Preservation, Extraction, and Analysis of Biomolecules in Complex Human Biofluids

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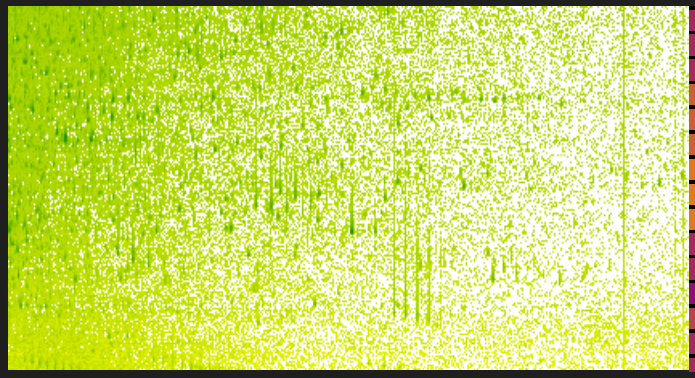
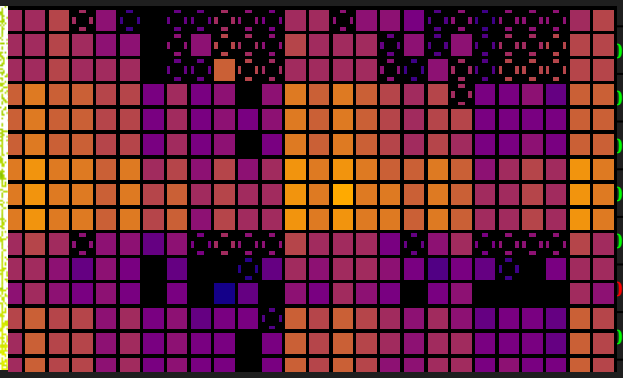




0013992	0.0206398	0.7917289	mir146b
0019657	0.0206398	0.7781608	mir221
0077541	0.0542787	0.7350238	let7a
0114304	0.0600096	0.7712901	mir155
0208282	0.0874784	0.8233863	mir175p
0279995	0.0979982	1.1724179	mir29c
0374234	0.1122702	0.8723669	mir27a

Blood/Plasma/Serum

- Cells/plasma (45/55 vol%)
- Total protein in plasma is ≈80 g/L, 50% albumin
- micro-RNA circulates in exosomes and as micro-RNP
- >>500-5,000 different proteins, conc. range 10^7
- ionic strength 154 mM
- pH 7.35-7.45
- Homeostatic fluid
- Reflects systemic or organ specific pathology
- Good buffering capacity

Spinal fluid (CSF)

- Few cells in normal CSF (if no blood contamination)
- Plasma ultrafiltrate
- Total protein is 0.3-0.8% of plasma (0.2-0.6 g/L), 60% albumin
- micro-RNA present at very low conc.
- Heterogeneous fluid (protein concentration gradient)
- Reflects CNS pathology
- Turns over 3-4 times a day
- Low buffering capacity, pH ↑ in samples exposed to air
- Antioxidant capacity 5x lower than plasma

Urine

- Few cells in normal urine
- Glomerular ultrafiltrate of plasma, normally ≈95% water
- Very low protein in normal urine, small molecules, metabolites abundant
- exosomal micro-RNA (tubular) present
- reflects kidney pathology etc.
- Variable volume & gravity, variable ioinic strength, variable pH
- i.e., non-homeostatic

- Spinal Fluid
- Urine
- Blood
  - serum proteome
  - microparticle proteome
  - circulating micro-RNA
  - dried blood spot specimens
    - DNA extractions and WGA
    - multiplex immunoassays

CSF - Transthyretin as an oxymeter

Immunoprecipitation LC-MS of intact transthyretin from crude CSF

32 hrs, 4 C

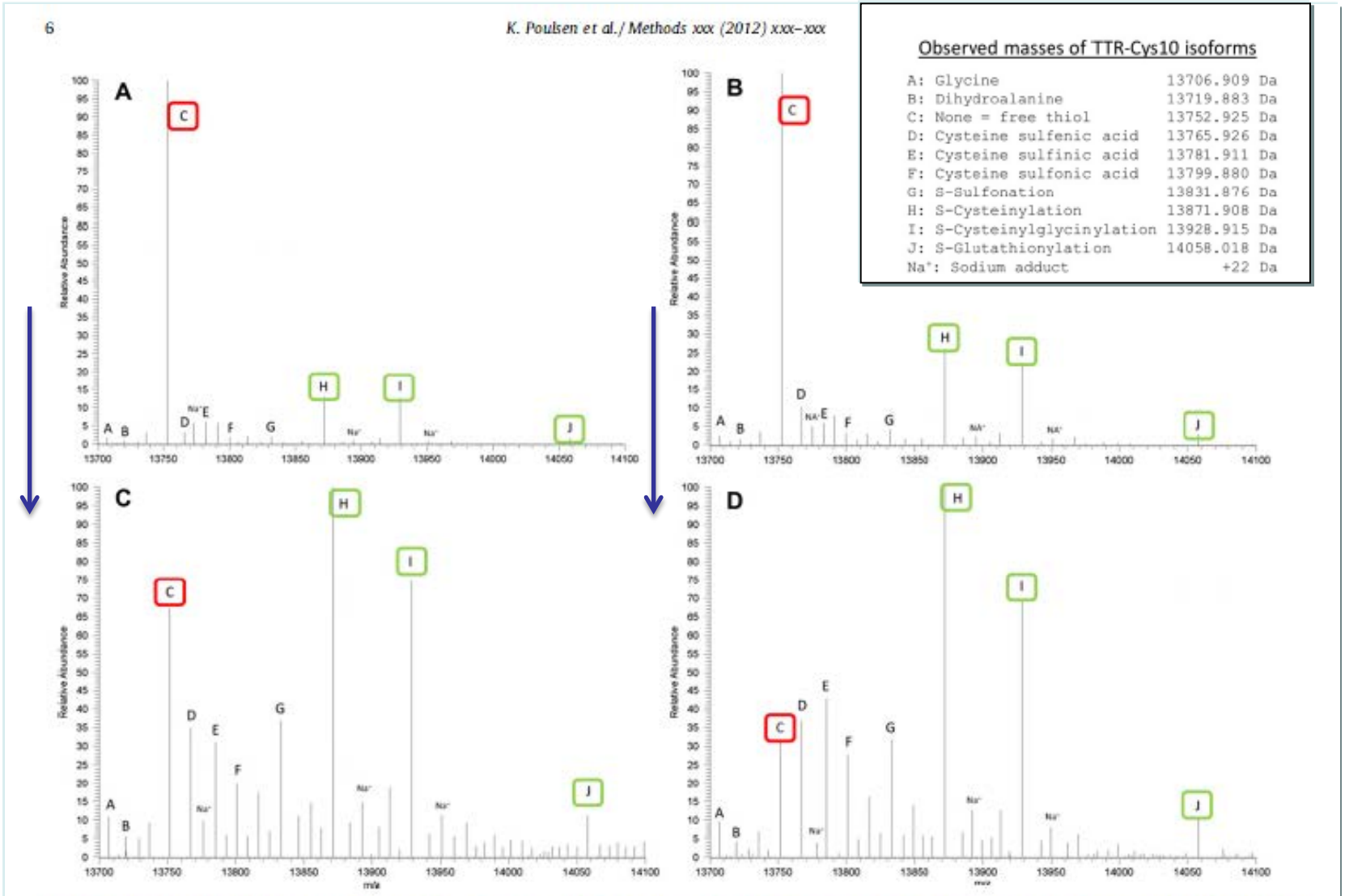
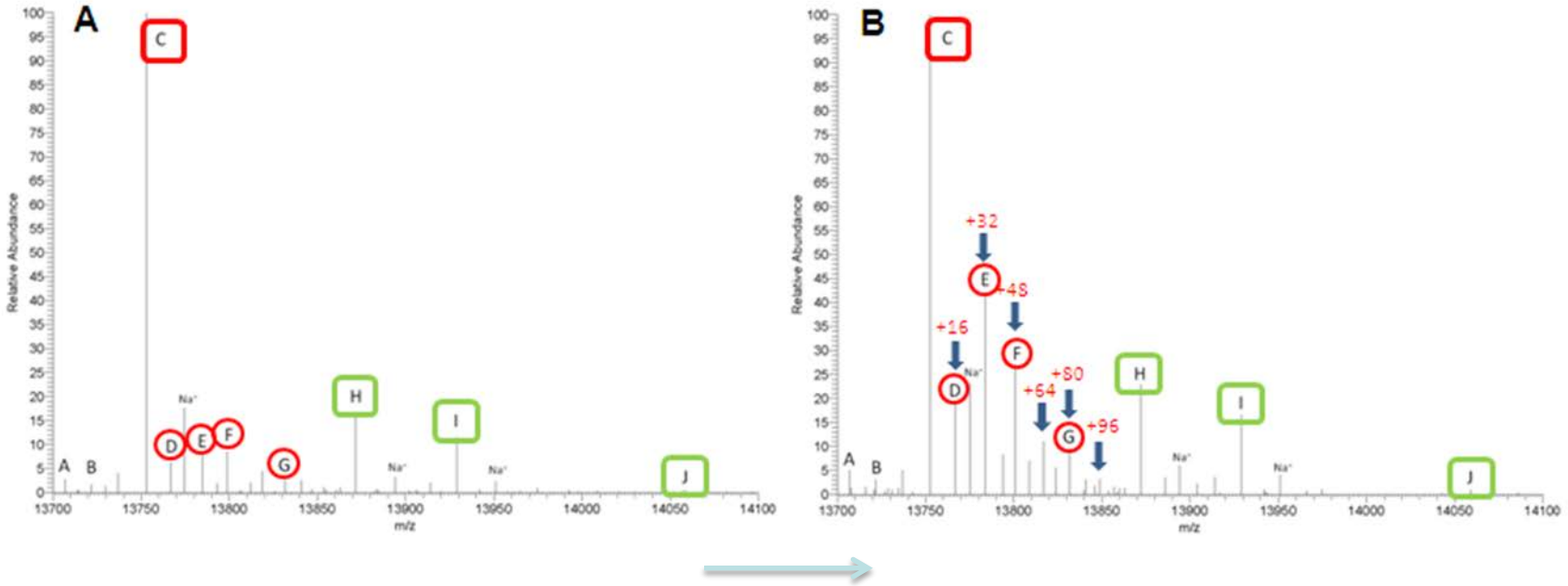


Fig. 3. Deconvoluted monoisotopic ESI-MS spectra calculated by Xtract, of immunoprecipitated TTR prior (sample 1 in 3**A** and sample 2 in 3**B**) and after CSF stability test (32 h at 4 °C, sample 1 in 3**C** and sample 2 in 3**D**).

Please cite this article in press as: K. Poulsen et al., Methods (2012), doi:10.1016/j.ymeth.2011.12.009

CSF - Transthyretin as an oxymeter



TTR dried down and kept under N_2 at RT, 4 days

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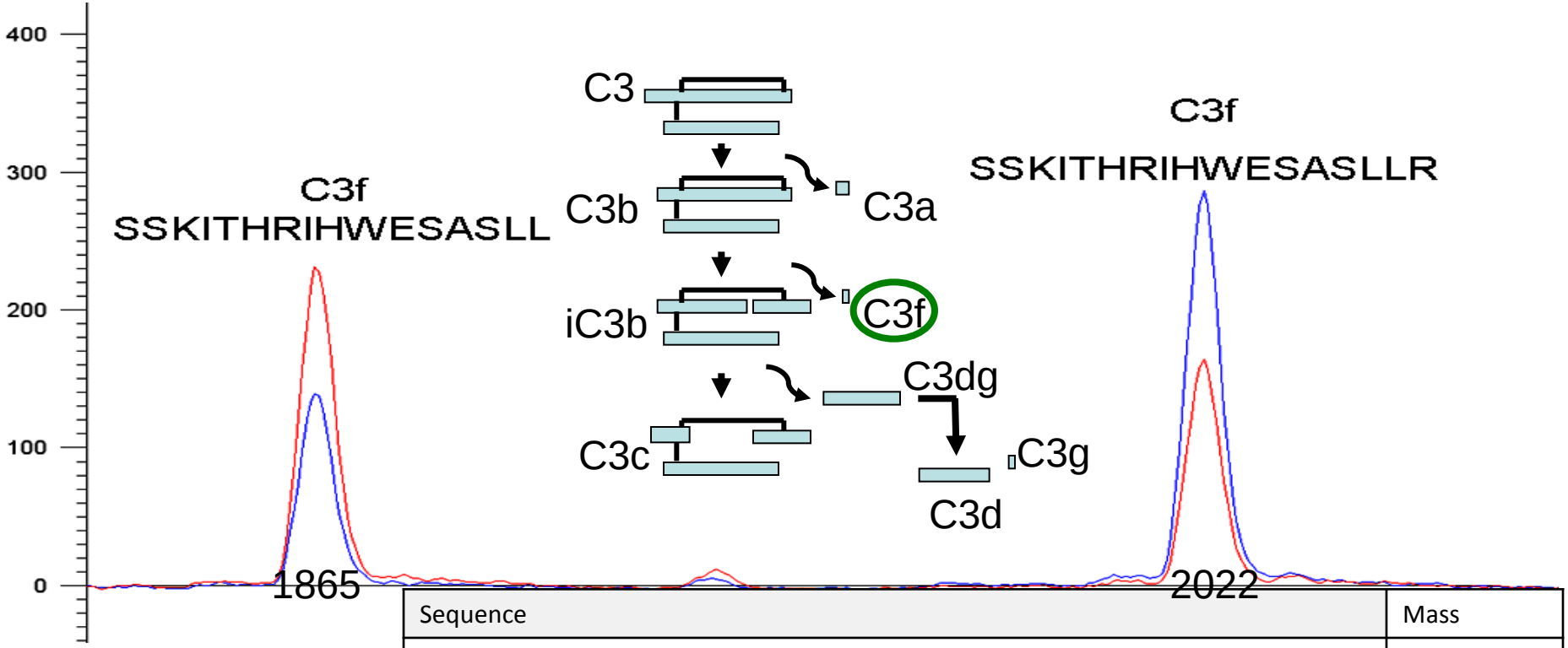
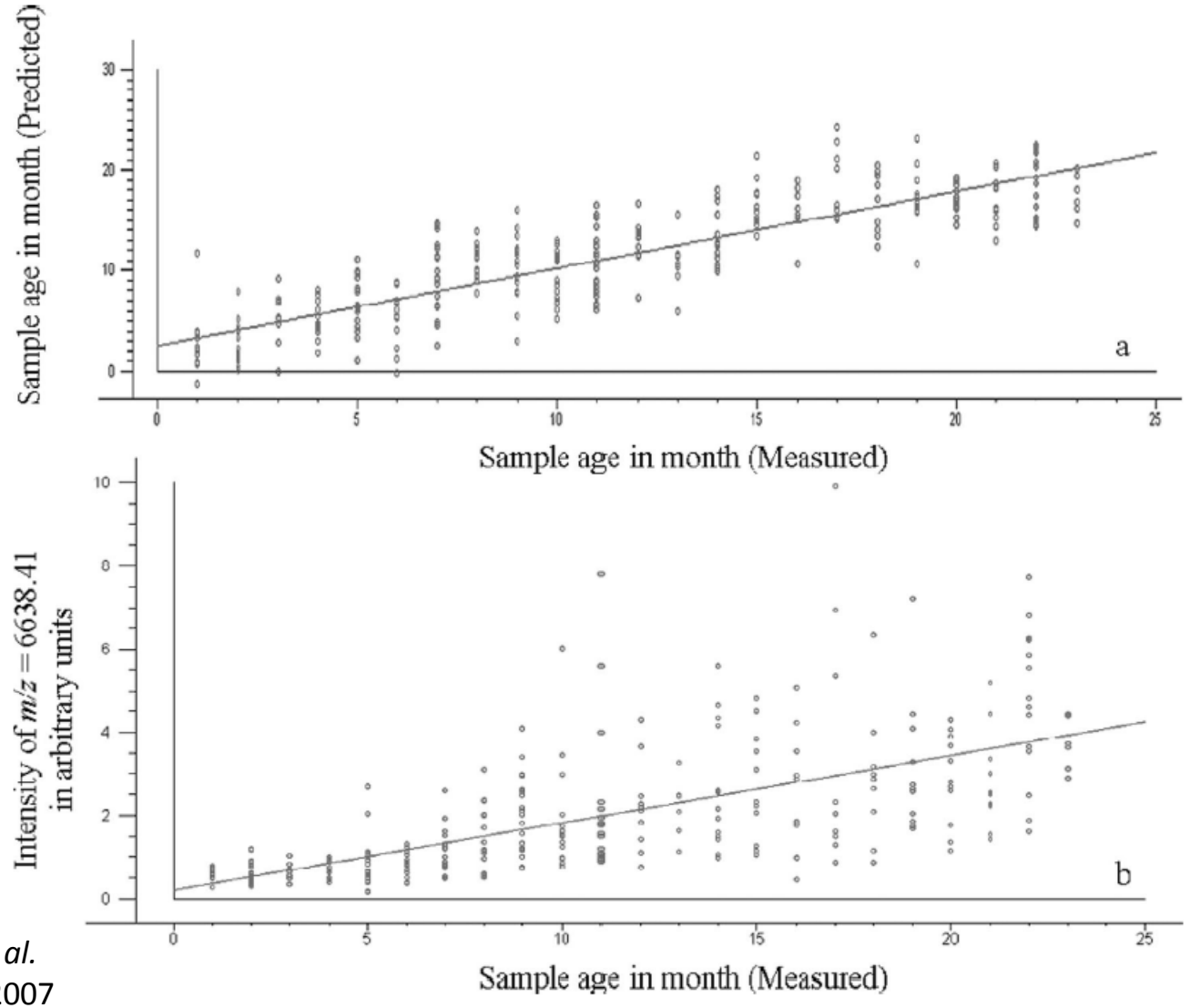
Serum proteomics – Sentinel polypeptides for degradation



Sequence	Mass
Complement C3f: aa 2-16 (SKITHRIHWESASLL**)**	1777.9
Complement C3f: aa 1-16 (SSKITHRIHWESASLL)	1864.9
Complement C3f: aa 1-17 (SSKITHRIHWESASLLR)	2021.7
Fibrin alpha C term fragment: aa 81-105 (SSSYSKQFTSSTSYNRGDSTFESKS)	2767.4
Fibrin alpha C term fragment: aa 81-106 (SSSYSKQFTSSTSYNRGDSTFESKSY)	2931.5
Fibrinopeptide A: aa 1-12 (EGDFLAEGGGVR**)**	1206.5
Fibrinopeptide A: aa 3-16 (SGEGDFLA EGGGVR)	1350.7
Fibrinopeptide A: aa 2-16 (DSGEGDFLAEGGGVR)	1465.5
Fibrinopeptide A (Modifications: 3 Phosphorylated): aa 1-16 (AD**S**GEGDFLAEGGGVR)	1616.9
Kininogen: aa 439-456 (HNLGHGHKHERDQGHGHQ)	2080.9

West-Nørager *et al.*
Anal Chem 2005,
J Chromatogr B 2007

Serum proteomics – storage effects -20ºC



West-Nørager *et al.*
J Chromatogr B 2007

Figure 3. Long-term storage effects at −20 °C. (a) Modeling sample age using PLS (validated). Samples were collected over a period of 23 months. The samples are grouped according to the month in which the sample was taken, 09-2004 is number 23 and 07-2006 is number 1 on the abscissa. The slope is 0.78 with correlation $r = 0.86$. (b) The figure shows a variable with m/z 6638.41 with intensity increasing as a function of storage time. The variance between samples becomes larger with increased storage time. For other variables, e.g., at 4205.71 Da, the opposite trend was observed (not shown), i.e., diminished intensity as a function of storage time.

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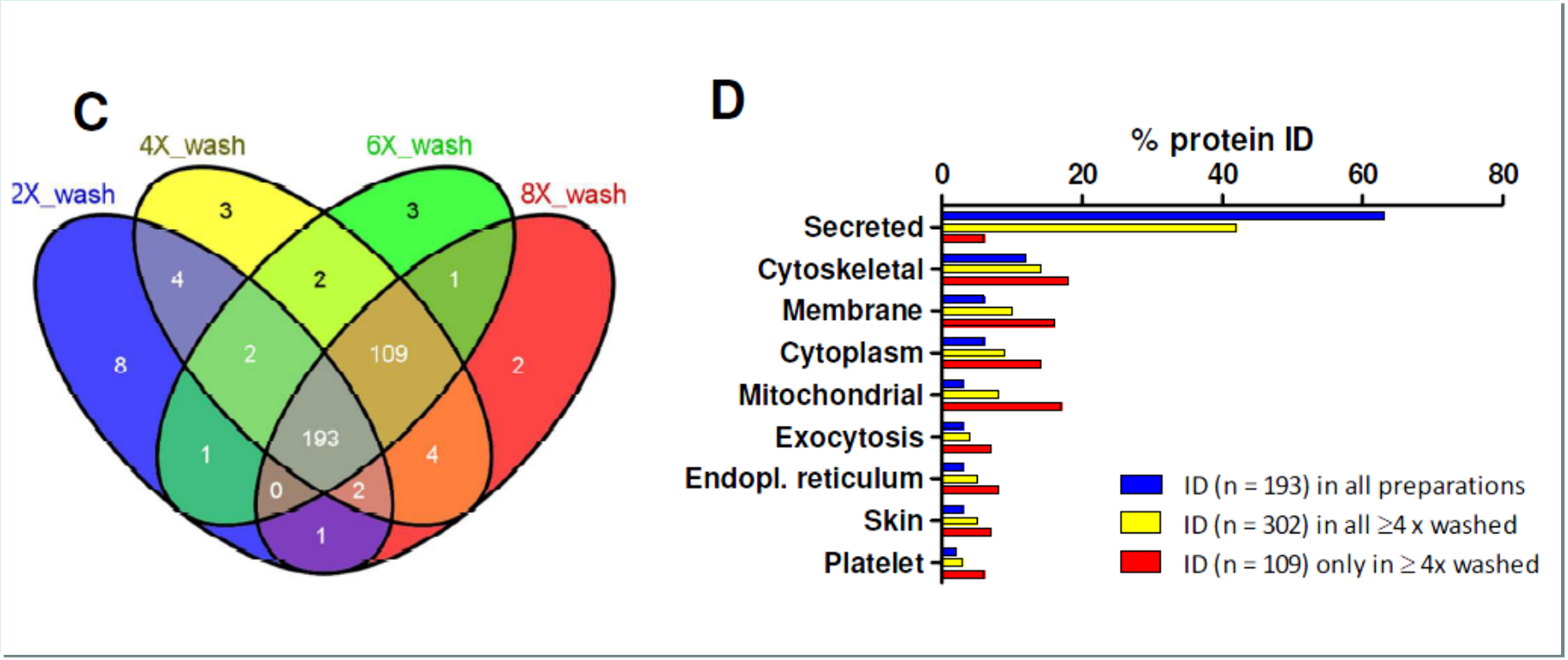
Circulating sub-proteomes
Subfraction-enrichment in blood opens new avenues



Oestergaard O *et al.* J Proteome Res 2012
Epub ahead of print

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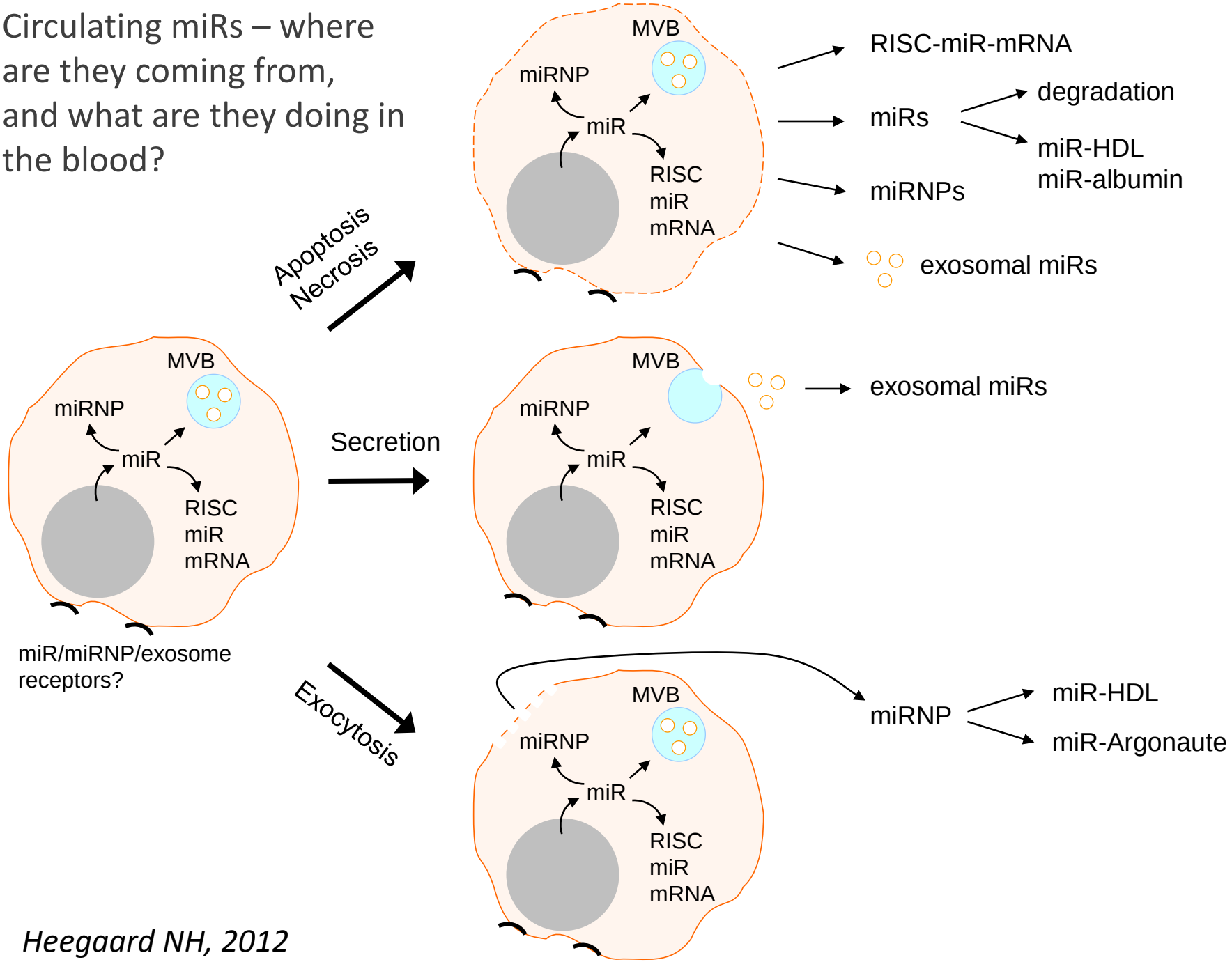
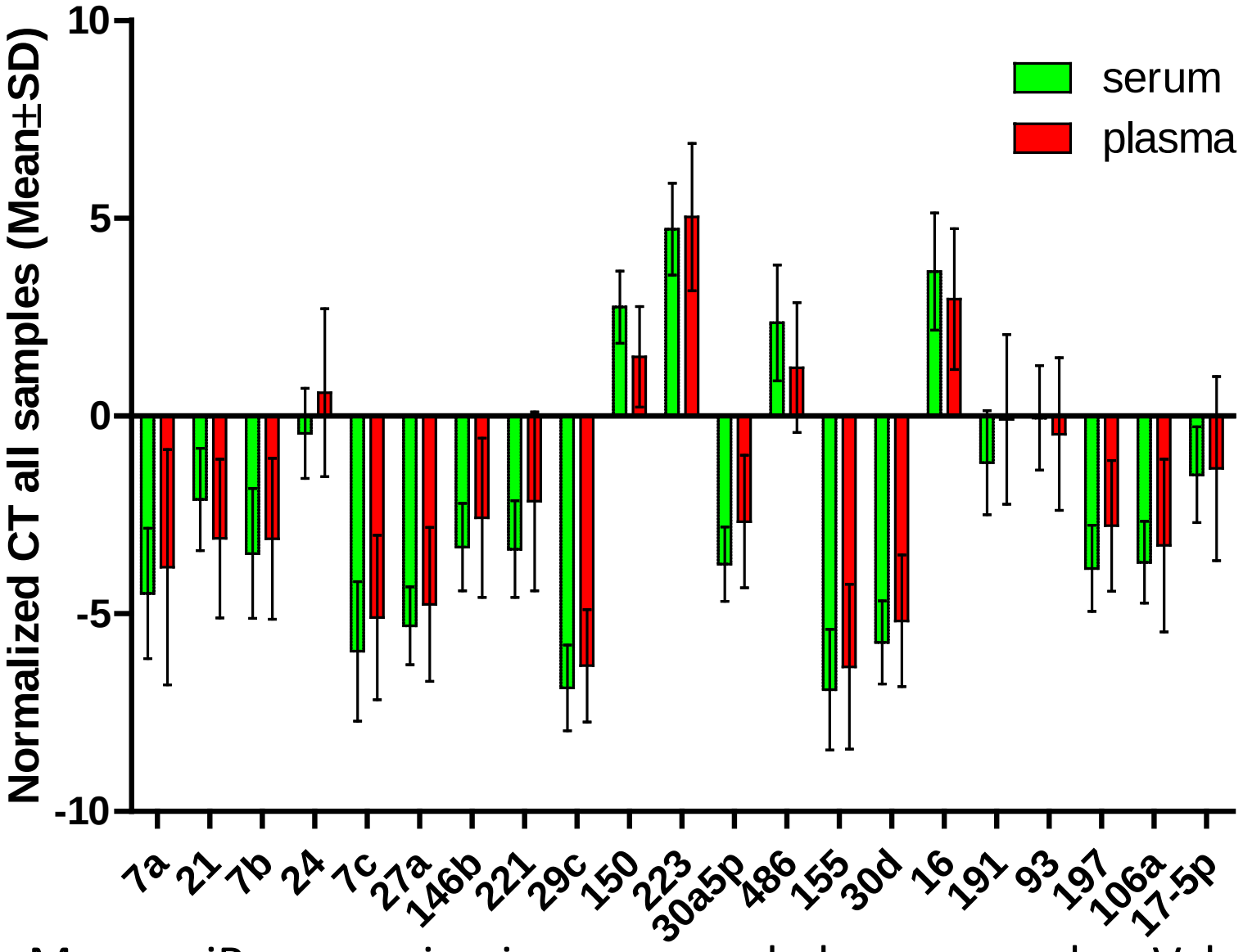
Circulating miRs – where are they coming from, and what are they doing in the blood?

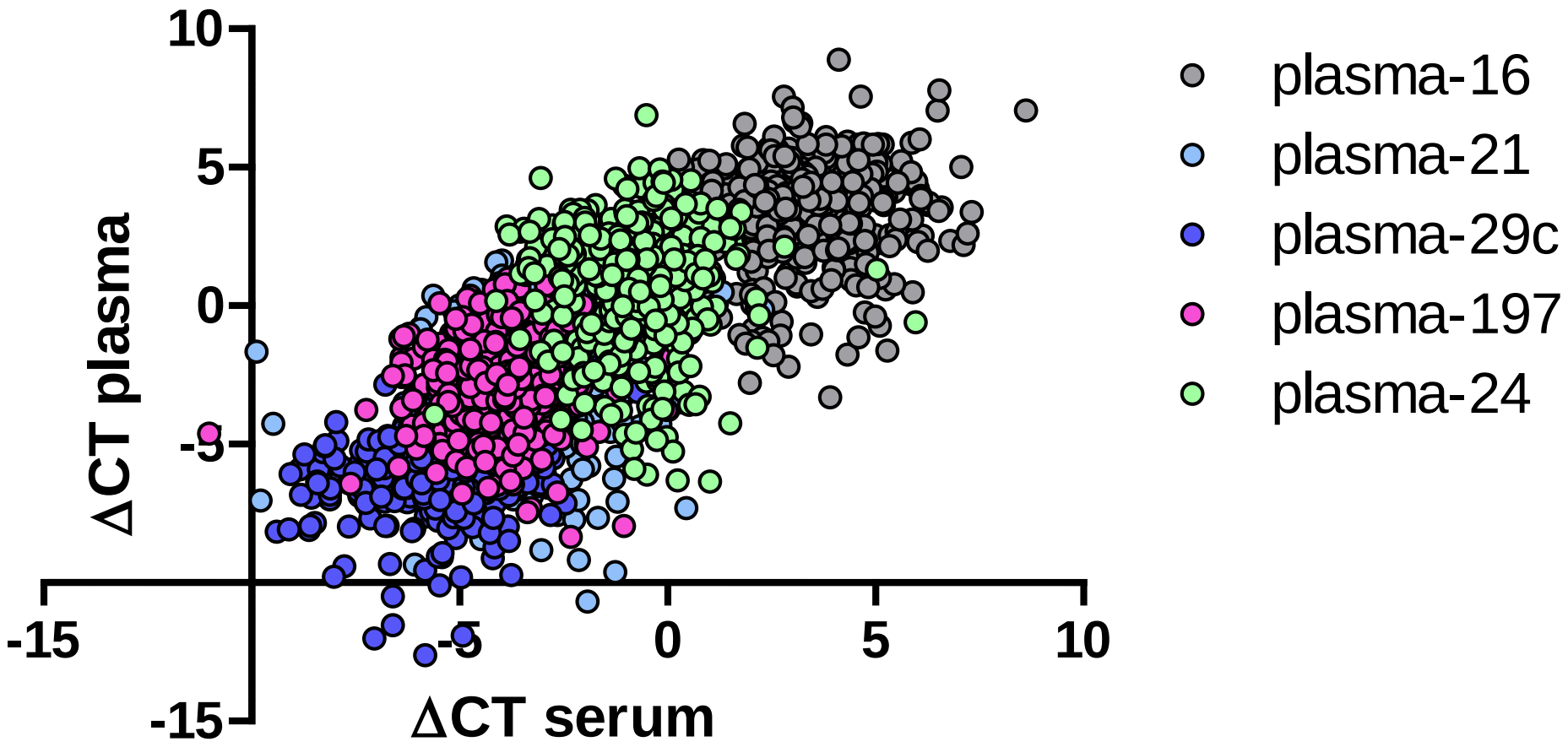


Heegaard NH, 2012



Mean miR-expression in serum and plasma samples. Values (± standard deviation) for each of the 21 miRs are shown.

Heegaard N *et al.* Int J Cancer 2012; 130: 1378-86



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Dried blood spot specimens (DBSS)

The Danish National Birth Cohort

3.2 mm punch
~3 µL blood

- **Heel blood samples** from all Danish newborns since 1981-82. Annual birth cohort
  ≈ 65,000 – i.e. ≈ 2 million samples in repository (-24°C)
- **Whole blood.** Erythrocytes, leukocytes, platelets and plasma
- **Dried blood in filter paper.**
  - Cells are more or less lyzed during drying
  - Analytes of interest must be extracted from the filter paper matrix
  - All biological processes are stopped immediately when the sample is dried.
- The very limited amount of sample material reduces analytical possibilities using conventional techniques

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Comprehensive genetic analysis on neonatal dried blood spot samples (DBSS)

1. Neonatal genetic screening for inherited disorders
2. Studies of genetic influence on complex disorders, using archived residual DBSS combined with clinical information from medical registries

SNP-analysis of material from dried blood spots. Genome scans by Illumina™ 610k chip of wgaDNA from 3.2 mm DBSS punch

BMC Genomics 2009, **10**:297
http://www.biomedcentral.com/1471-2164/10/297
Hollegaard MV et al.

Table 1: Robustness of the GPlex4 and REPLI-g WGA kits.

ID	GPlex4			REPLI-g		
	Call-rate A[1]	Call-rate B[2]	WGAout[3]	Call-rate A[1]	Call-rate B[2]	WGAout[3]
1	97.74%	99.24%	4.86	99.42%	99.65%	6.55
2	97.41%	99.30%	4.85	99.56%	99.64%	7.22
3	98.21%	99.42%	5.06	98.54%	99.33%	3.53
4	97.80%	99.38%	4.93	99.14%	99.56%	3.00
5	97.90%	99.41%	5.20	99.00%	99.51%	4.47
6	98.04%	99.42%	4.92	99.73%	99.73%	3.74
7	97.83%	99.38%	5.00	99.72%	99.73%	6.02
8	97.62%	99.29%	5.01	99.49%	99.66%	6.00
9	97.56%	99.29%	5.15	99.55%	99.64%	7.64
10	97.30%	99.26%	5.33	95.91%	99.15%	7.86
11	98.17%	99.32%	5.41	99.43%	99.60%	7.48
12	98.13%	99.38%	5.49	99.38%	99.56%	1.56
13	96.62%	99.08%	5.15	99.43%	99.60%	6.71
14	97.55%	99.35%	5.48	99.22%	99.63%	1.40
15	96.75%	99.17%	5.14	98.97%	99.53%	4.98
16	96.53%	*99.16%*	4.13	98.49%	99.43%	2.72
Median	*97.68%*	*99.31%*	*5.10*	*99.40%*	*99.60%*	*5.49*
Std. Dev.	*0.53%*	*0.10%*	*0.33*	*0.92%*	*0.15%*	*2.18*

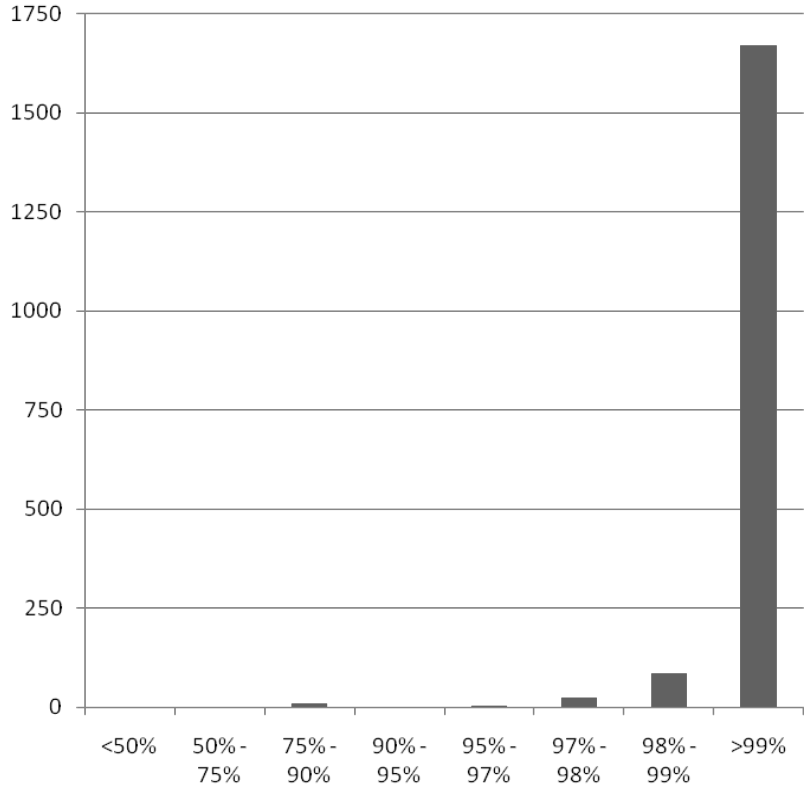
[1]Call-rate (percent) using the Illumina Human610-Quadv1B cluster file.
[2]Call-rate (percent) using the WGA kit custom cluster file.
[3]wgaDNA (μg) produced per reaction.

Conflict rates 0.02-0.03%

Robustness of SNP genotyping

610,000 genetic variants in 1806 DBSS

Call rate	Number of DBSS
<50%	1
50% - 75%	2
75% - 90%	10
90% - 95%	1
95% - 97%	3
97% - 98%	24
98% - 99%	87
>99%	1670
Total samples	1806



1806 DBSS from 1982-1990 stored in The Danish Neonatal Screening Biobank.
SNP genotyping performed on wgaDNA (MDA (REPLI-g) method) using the Illumina™ Infinium HD Human610-Quad BeadChip. Calls were made from a custom made cluster file based on 400 wgaDNA samples.

Common variants near *MBNL1* and *NKX2-5* are associated with infantile hypertrophic pyloric stenosis

Bjarke Feenstra[1,4], Frank Geller[1,4], Camilla Krogh[1], Mads V Hollegaard[2], Sanne Gørtz[1], Heather A Boyd[1], Jeffrey C Murray[3], David M Hougaard[2] & Mads Melbye[1]

Nature Genetics. Advance online publication 2012
http://www.nature.com/doifinder/10.1038/ng.1067

Discovery: Samples from 1,001 cases were selected and successfully genotyped. The control group consisted of 2,401 Danish children without IHPS.
Replication: Samples from 796 cases and 876 controls drawn from the same population.
All samples were drawn from the Danish Newborn Screening Biobank and the biobank of the Danish National Birth Cohort, both of which are part of the Danish National Biobank. Sampling and genotyping (using the Illumina Human660W-Quad v1_A chip) was undertaken in two rounds. In total, genotypes for 559,390 SNPs were released in both genotyping rounds. For the association analysis, we used the data from 523,420 SNPs

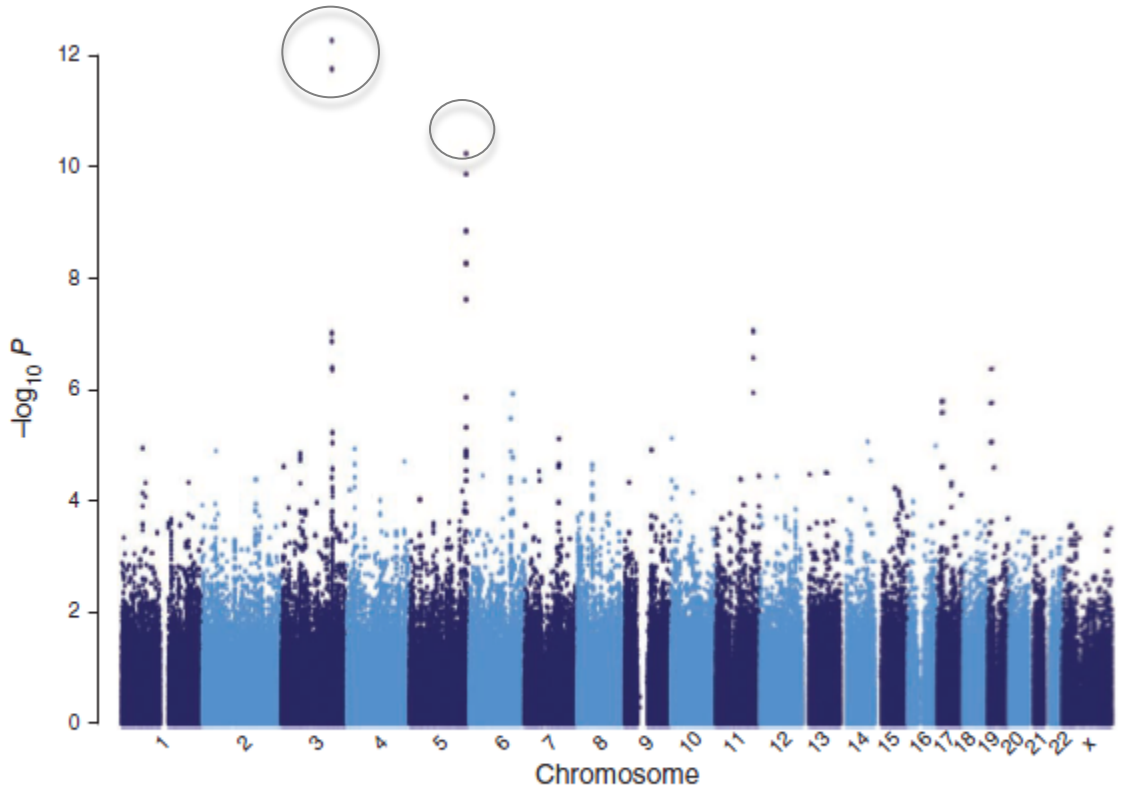


Figure 1 Manhattan plot of the IHPS GWAS. The genome-wide distribution of $-\log_{10} P$ values after correction by the genomic control factor ($\lambda = 1.06$) is shown across the chromosomes.

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Simultaneous Measurement of 25 Inflammatory Markers and Neurotrophins in Neonatal DBSS by Immunoassay with Luminex xMAP Technology

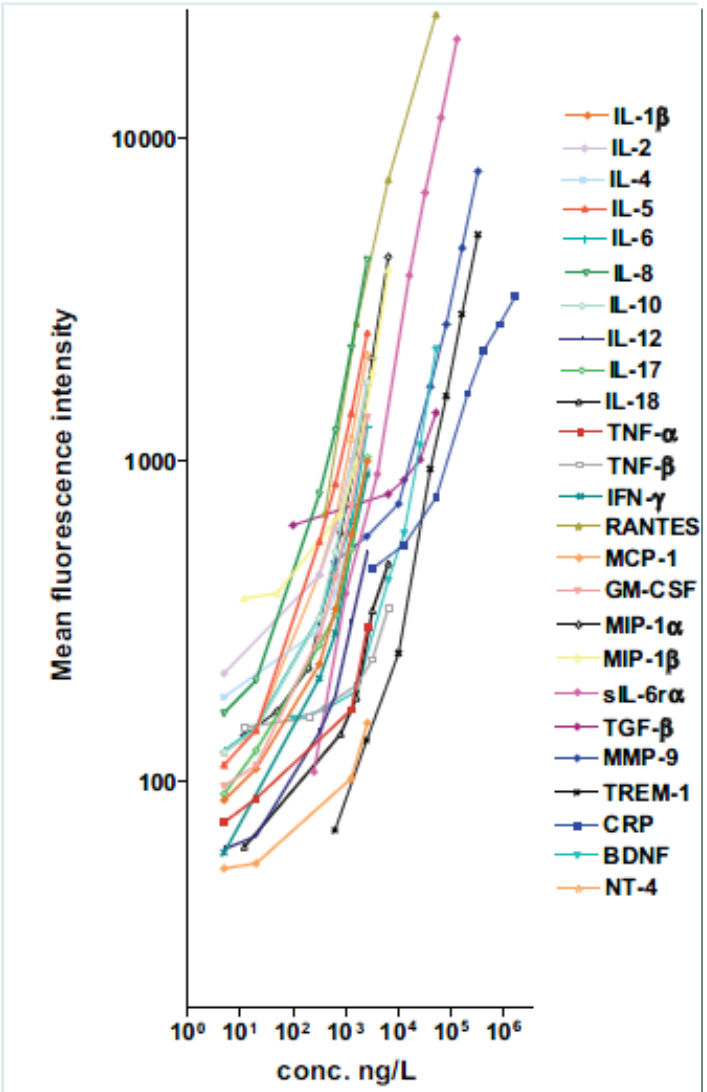

Fig. 2. Calibration curves for quantification of 25 analytes in DBSS.

Calibrators were dissolved at appropriate concentrations in pig/guinea pig serum (1:1), spotted on filter paper, and dried. Calibrators were extracted from two 3.2-mm punches and analyzed as described. Blank values are not subtracted.

Table 6. Concentrations of analytes detectable in DBSS stored at −24 °C.[a]

	Relative concentration, %		
Analytes	23 years	3 years	1 month
IL-1β	44	43	93
IL-2	116	115	113
IL-4	91	91	107
IL-5	105	116	122
IL-6	95	101	108
IL-8	28	38	64
IL-10	124	103	129
IL-12	95	108	107
IL-17	94	100	107
IL-18	138	113	129
TNF-α	92	101	109
TNF-β	88	94	93
IFN-γ	117	119	121
RANTES	87	89	90
MCP-1	94	112	112
GM-CSF	102	107	108
MIP-1α	85	88	98
MIP-1β	59	76	79
SIL-6ra	48	101	113
TGF-β	111	100	95
MMP-9	57	49	93
TREM-1	68	84	129
CRP	73	123	110
BDNF	22	54	58
NT-4	54	63	111

[a] Results are expressed as percentage of concentration detectable in 2-week-old DBSS not yet put into storage in the PKU-biobank. Notice that detected concentrations of some analytes are decreased after prolonged storage.

Skogstrand K *et al.* Clin Chem 2005; 51: 1854-66

Antigen-induced cytokine and chemokine release test for tuberculosis infection using adsorption of stimulated whole blood on filter paper and multiplex analysis by Skogstrand K *et al.*

Scand. J. Clin. Lab. Invest. Online publication 2012
DOI 10.3109/00365513.2011.649014

Table I. Concentrations measured in unstimulated plasma and DBSS from healthy controls. The values are medians (range).

	Plasma	DBSS
IL-8	11 (<4–15)	87 (15–130)
IL-18	18 (<10–307)	3109 (1953–3559)
RANTES ng/mL	20 (15–26)	102 (78–105)
MMP-9 ng/mL	213 (89–303)	519 (464–4561)
IFN-γ	<4 (<4–18)	33 (18–43)
GM-CSF	55 (38–77)	<10 (<10–56)
IL-2	12 (<4–87)	<4 (<4–<4)
sIL-6rα ng/mL	85 (73–111)	43 (37–44)
MIP-1α	2811 (478–3980)	404 (122–716)
IL-17	203 (<4–249)	121 (86–305)
IL-4	<4 (<4–<4)	11 (<4–17)
IL-5	<4 (<4–44)	<4 (<4–79)
IL-6	22 (9–32)	33 (13–48)
IL-10	60 (<4–246)	74 (<4–198)
IL-12	25 (24–40)	31 (12–81)
TNF-α	117 (<4–1316)	<4 (<4–59)
IL-1β	26 (9–34)	141 (65–261)
TNF-β	170 (28–349)	1303 (65–1598)
MCP-1	40 (<10–963)	293 (49–1537)
TGF-β	171 (102–310)	1397 (970–1547)
TREM-1	<488 (<488–1152)	<488 (<488–1285)

Whole blood was drawn from 5 healthy controls and put on ice. After all samples were drawn, plasma and DBSS was prepared and frozen. All concentrations are in pg/mL unless otherwise indicated. < indicates less than the lowest concentration in the working range.

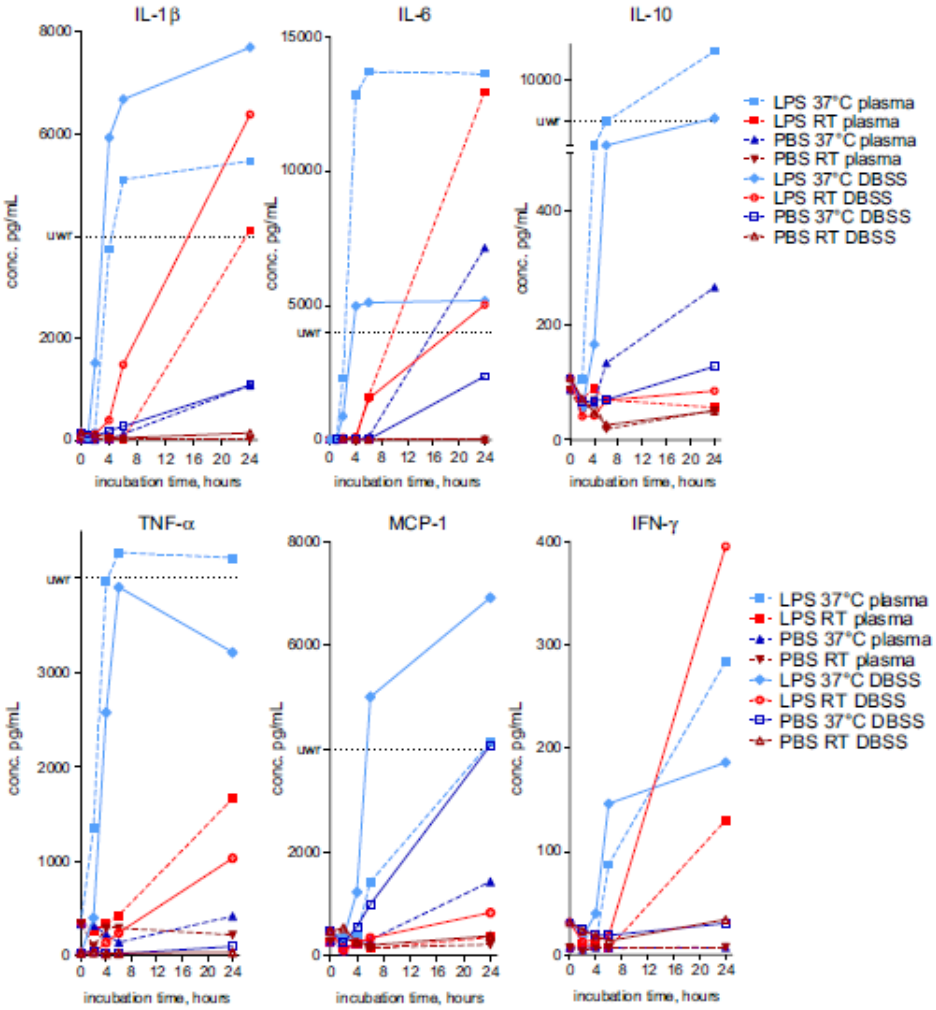

Figure 1. Selected inflammatory markers responses after stimulation with LPS and isotonic saline. The figure shows the mean concentration of selected inflammatory markers after whole blood incubation (blood from 5 healthy volunteers) with either LPS or isotonic saline at room temperature (RT) or 37°C for 5 min, 2, 4, 6 and 24 h before centrifugation (dotted lines) or spotting whole blood on filter paper (continuous lines). Uwr, upper working range.

SUMMARY

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- CSF samples are prone to post-sampling artifacts. On the protein level oxidation may be monitored by high-resolution MS
- Urine requires removal of salt for urine proteome analysis in kidney disease studies
- Plasma polypeptides are sensitive indicators of storage time and postsampling differences
- Circulating microparticles are a promising subproteome of blood
- Circulating micro-RNA are robustly analyzed but do not show good concordance between serum and plasma values. More data on normal range, intra- and interindividual variability, ethnicity etc. needed
- It is possible to perform comprehensive genetic analyses on DBSS material using a fraction of a 3.2 mm punch
- Whole genome-amplified DNA from neonatal DBSS is well suited for different genotyping methods, including chips.
- Storage of DBSS for 25 years at -20^{o}C does not affect the quality of wgaDNA
- DBSS may also be used for quantitative RNA microarrays detecting up to 3000 genes and quantitative DNA methylation analysis
- DBSS material can also be used for multiplex immunoassays after extraction of punch in buffer