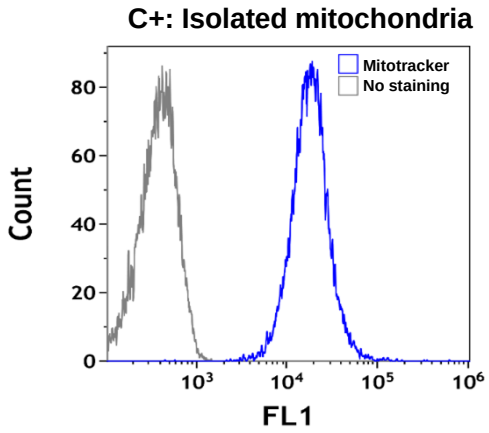
FASEB J. 2020 Mar;34(3):3616-3630,

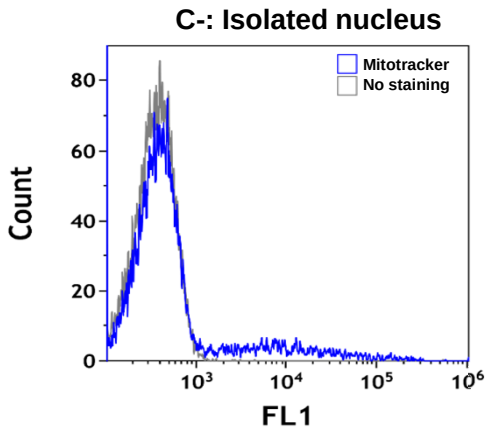
Study of the Topology of circulating mitochondrial DNA

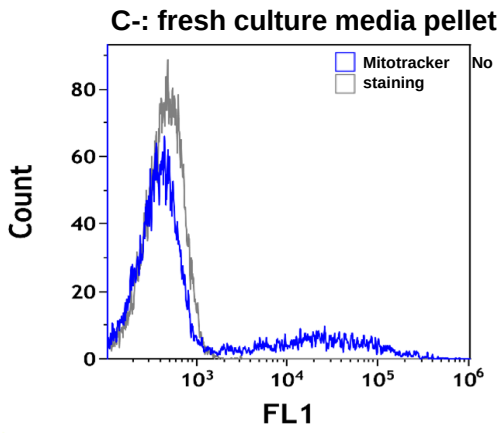
Mitochondria FACS analysis

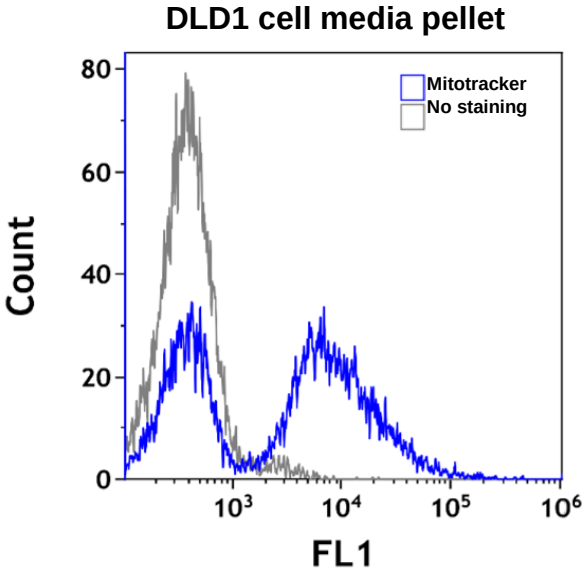
The presence of mitochondrial material in the plasma and cell media supernatant

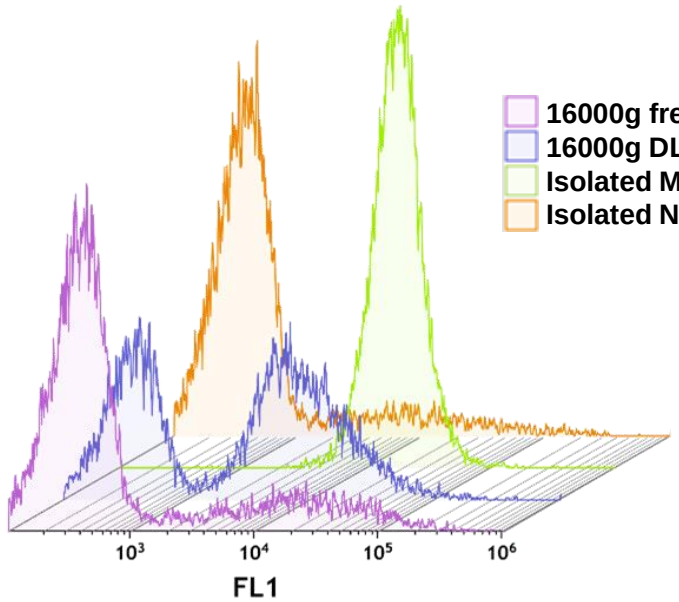
Fluorescence signal of mitotracker detected in cell media and plasma pellet by flow cytometer:

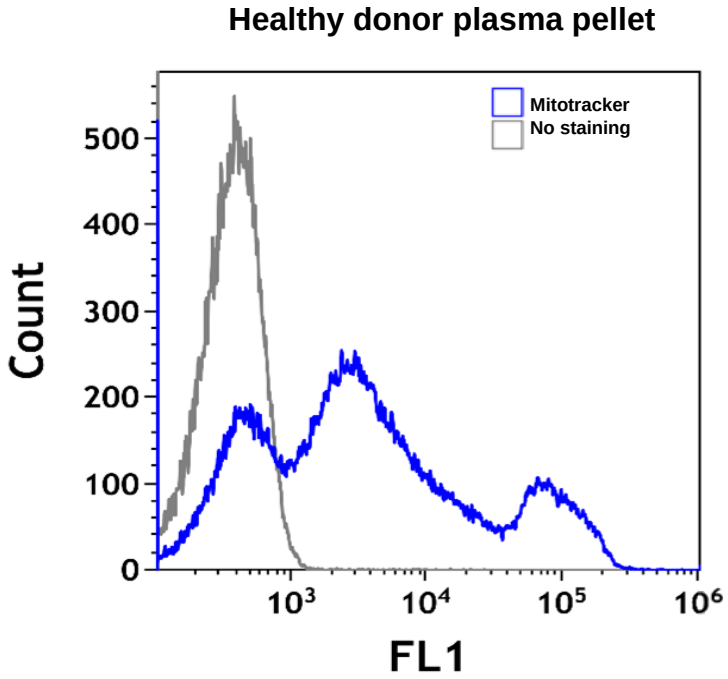












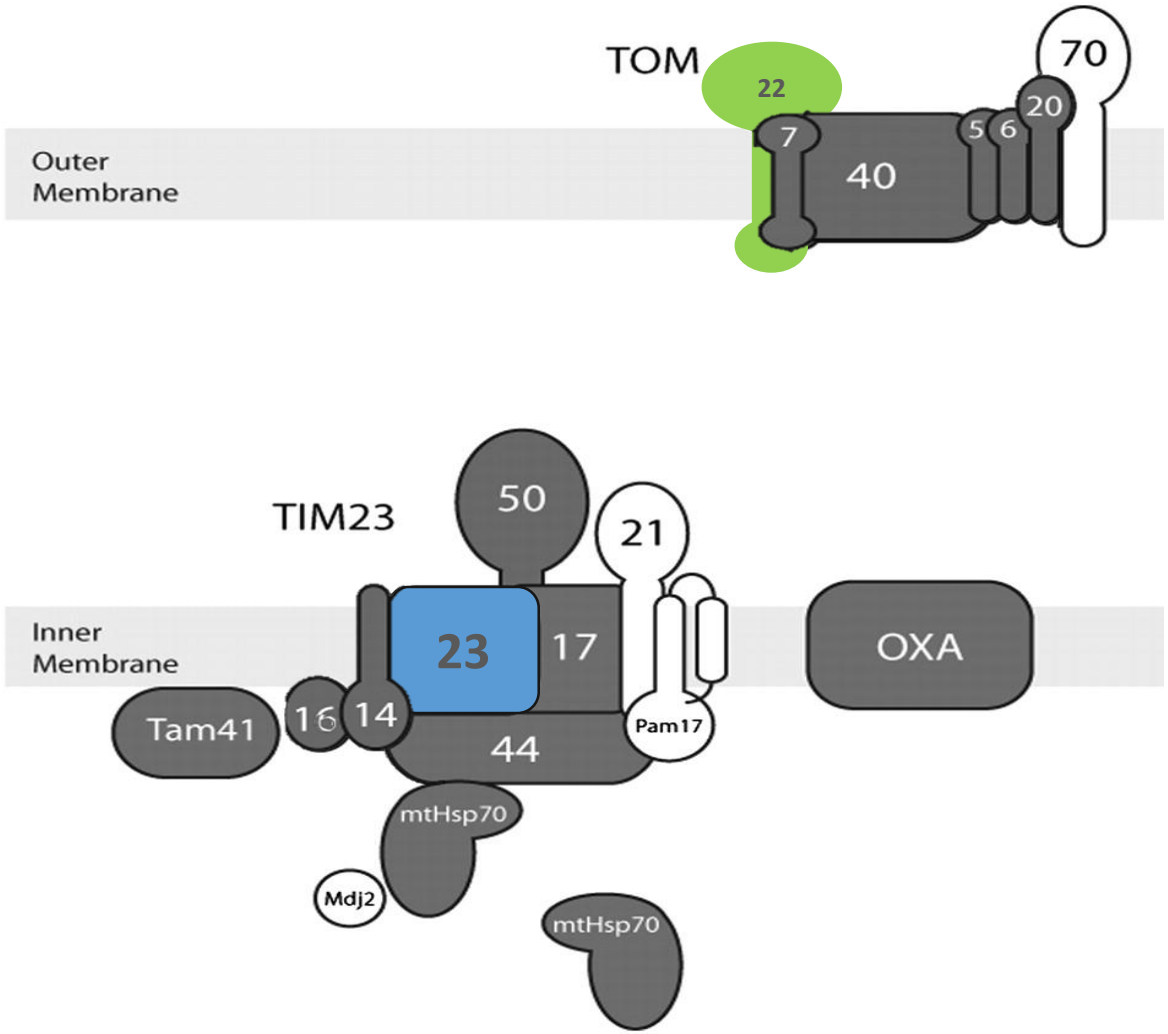
FASEB J. 2020 Mar;34(3):3616-3630,

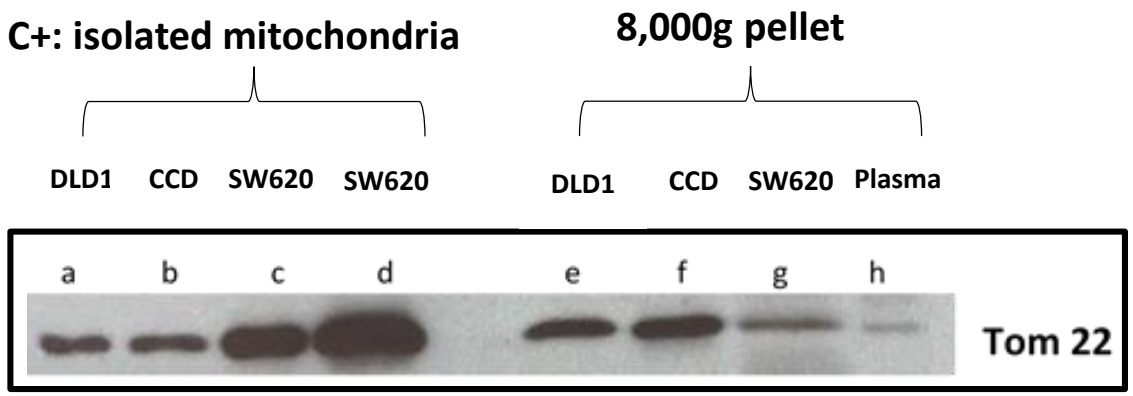
Study of the Topology of circulating mitochondrial DNA

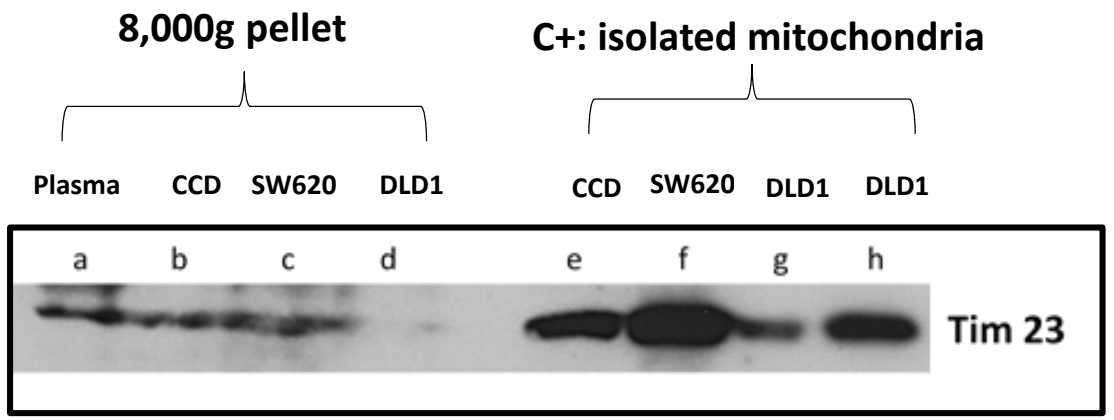
Cell biology analysis

The presence of mitochondrial transporter TOM22 and TIM23 in the plasma and cell media supernatant

Mitochondria





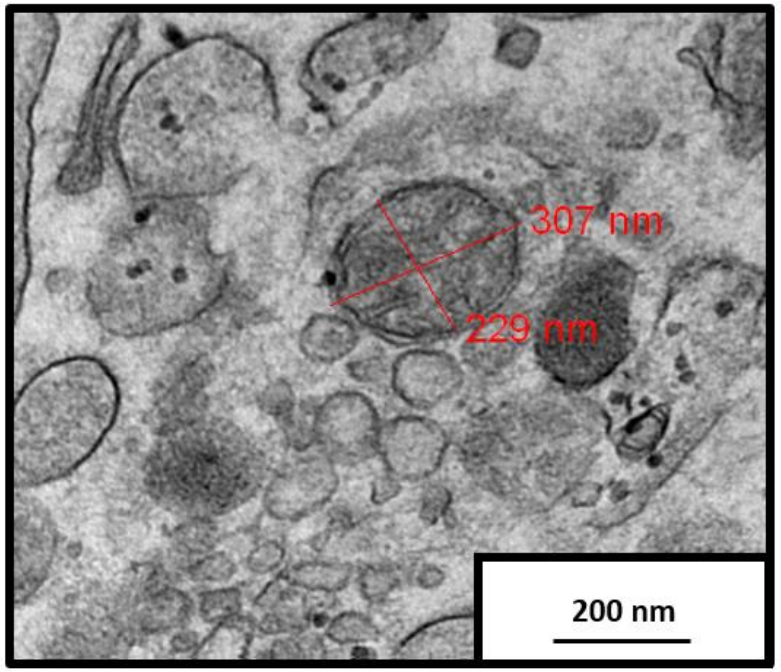


Study of the Topology of circulating mitochondrial DNA

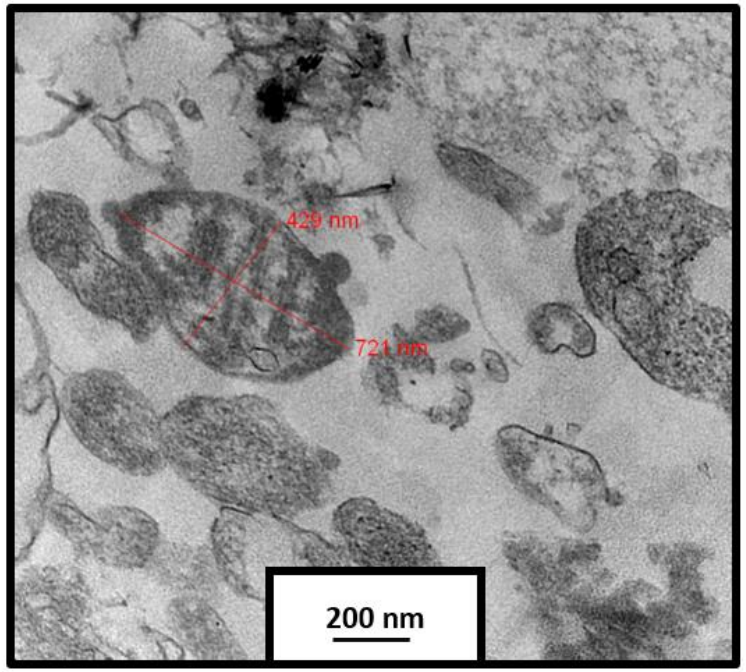
Microscopy analysis

Confirmation of double membrane structure using electron microscopy

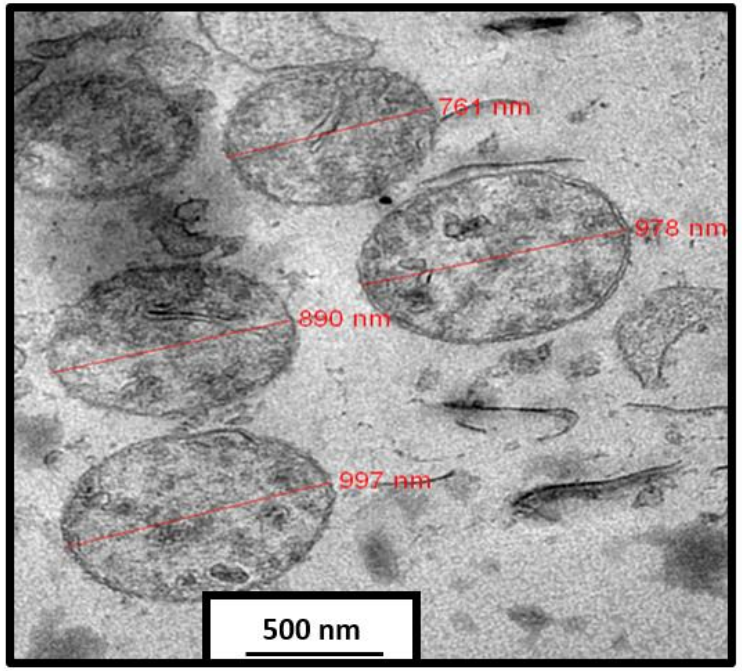
healthy donor's plasma pellet:



SW620 Cell media pellet:



DLD1 Cell media pellet:



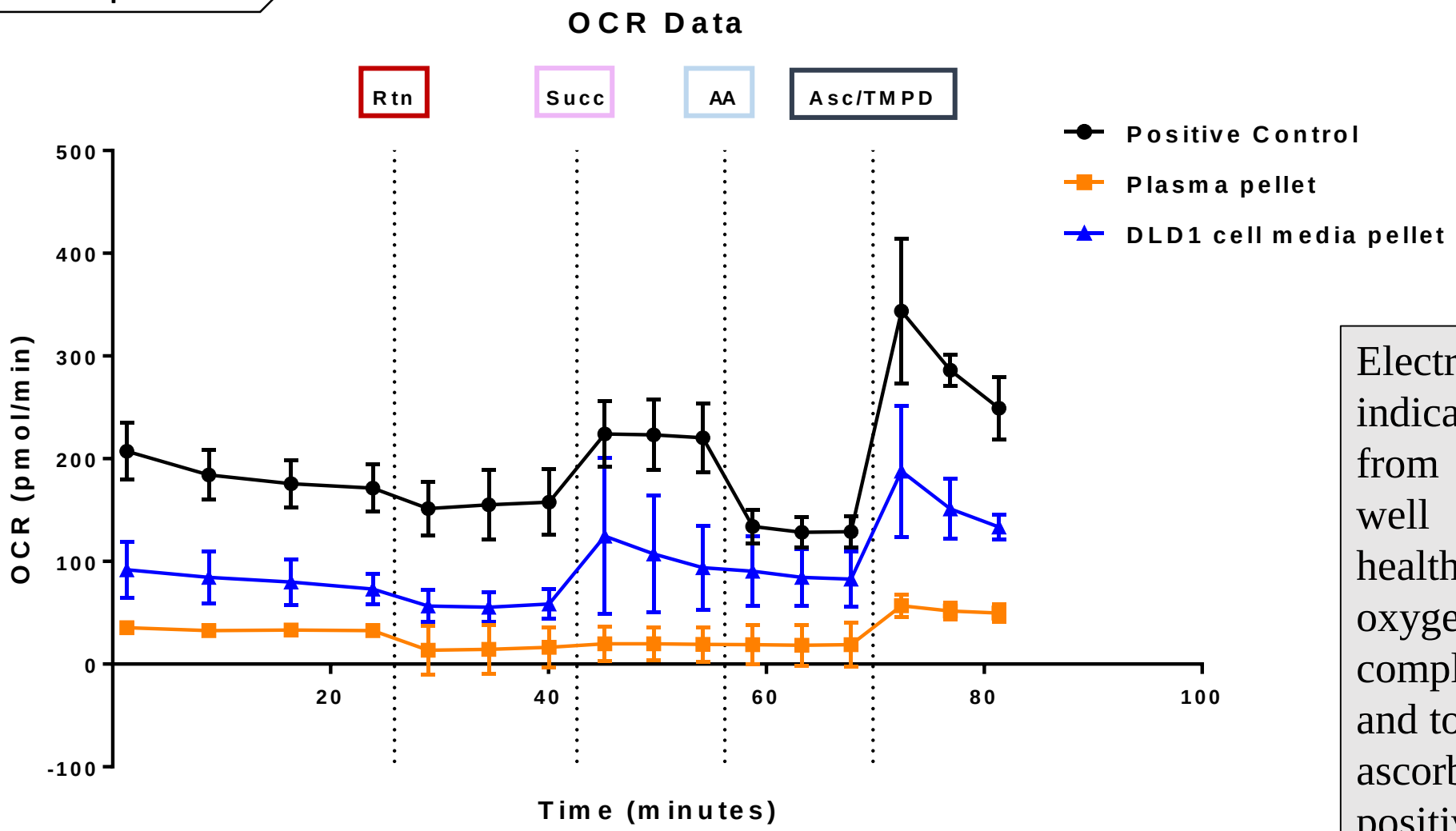
FASEB J. 2020 Mar;34(3):3616-3630,

Study of the Topology of circulating mitochondrial DNA

Functional assay

Confirmation of respiratory-competent mitochondria in the plasma of healthy individuals and DLD-1 cell media supernatant

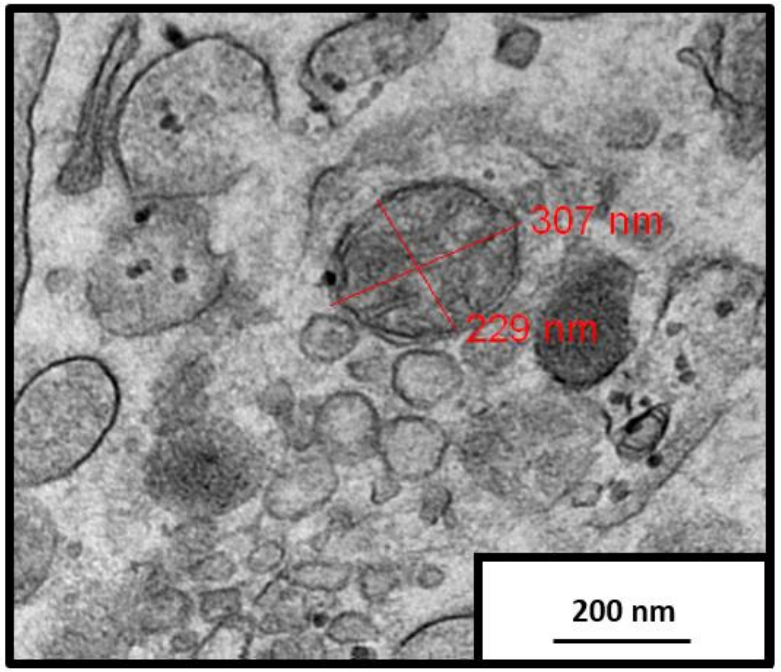
Seahorse technology
Metamontp Platform



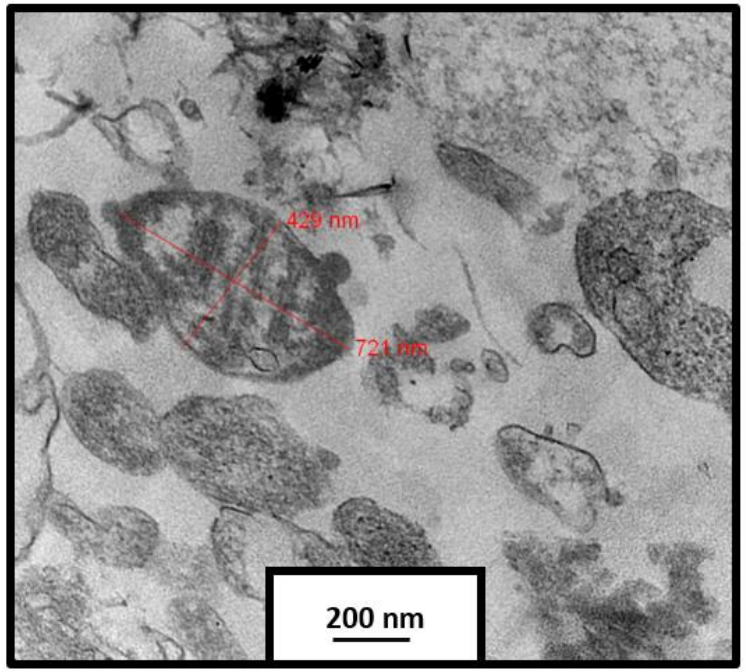
Electron flow assay clearly indicates that the pellets isolated from DLD-1 culture media, as well as from plasma pool of healthy individuals, consume oxygen and are sensitive to complex I inhibition by rotenone, and to complex IV stimulation by ascorbate/TMPD, like the positive control.

Blood contains circulating Extracellular respiratory competent mitochondria in blood circulation

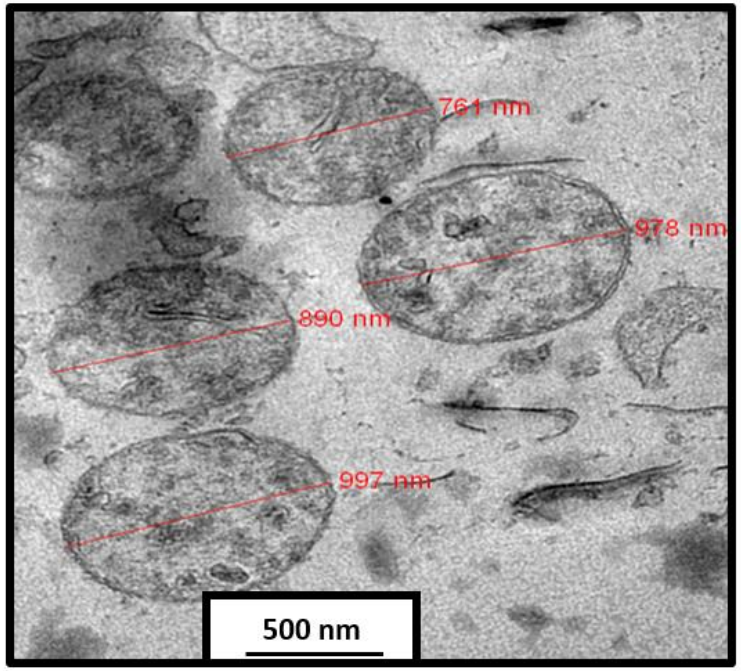
healthy donor's plasma pellet:



SW620 Cell media pellet:



DLD1 Cell media pellet:

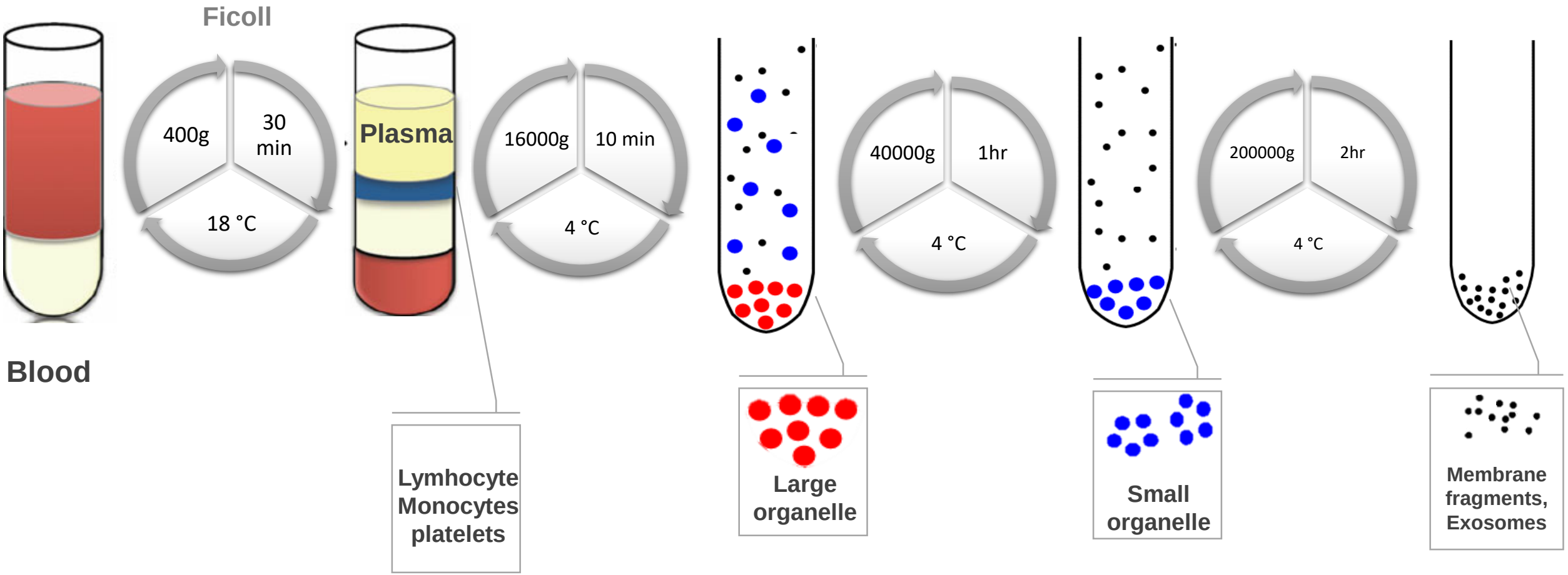


Upon our estimation, there are between 0.1 to 1.8 millions of circulating cell-free mitochondria per mL of blood

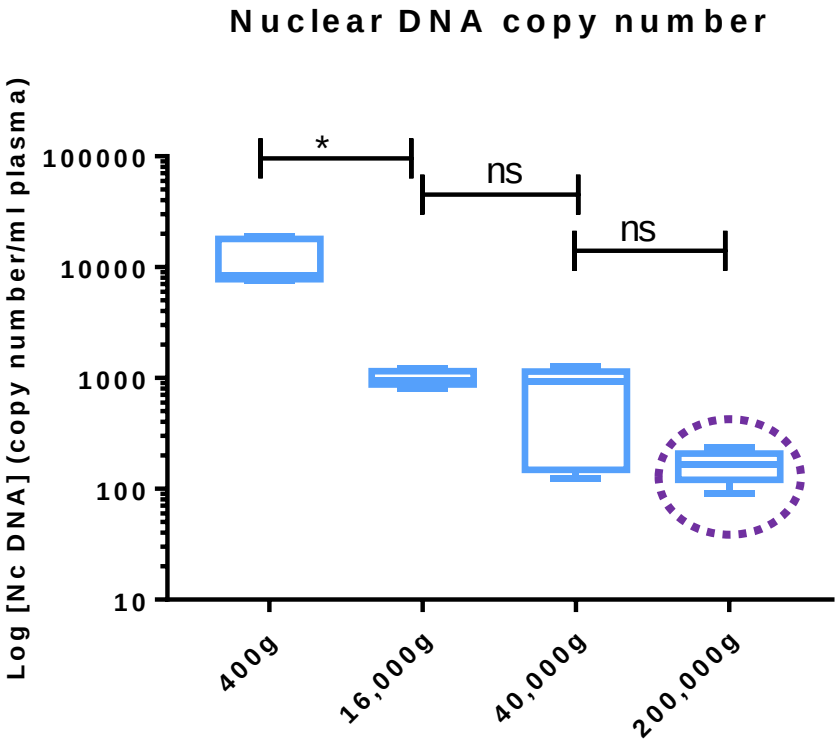
Study of the Topology of circulating mitochondrial DNA

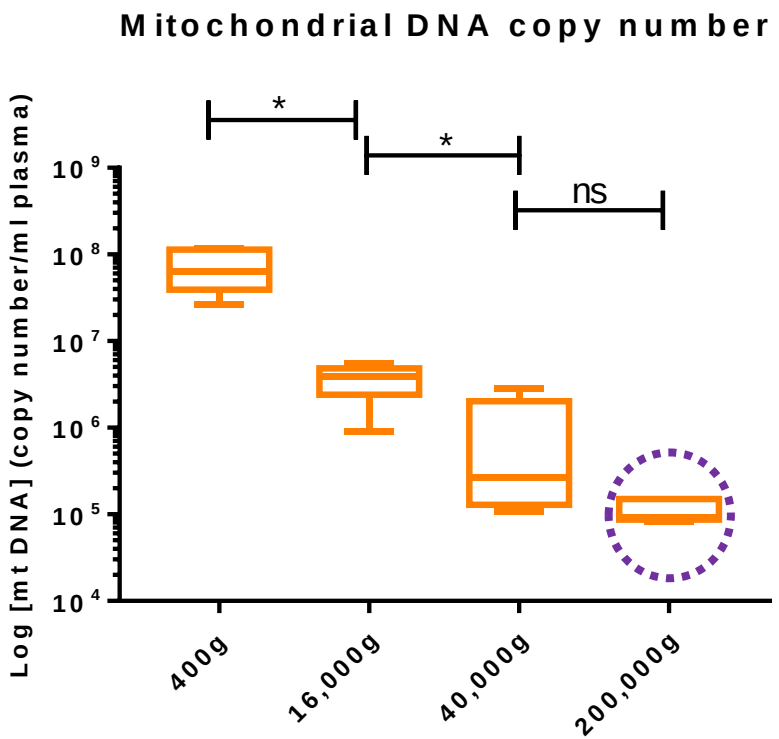
Differential centrifugation

Differential centrifugation effect on plasma



Differential centrifugation effect on plasma



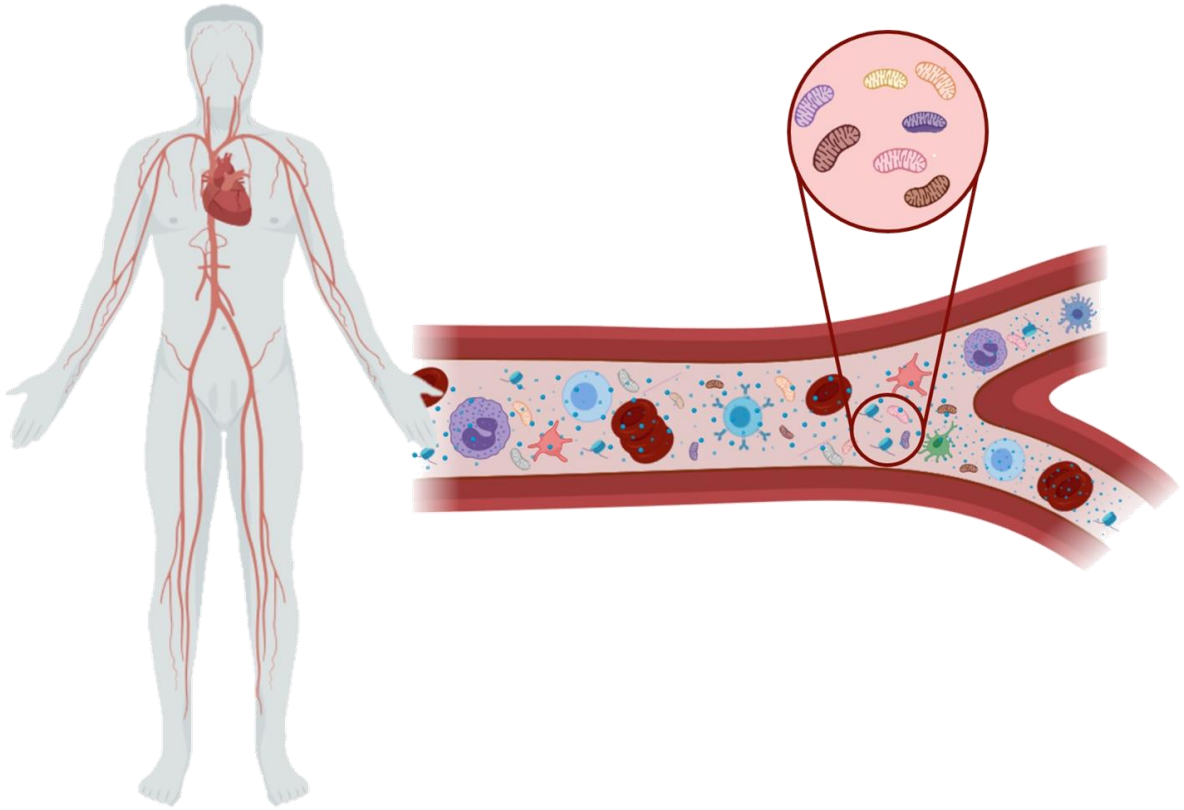


The presence of microvesicle free circulating mitochondrial and nuclear DNA

Study of the Topology of circulating mitochondrial DNA

Conclusion

The presence of cell-free intact mitochondria in the blood under physiological state



Received: 29 July 2019 | Revised: 27 November 2019 | Accepted: 29 November 2019

DOI: 10.1096/fj.201901917RR

RESEARCH ARTICLE

THE FASEB JOURNAL

Blood contains circulating cell-free respiratory competent mitochondria

Zahra Al Amir Dache[1] | Amaëlle Otandault[1] | Rita Tanos[1] | Brice Pastor[1] | Romain Meddeb[1] | Cynthia Sanchez[1] | Giuseppe Arena[2] | Laurence Lasorsa[1] | Andrew Bennett[3] | Thierry Grange[3] | Safia El Messaoudi[1] | Thibault Mazard[1] | Corinne Prevostel[1] | Alain R. Thierry[1]

Confirmation by:

Existence of Circulating Mitochondria in Human and Animal Peripheral Blood

Xiang Song, Wei Hu, Haibo Yu, Honglan Wang, Yelu Zhao, Robert Korngold, and Yong Zhao*

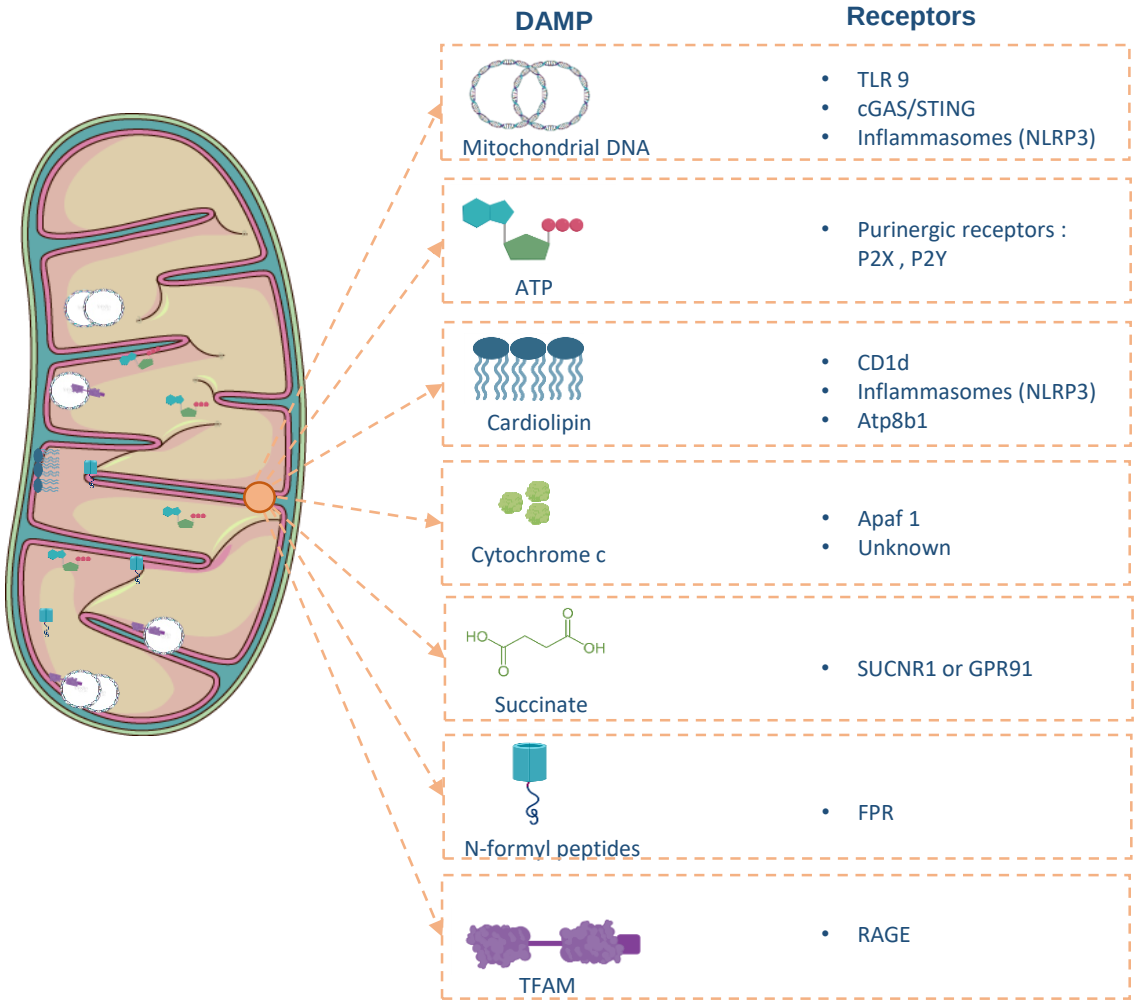
Characterization and origins of cell-free mitochondria in healthy murine and human blood

Olivia R. Stephens[a], Dillon Grant[a], Matthew Frimel[a], Nicholas Wanner[a], Mei Yin[b], Belinda Willard[c], Serpil C. Erzurum[a], Kewal Asosingh[a,d,*]

What could be the impact of intact free mitochondria presence in the circulation?

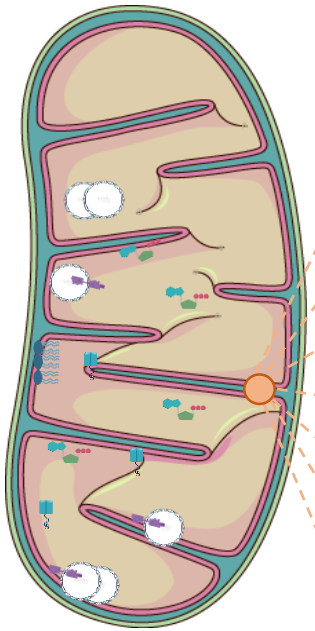
Fundamental research

Inflammation:



Fundamental research

Inflammation:



DAMP	Receptors
Mitochondrial DNA	• TLR 9 • cGAS/STING • Inflammasomes (NLRP3)
ATP	• Purinergic receptors : P2X , P2Y
Cardiolipin	• CD1d • Inflammasomes (NLRP3) • Atp8b1
Cytochrome c	• Apaf 1 • Unknown
Succinate	• SUCNR1 or GPR91
N-formyl peptides	• FPR
TFAM	• RAGE

Mitochondrial Damage-Associated Molecular Patterns: From Inflammatory Signaling to Human Diseases

Serge Grazioli[1,2] and Jérôme Pugin[3]*

Mitochondria Are a Subset of Extracellular Vesicles Released by Activated Monocytes and Induce Type I IFN and TNF Responses in Endothelial Cells

Florian Puhm, Taras Afonyushkin, Ulrike Resch, Georg Obermayer, Manfred Rohde, Thomas Penz, Michael Schuster, Gabriel Wagner, Andre F. Rendeiro, Imene Melki, Christoph Kaun, Johann Wojta, Christoph Bock, Bernd Jilma, Nigel Mackman, Eric Boilard, Christoph J. Binder

Nat Immunol. 2017 Apr 18;18(5):488-498. doi: 10.1038/ni.3704.

Mitochondria are the powerhouses of immunity.

Mills EL[1], Kelly B[2], O'Neill LAJ[1].

Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A_2 to promote inflammation

Luc H. Boudreau,[1] Anne-Claire Duchez,[1] Nathalie Cloutier,[1] Denis Soulet,[2] Nicolas Martin,[3] James Bollinger,[4] Alexandre Paré,[2] Matthieu Rousseau,[1] Gajendra S. Naika,[4] Tania Lévesque,[1] Cynthia Laflamme,[1] Geneviève Marcoux,[1] Gérard Lambeau,[5] Richard W. Farndale,[6] Marc Pouliot,[1] Hind Hamzeh-Cognasse,[7] Fabrice Cognasse,[7] Olivier Garraud,[7] Peter A. Nigrovic,[8] Helga Guderley,[3] Steve Lacroix,[2] Louis Thibault,[9] John W. Semple,[10] Michael H. Gelb,[4] and Eric Boilard[1]

Cell Stress, Vol. 3, No. 6, pp. 195 - 207; doi: 10.15698/cst2019.06.190

The sensing of mitochondrial DAMPs by non-immune cells

Aida Rodríguez-Nuevo[1,2,3] and Antonio Zorzano[1,2,3]

Journal of Neuroimmune Pharmacology

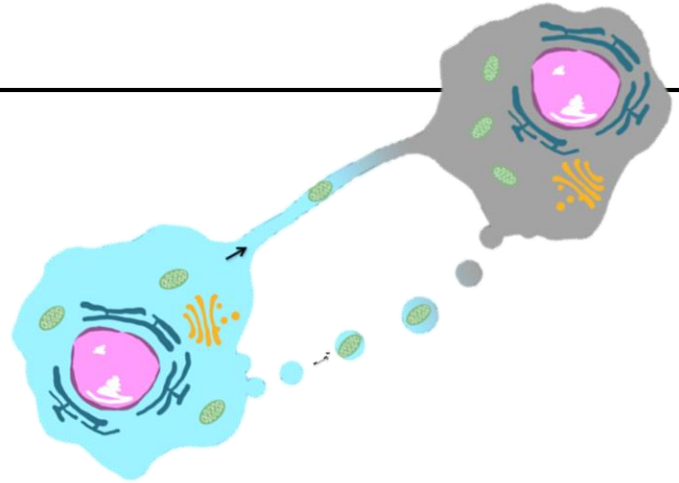
December 2016, Volume 11, Issue 4, pp 622–628 | Cite as

Extracellular Mitochondria and Mitochondrial Components Act as Damage-Associated Molecular Pattern Molecules in the Mouse Brain

Emerging Roles of Blood-Borne Intact and Respiring Mitochondria as Bidirectional Mediators of Pro- and Anti-Inflammatory Processes

Article in Medical science monitor: international medical journal of experimental and clinical research · March 2020

Fundamental research



Cell-cell communication:

Intercellular mitochondrial transfer rescues injured cells :

- *Mitochondrial transfer between cells can rescue aerobic respiration, Proc. Natl. Acad. Sci. U.S.A., 2006*
- *Mesenchymal stem cells sense mitochondria released from damaged cells as danger signals to activate their rescue properties, Cell death and differentiation, 2017*
- *Mitochondria are transported along microtubules in membrane nanotubes to rescue distressed cardiomyocytes from apoptosis , Cell death and disease, 2018*

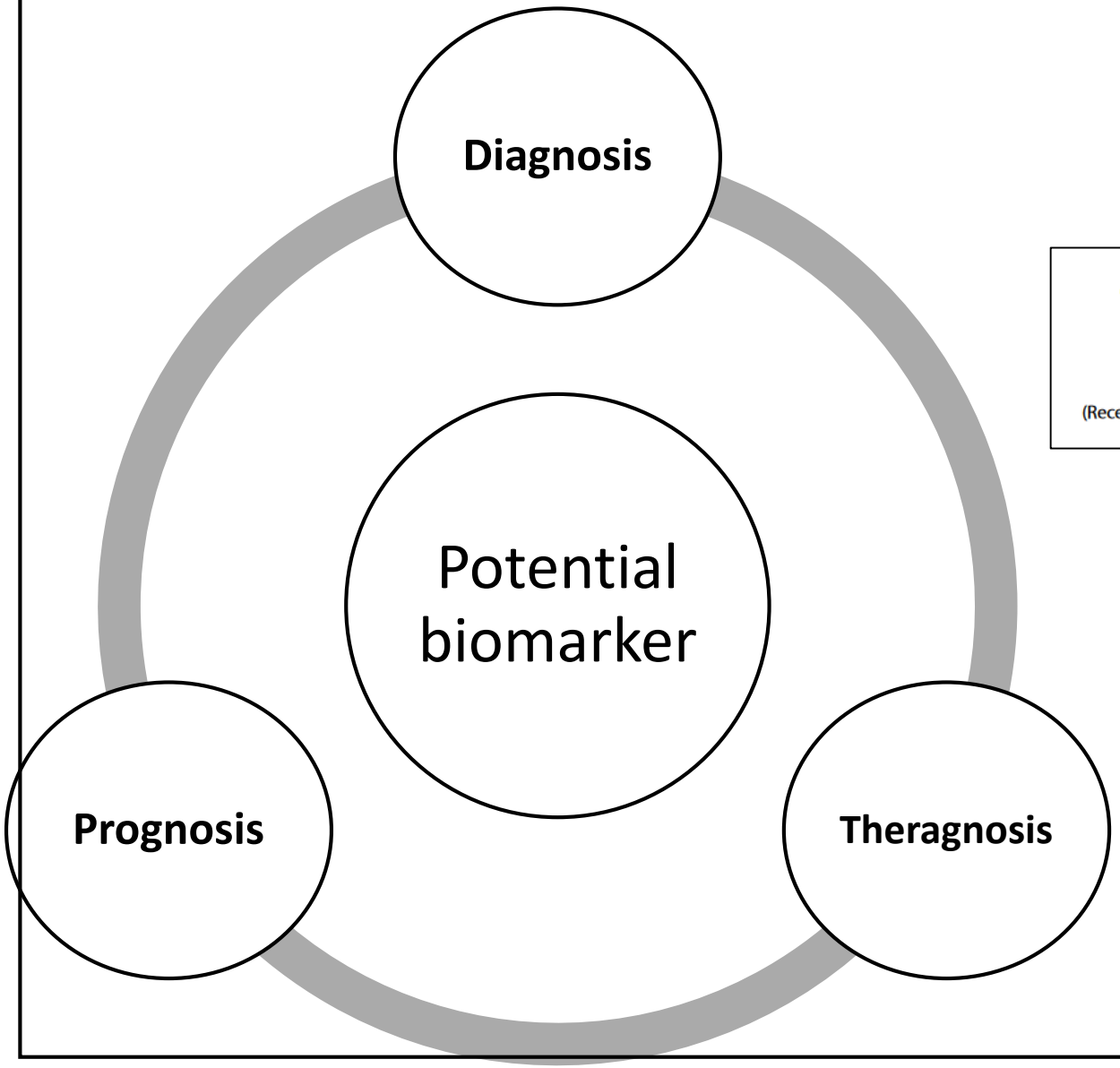
Intercellular mitochondrial transfer promotes cancer malignancy :

- *Cancer-associated fibroblasts promote prostate cancer malignancy via metabolic rewiring and mitochondrial transfer. Oncogene, 2019.*
- *Mitochondrial genome acquisition restores respiratory function and tumorigenic potential of cancer cells without mitochondrial DNA. Cell Metab, 2015*
- *Preferential transfer of mitochondria from endothelial to cancer cells through tunneling nanotubes modulates chemoresistance. J Transl Med, 2013*

Artificial incorporation of mitochondria into mammalian cells:

- *Mitochondrial transformation of mammalian cells. Nature, 1982.*
- *Injection of mitochondria into human cells leads to a rapid replacement of the endogenous mitochondrial DNA. Cell, 1988.*
- *MitoCeption as a new tool to assess the effects of mesenchymal stem/stromal cell mitochondria on cancer cell metabolism and function. Scientific Reports, 2015.*
- *Characteristics of Mitochondrial Transformation into Human Cells, Scientific reports, 2016*

Clinical research



Perspectives

Mitochondrial DNA Mutations in Cancer

Stefanie Zanssen*, Eric A. Schon

Mitochondrial DNA Somatic Mutation in Cancer

Aekyong Kim

School of Pharmacy, Catholic University of Daegu, Gyeon

(Received November 19, 2014; Revised December 20, 2014; Accept

The Landscape of mtDNA Modifications in Cancer: A Tale of Two Cities

*Kate L. Hertweck[1] and Santanu Dasgupta[2]**

[1]Department of Biology, The University of Texas at Tyler, Tyler, TX, United States, [2]Department of Cellular and Molecular

Review

Current and upcoming mitochondrial targets for cancer therapy

Hyoung Kyu Kim [a, b], Yeon Hee Noh [a], Bernd Nilius [c], Kyung Soo Ko [a], Byoung Doo Rhee [a], Nari Kim [a], Jin Han [a]

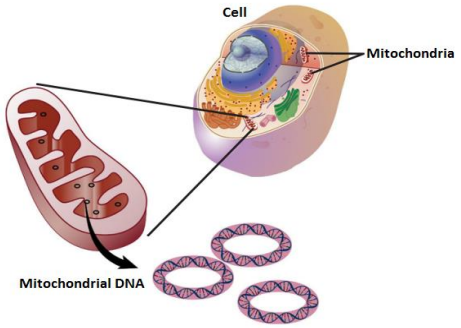
Mitochondria and Cancer

Sejal Vyas, **Elma Zaganjor**, and **Marcia C. Haigis***

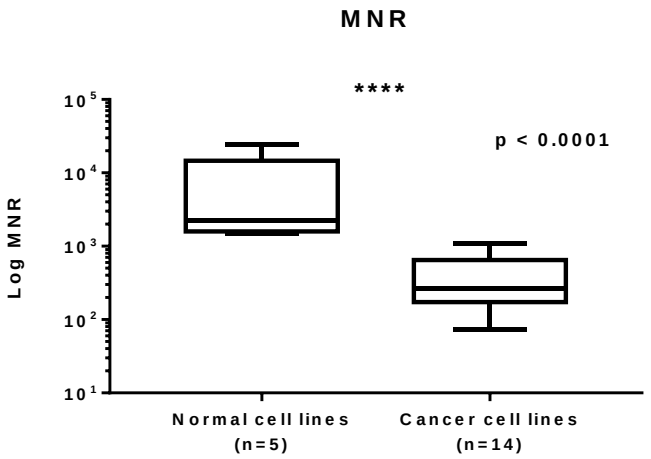
Department of Cell Biology, Ludwig Center at Harvard, Harvard Medical School, Boston, MA 02115

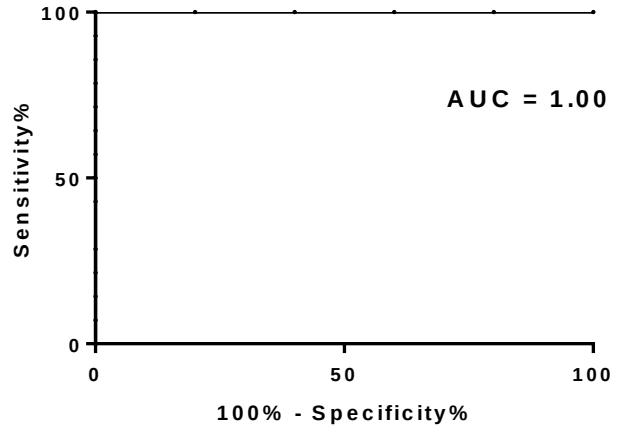
Team’s study on the diagnostic value of vesicular circulating mitochondrial DNA

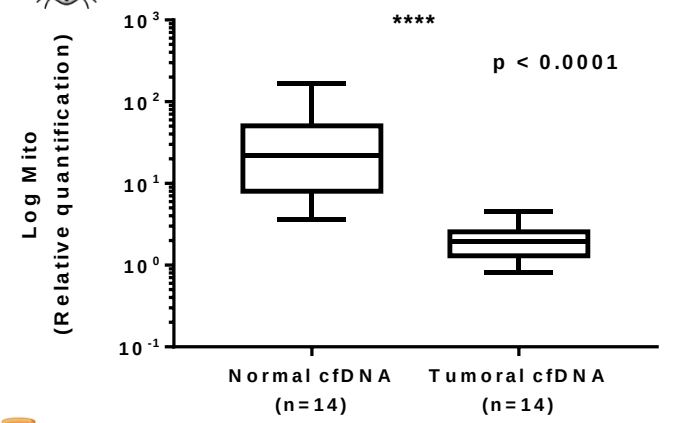
Mitochondrial cfDNA for cancer screening

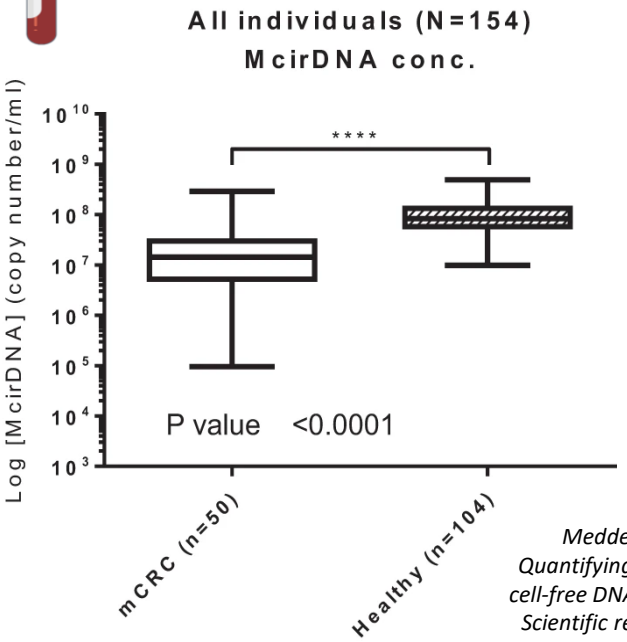


[mtcfDNA] in cancer cases < [mtcfDNA] in normal cases





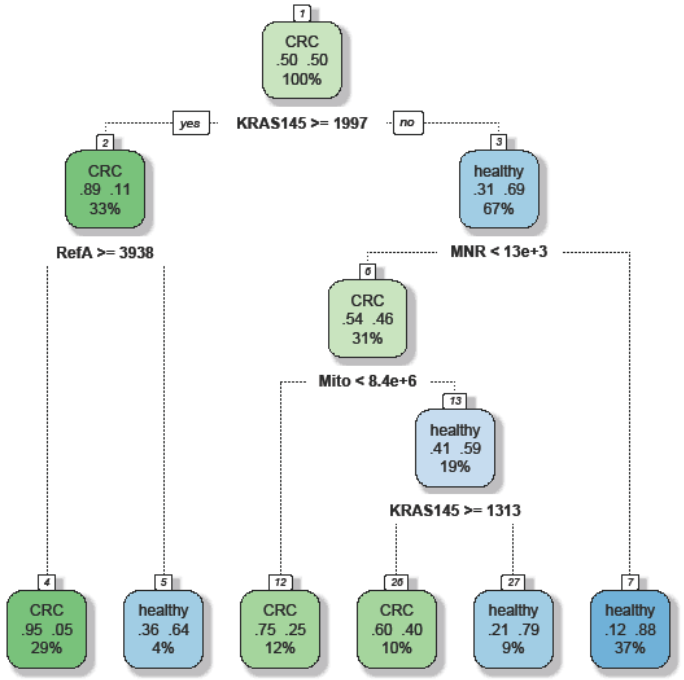


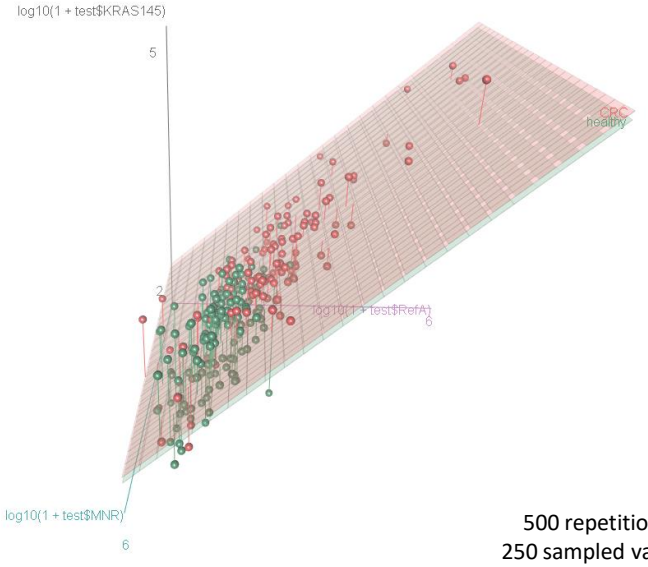


Meddeb et al. Quantifying circulating cell-free DNA in humans. Scientific reports 2019

Use of machine learning for selecting the best combinaison

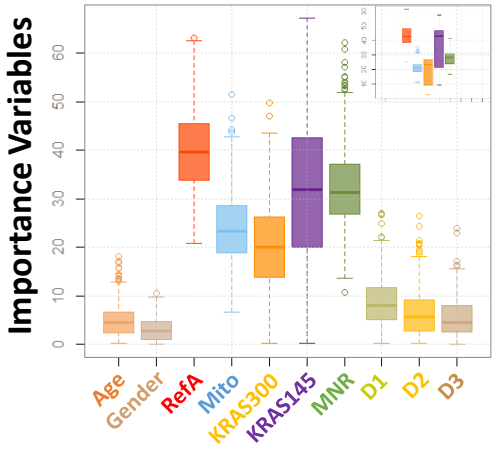
Selected decision tree From resampling





Jacques COLLINGE
Guillaume TOSATO
IRCM

500 repetitions
250 sampled values



Over all these repetitions : RefA seems to be the more often the most important variable amongs all the others, then KRAS145, then MNR, then KRAS300 and finally Mito. The other variables may be not relevant.

All n=1000

Se = 0.751 [0.724 – 0.787]
Sp = 0.921 [0.893 – 0.957]

Stage 0, 1, 2 VS Healthy

Se = 0.715 [0.678 – 0.892]
Sp = 0.921 [0.892 – 0.948]

Tanos et al, Adv. Science, 2020

Biomarkers for Precision Oncology

- **Alain R. Thierry**
- Director of Research, INSERM - Institute of Research on Oncology de Montpellier
- Head of the “Biomarkers for Precision Oncology” team
- Inserm U1194 – IRCM, Montpellier

THIERRY, Alain	DR1, INSERM/ Head
BLACHE, Philippe,	DR2, CNRS
PREVOSTEL, Corinne,	CRCN, INSERM
YCHOU, Marc	PU PH, ICM
ADENIS, Antoine	PU PH, ICM
MAZARD, Thibault	PH, ICM
CRAPEZ,Evelyne,	IR, 25% ICM
LASORSA,Laurence,	IE, ICM
FRAYSSINOUX, Florence	TR, INSERM

AMIR DACHE Zahra	Post Doc
PISAREVA, Ekaterina	Post Doc, EU H2020
SANCHEZ, Cynthia	IE, IRCM CDD
KUDRIAVTSEV, Andrei	IE, IRCM CDD
MIRANDOLA, Alexia	IE, IRCM CDD
GROSGEORGES Marie	IE, IRCM CDD

PASTOR, Brice	MD, PhD student
ROCH, Benoit	PhD student UM