PSTC Japan Safety Biomarker Conference April 18, 2019

Total Laboratory Harmonization for Precision Laboratory Medicine

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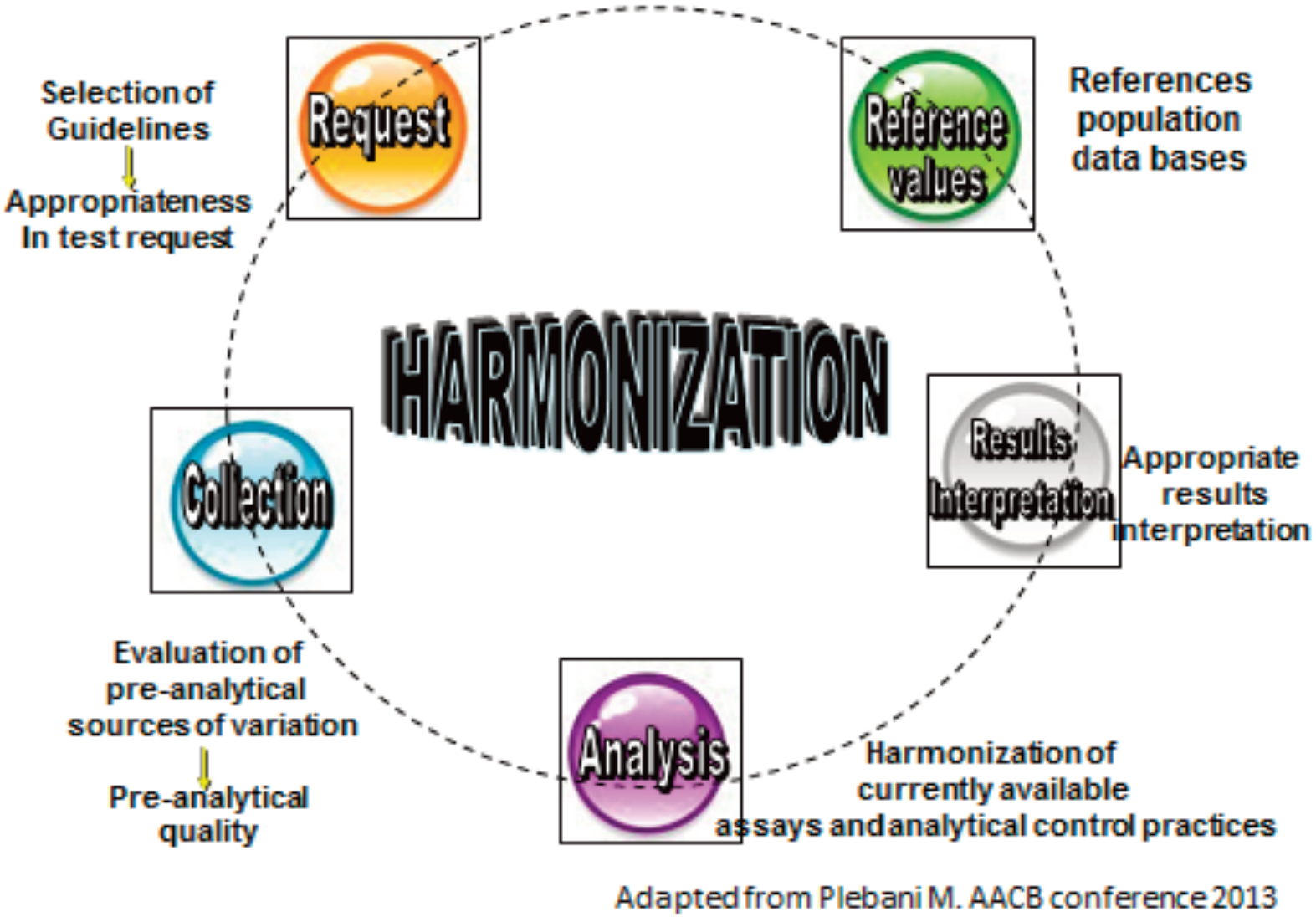
Laboratory medicine:
A hidden treasure in health care

94%	objective data in medical records
60-70%	clinical decisions influenced
37%	of practice guidelines
23 %	different disease areas & growing number of companion diagnostics

Sources: IMS Report 2003, www.VDGH.de / Forsman, R.W. (2002) ***Clin. Leadersh. Manag. Rev., 16, 370 / Forsman, R.W.***(2000) *Clin. Leadersh. Manag. Rev.,* ***14, 292 / Gibler et al. 1992, Annals of Emergency Medicine, 21, 504 / Herrmann et al., 2001*** Med. Klinik, 144 / Clinica 19.7. + 13.9.2002, 11.04.2002

IFCC HP から

Laboratory total testing procedure that enables harmonisation



When laboratory errors are happened?

- Preanalytical errors still account for nearly **60%–70%** of all mistakes occurring in laboratory diagnostics, most of them attributable to mishandling during collection, handling and preparing the specimens for testing.

Pre-analytical errors

1. Missing sample and/or test request
2. Wrong/missing identification
3. In vitro haemolysis
4. Undue clotting
5. Wrong container
6. Contamination from infusion route
7. Insufficient sample
8. Inappropriate blood to anticoagulant ratio
9. Insufficient mixing of the sample
10. Inappropriate transport and storage conditions
11. Inappropriate centrifugation conditions

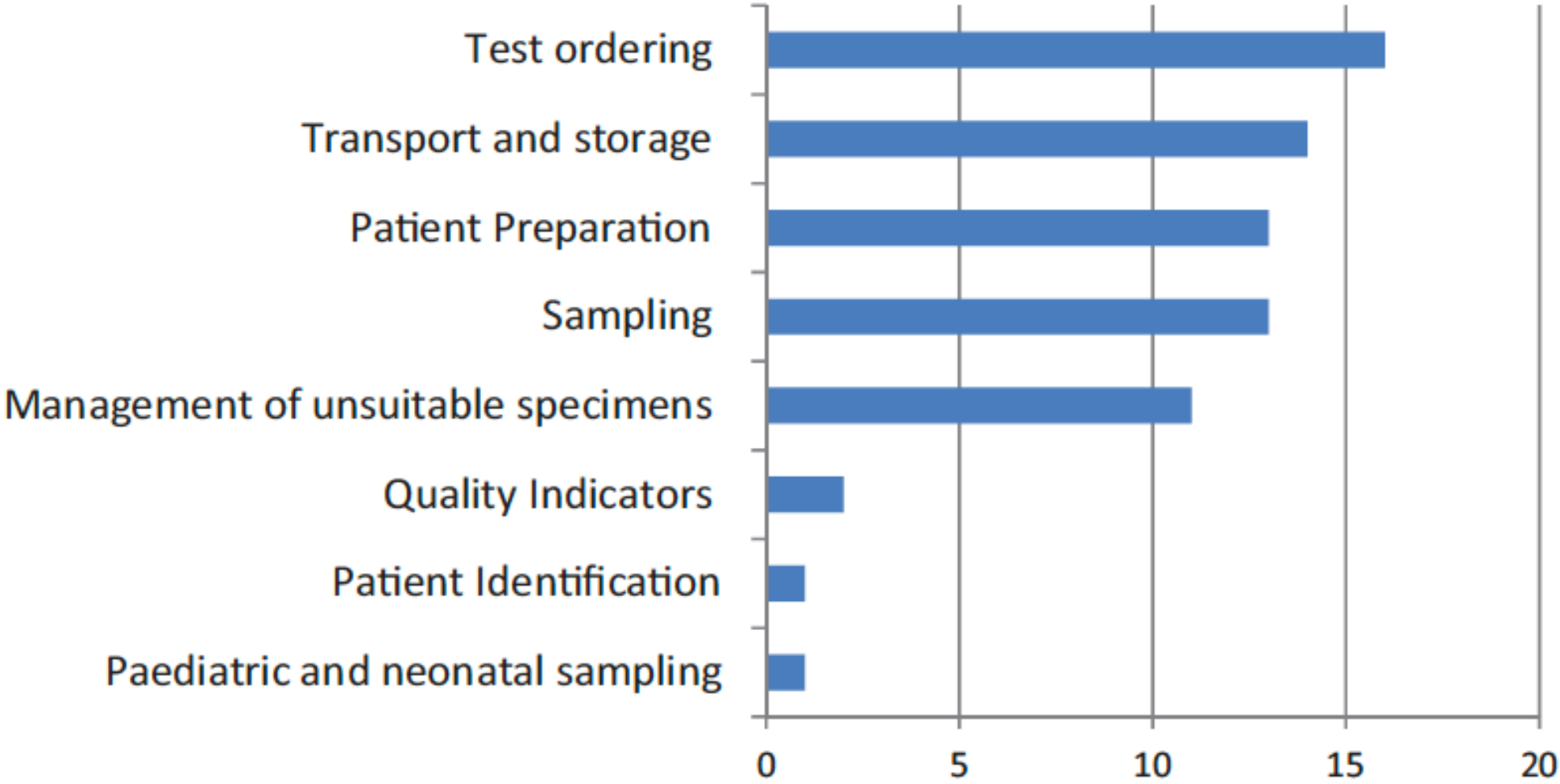
Lippi G, et al. Preanalytical quality improvement: from dream to reality. CCLM 2011

Sample problems

Type of sample error	Total (%)	Outpatients (%)	Inpatients (%)	
			Routine	Emergency
Hemolyzed	49.7	4.7	43.7	51.6
Clotted	9.1	1.8	53.1	43.1
Icteric/lipemic	2.0	4.0	96.0	0
Incorrect filling level	7.3	0	42.9	57.1
Incorrect	3.8	8.5	78.7	12.8
Inadequate quantity	24.3	14.8	84.3	1.0
Lost/not received	3.5	9.1	90.9	0

Plebani M, et al. Quality indicators to detect pre-analytical errors in laboratory testing. CCA 432 (2014) 44–48

Key preanalytical steps identified by EFLM NS **as the most critical and in need of immediate harmonization**



Cornes MP, et al. Ann Clin Biochem 2016

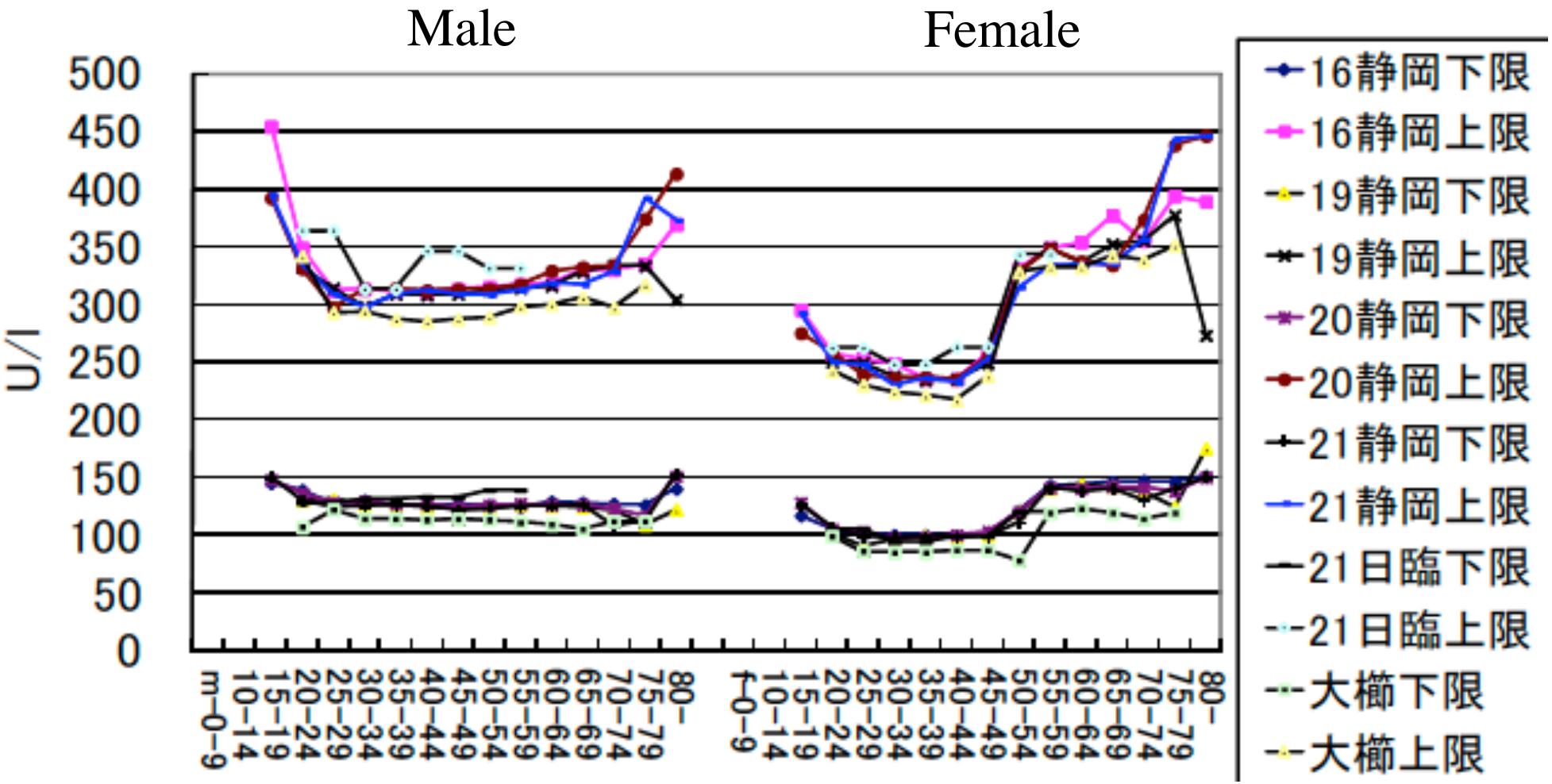
Variation factors of laboratory data

1. Physiological variation (Inter-individual and intra-individual)
2. Specimen sampling, handling and storage
3. Patients' conditions
4. Measurement procedure

1. Physiological variation

- Age and gender
- Life style
- Circadian rhythm
  - Serum iron is higher in the morning and lower in the evening. It is dramatically flucturated.
  - Serum inorganic phosphorus is lower in the morning.
- Daily and seasonal variation, menstrual cycle
- Diet: blood glucose, triglyceride, insulin, etc.
- Exercise: skeletal muscle injury (CK), leukocytes
- Posture
  - High molecular weight molecules: standing > sitting > spine

Reference Interval of Alkaline Phosphatase（ALP）



By health check-up in Shizuoka prefecture

http://www.shizuoka.med.or.jp/documents/240208.pdf

Example of the Biological Variation (BV)

Testing items	Within-subject BV	Between-subject BV	Reference change value
AFP	12.2	45.6	0.27
CA19-9	16.0	130.5	0.12
CEA	12.7	55.6	0.23
CA15-3	6.1	62.9	0.10
CA125	24.7	54.6	0.45
ALP	6.45	26.1	0.25
TC	5.95	15.3	0.39
Na	0.6	0.7	0.86

https://www.westgard.com/biodatabase1.htm#11

Adequate Laboratory Conditions

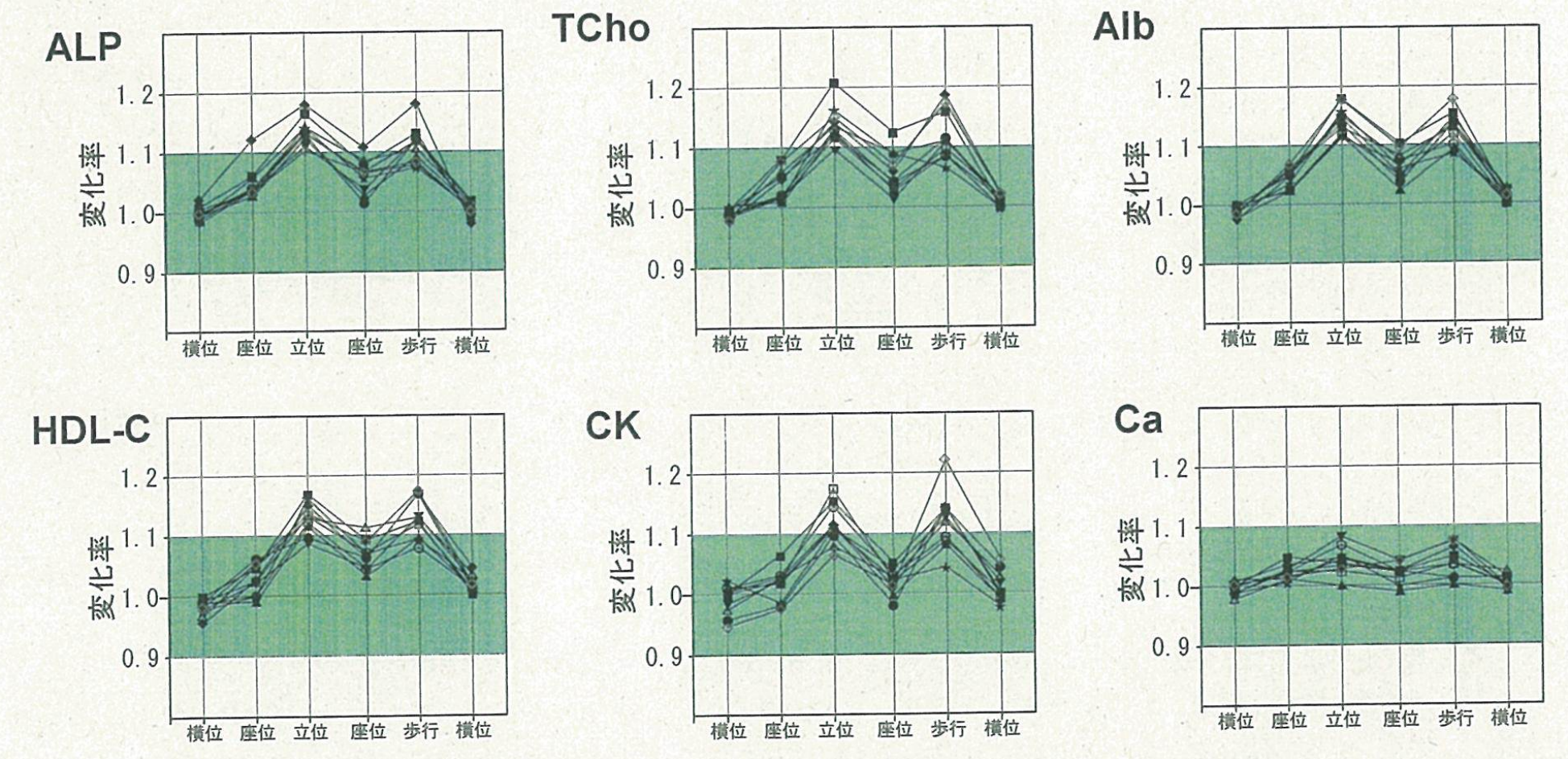
- Patients' conditions (including medication)
- Physiological variation and circadian rhythm
  - Medication
  - Diet, exercise, smoking, alcohol drinking
  - Circadian rhythm, seasonal variation
  - Age and gender
  - Posture

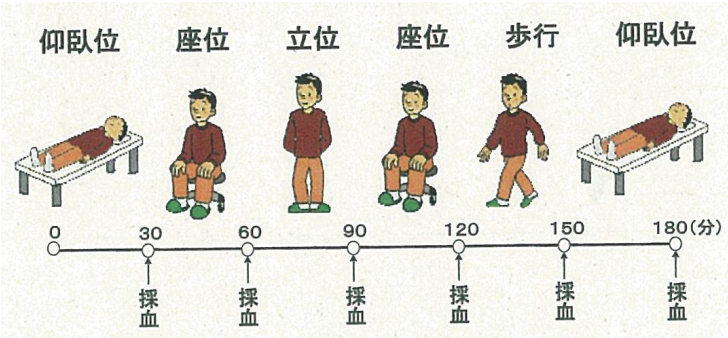
Individual variations can be diminished by adequate sampling conditions all times, and thus petit abnormalities can be detected earlier.

Recommendation: Precision laboratory medicine needs individual reference interval

- Not reference interval from groups, from the second examination
  - but needs harmonized results
- Use individual reference interval
  - it is useful for small reference change value (RCV)

Posture variation of laboratory testings

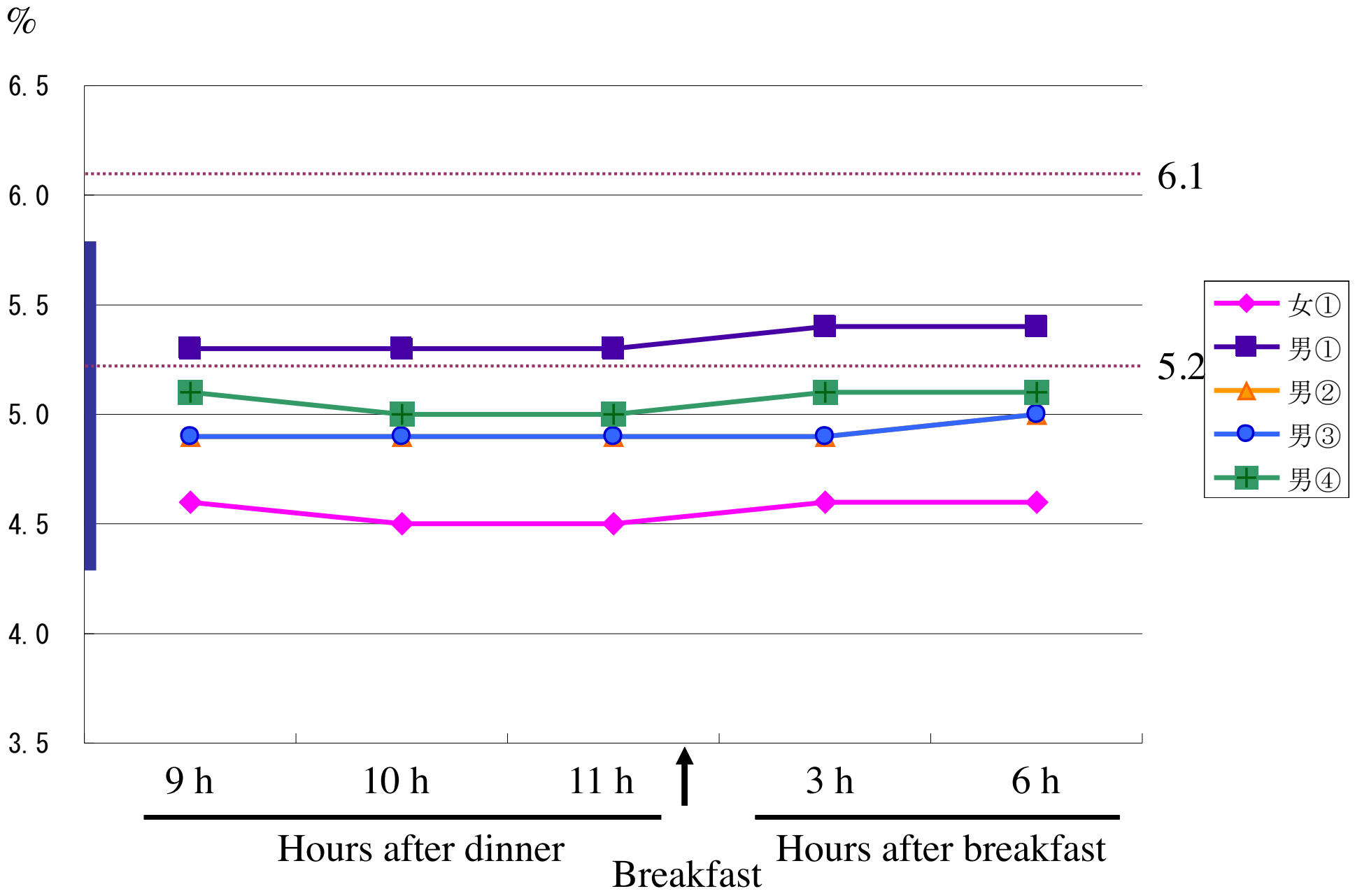




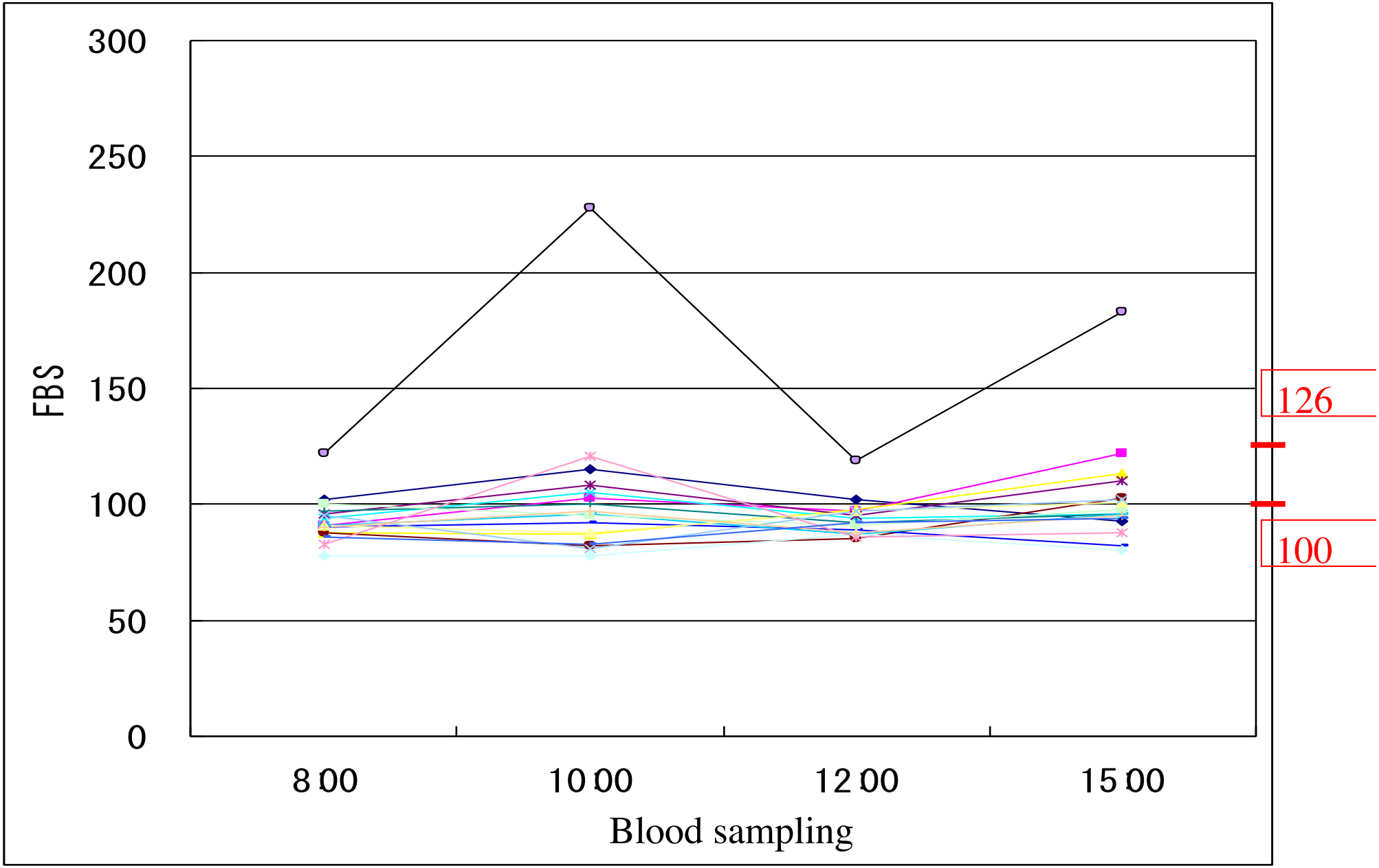
Standing > Sitting > Spine posture

Ichihara and Kohguchi (edited): Laboratory Diagnosis Manual. 2011

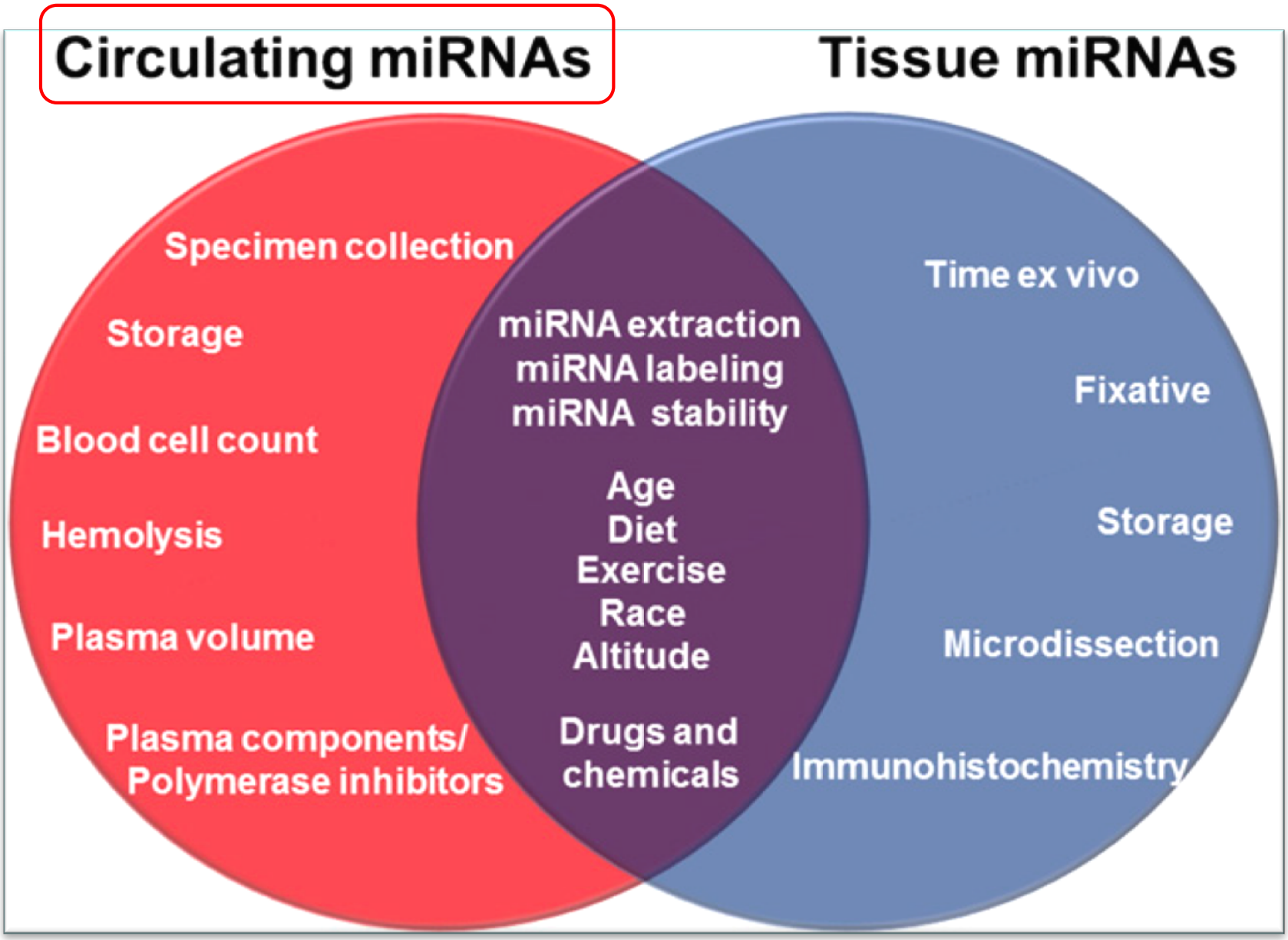
Dietary interference on HbA1c



Dietary interference on blood glucose

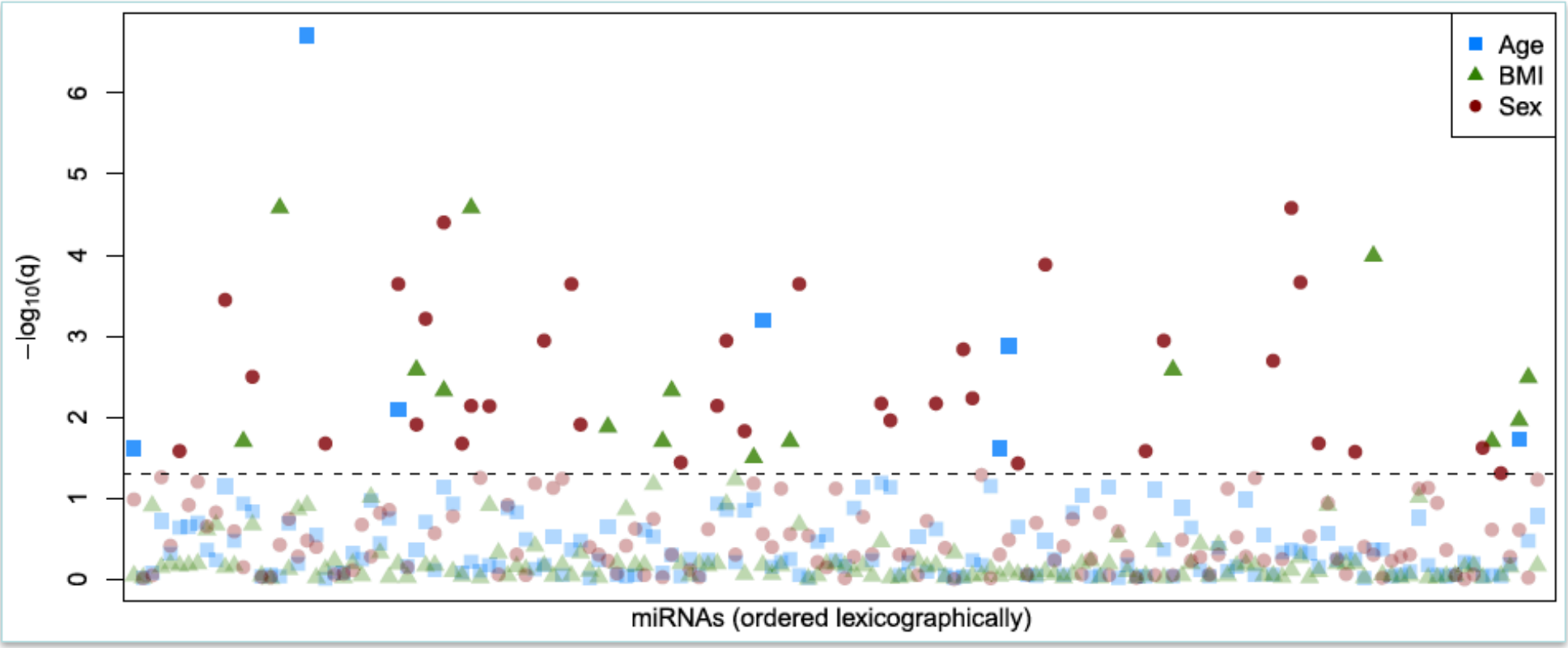


Preanalytical variables in miRNA analysis



N. Becker, C.M. Lockwood / Clinical Biochemistry 46 (2013) 861–868

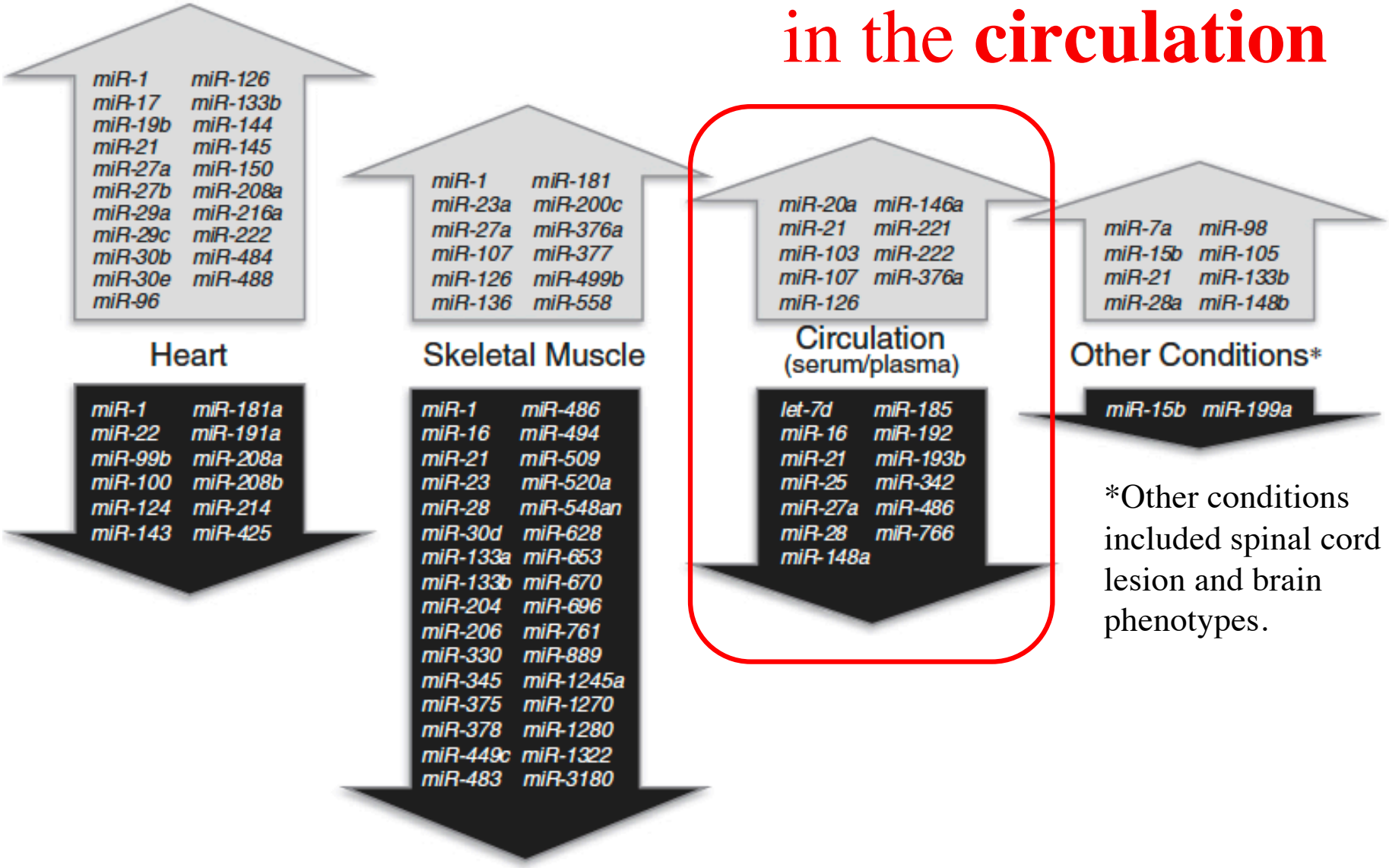
Associations of miRNAs with age, BMI and sex



Association q-values of miRNAs in two-step regression models with adjustment for technical and biological parameters. The -log10(q) values of the linear regression analysis of miRNA levels and phenotypes age (blue rectangle), BMI (green triangle) and sex (red circle) are depicted. Q-values were obtained via Benjamini-Hochberg (BH) multiple testing correction of raw p-values. The dotted line marks the significance threshold of q = 0.05. Plasma miRNAs are lexicographically arranged on the x-axis (though not labelled individually)

Ameling et al. Associations of circulating plasma microRNAs with age, body mass index and sex in a population-based study. BMC Medical Genomics (2015) 8:61

miRNAs up and down regulated by **exercise training** in different tissues and in the **circulation**



*Other conditions included spinal cord lesion and brain phenotypes.

MicroRNAs as Important Regulators of Exercise Adaptation. Prog Cardiovascular Diseases 2017; 60: 130-151.

2. Specimen sampling, handling and storage

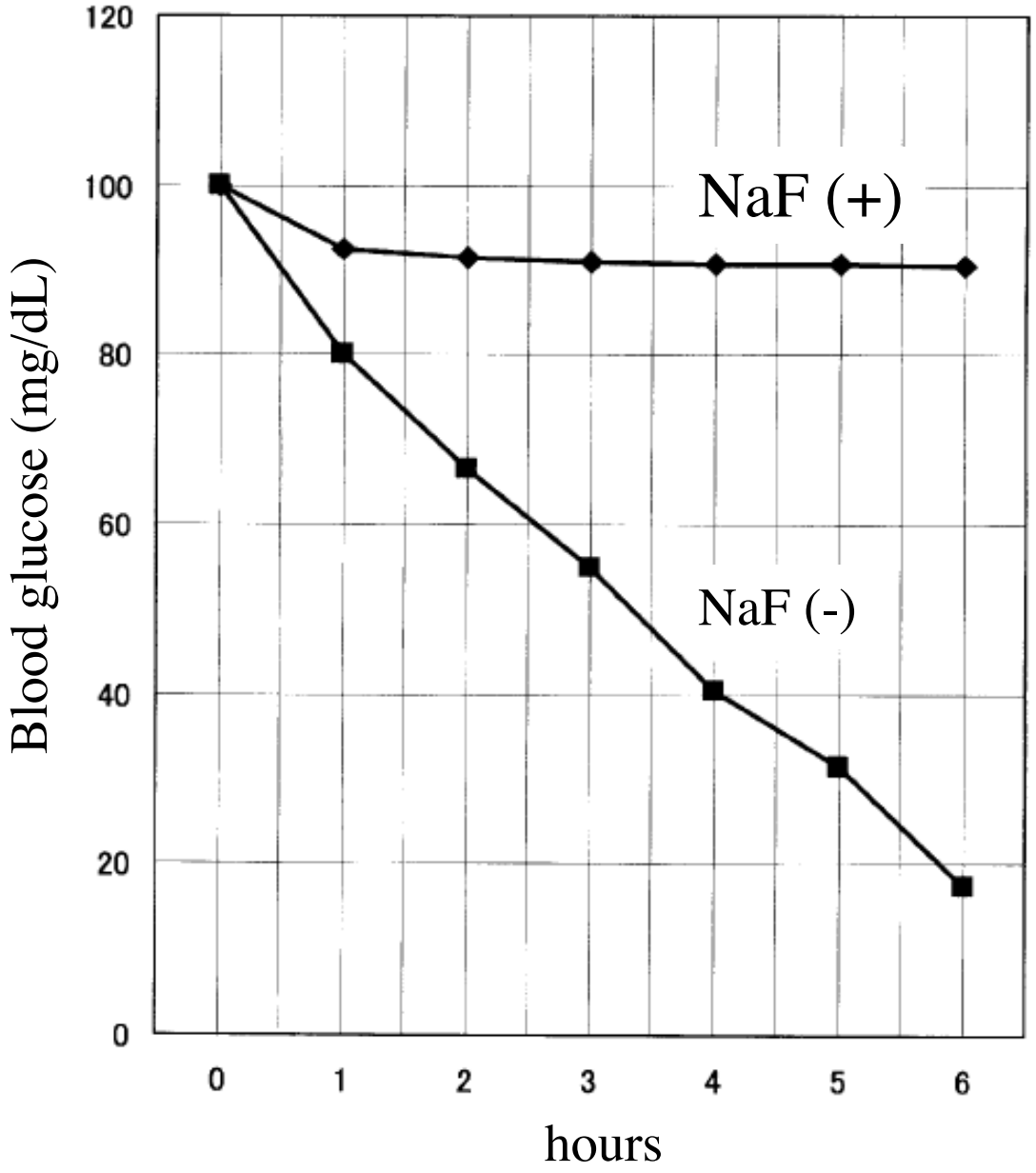
- Specimen
  - Whole blood, plasma, serum
  - RBC (hemolysis), WBC, Plt, anti-coagulant
  - Other biological fluid
- Stability
- Infusion, transfusion, medication
- Incorporation of tissue fluid

- Patient identification
- Transportation (temperature, time, vibration)
- Centrifugation (temperature, speed and gravity, time)
- Deposit and storage (temperature, time and period, tube, condition, freeze and thaw counts, etc.)

Anti-coagulants and their purpose

Anti-coagulant and others	Purpose
heparin	Blood gas
Sodium fluoride	Blood glucose
EDTA-2K	Blood counts
EDTA-2Na	Renin, ACTH
Sodium citrate	Coagulation, ESR

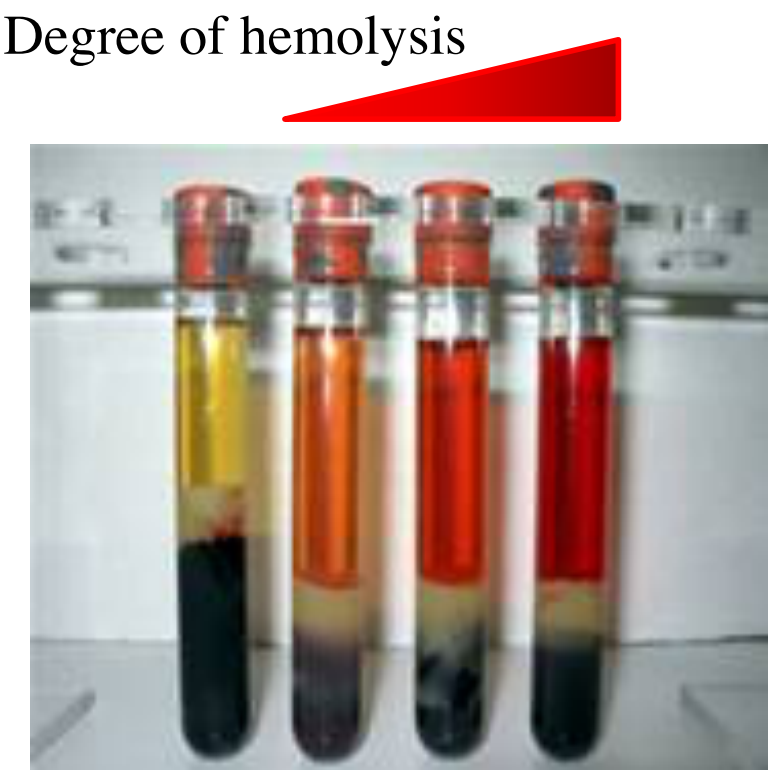
Effect of NaF on blood glucose



Hemolysis

- Molecules rich in red blood cells strongly affect laboratory data.

Test	RBC／plasma
LD	160
K	23
AST	20

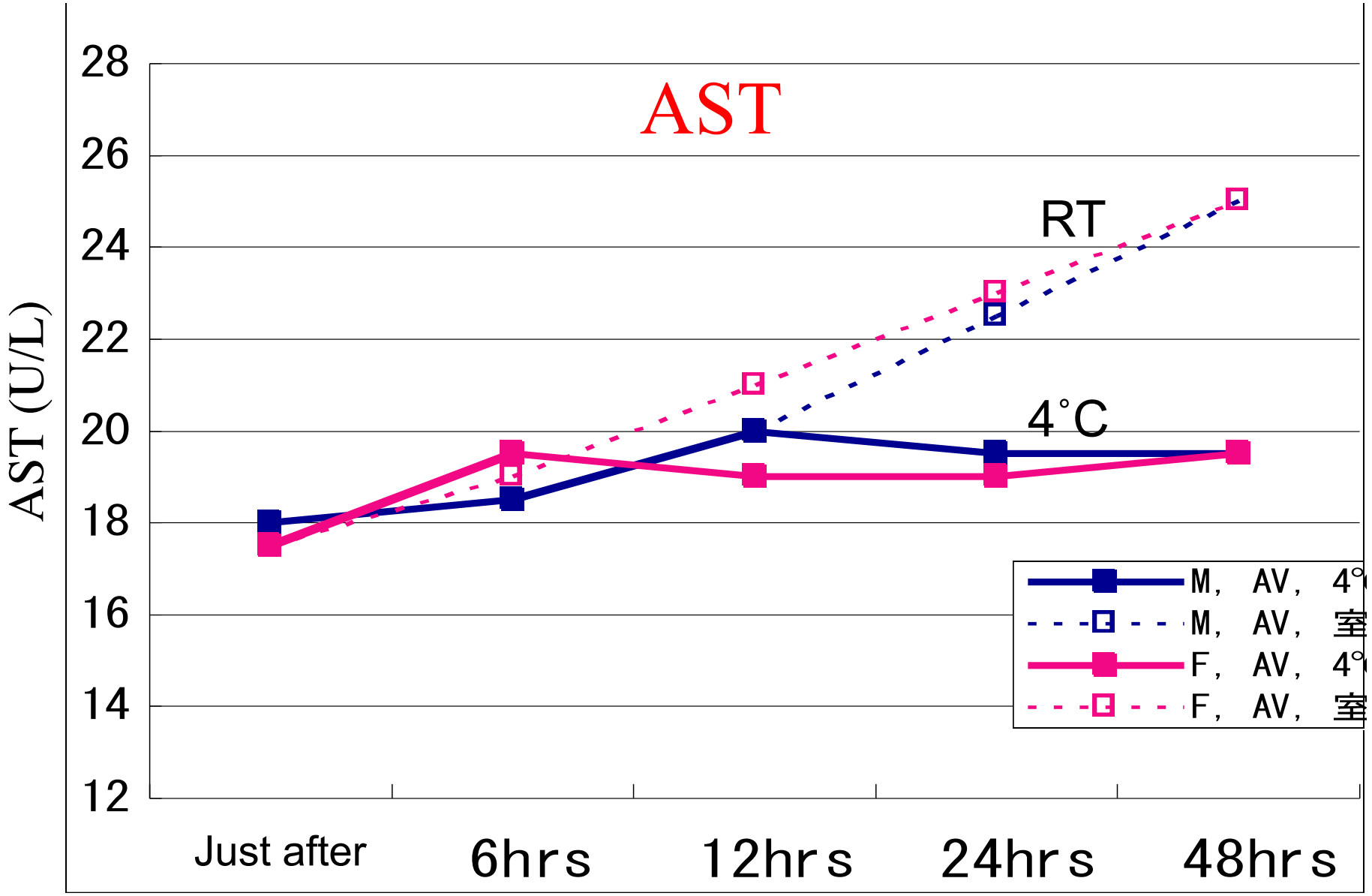


What happened?

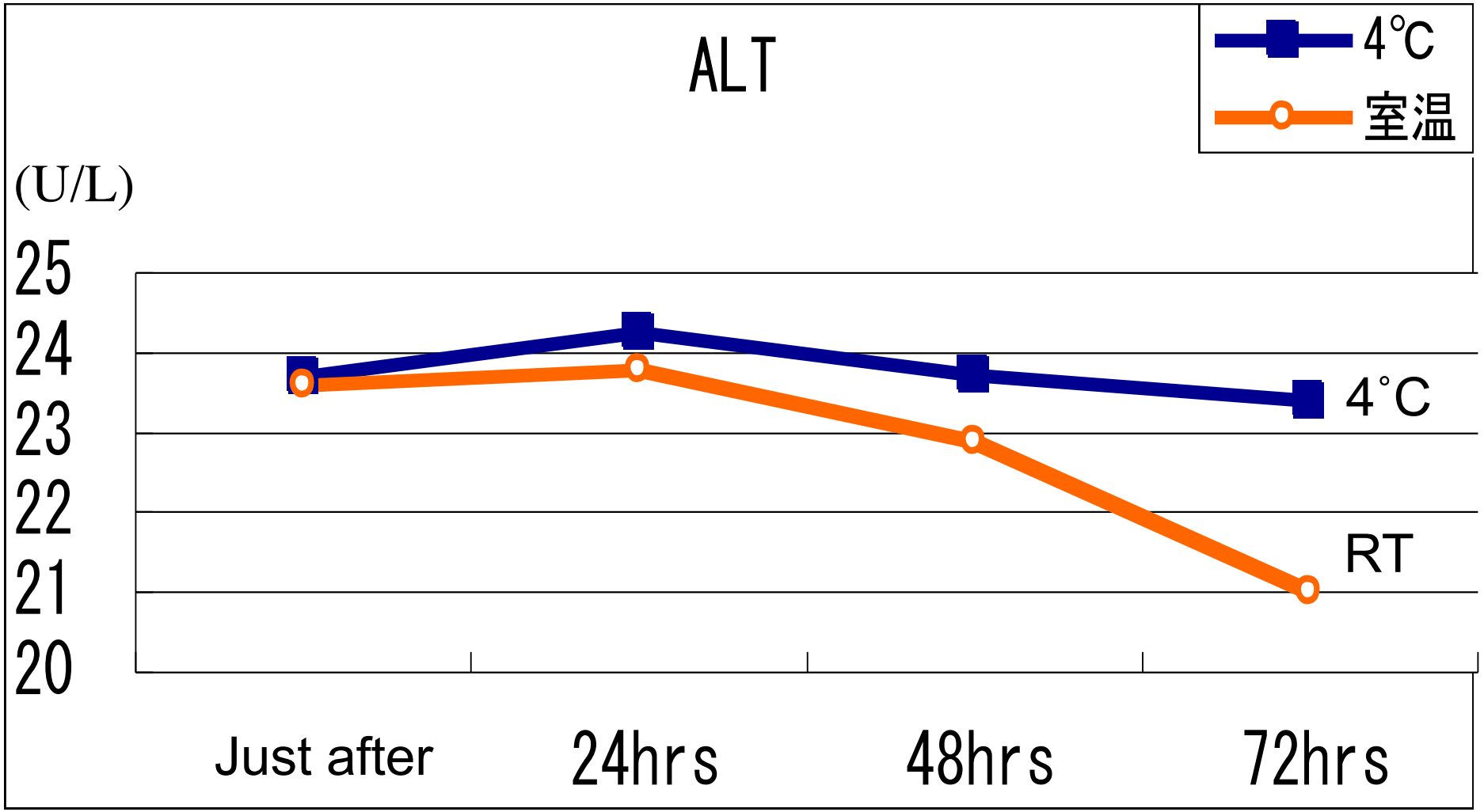
	Previous	Present	Re-sampling
Na	126	**107**	127
K	5.0	**4.1**	4.5
Cl	95	**80**	97
Glucose	199	**667**	227

Contamination of infusion fluid containing glucose might lead to misdiagnosis and unnecessary therapy.

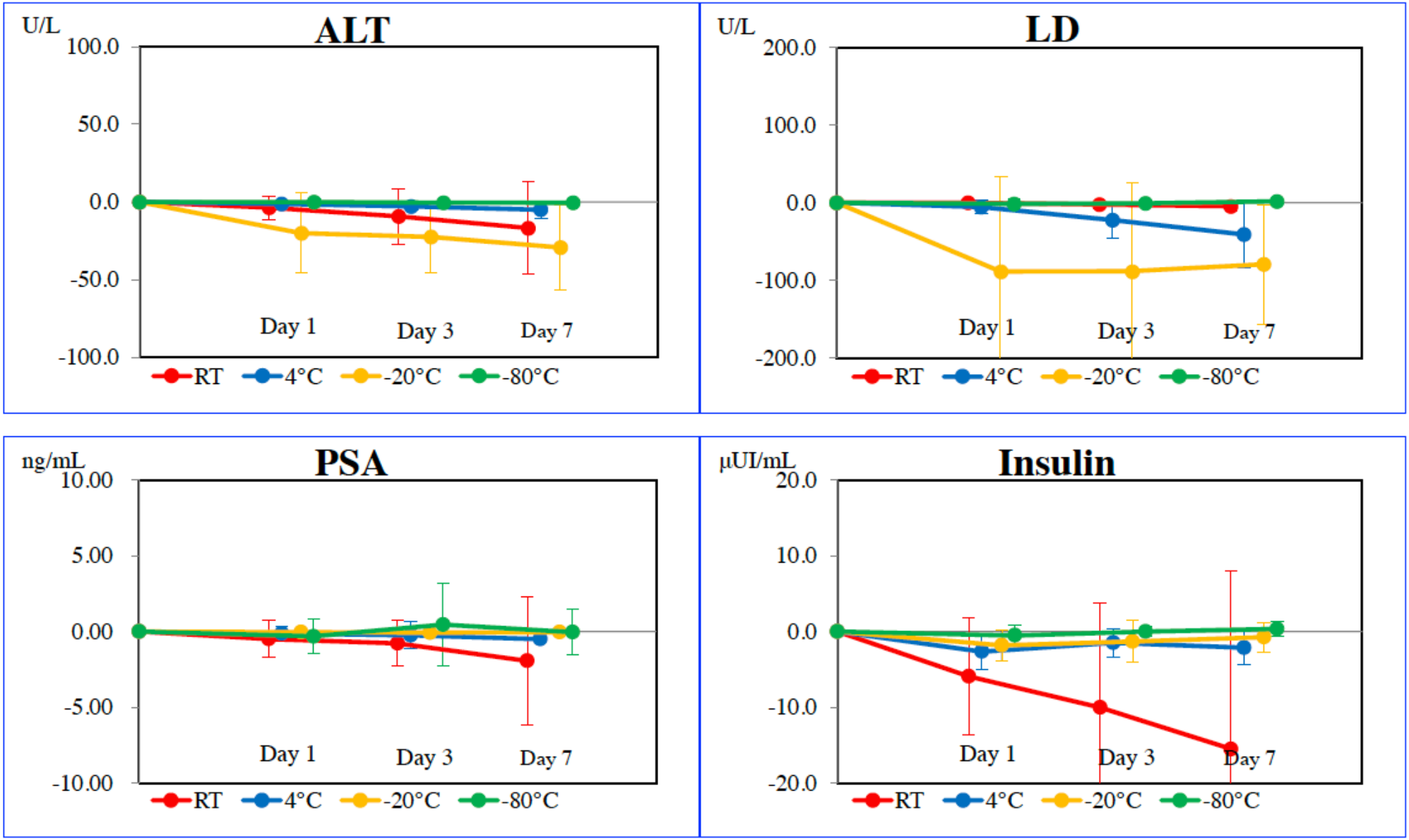
Effect of time from blood sampling to centrifugation at 4˚C and room temperature



Effect of time from centrifugation to analysis at 4˚C and room temperature



Storage stability on different temperatures



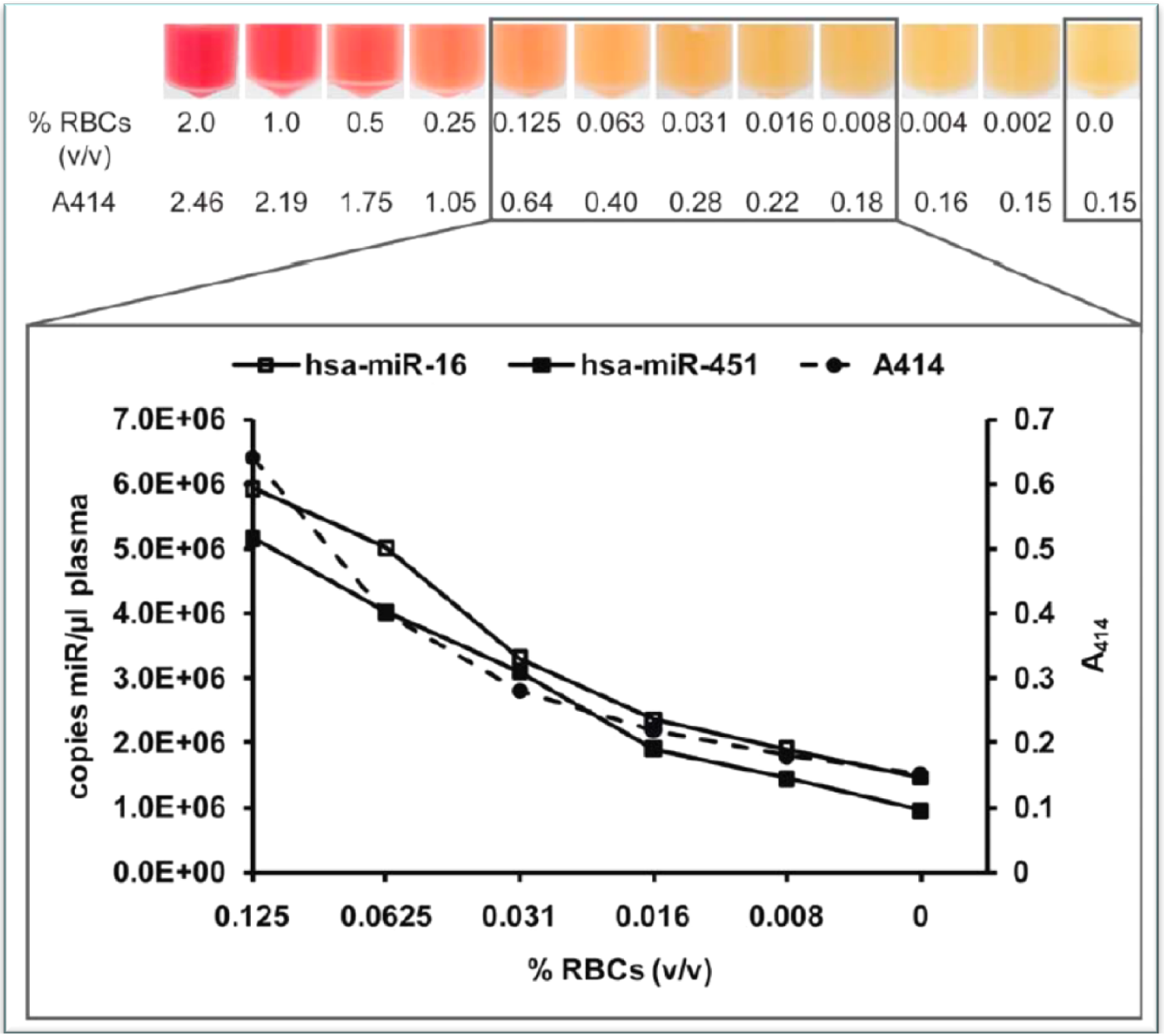
Ikeda K: Specimen stability. Guideline of clinical laboratory testings. 2015

Sample matrix effect on circulating miRNAs determination

Sample matrix	miRNA behavior	Reference
Plasma-EDTA	Best anticoagulant for PCR-based (miRNA) profiling	[84]
	Higher levels of miR-223 compared to the other matrices	[85]
Plasma-heparin	Interferes with enzyme activity in PCR-based assays	[14]
	Lowest levels of both miR-16 and miR-223	[85]
Plasma NaF/KOx	Suitable alternative to EDTA, but it can determine increased miRNA detection compared to the other matrices	[85]
	Higher levels of miR-223 compared to the other matrices	[85]
	When NaF/KOx are added to frozen samples, levels of miR-16 doubled in EDTA-plasma, and tripled in serum	[85]
Plasma-citrate	Interferes with enzyme activity in PCR-based assays	[14]
Serum	Stable and reproducible	[17]
	Higher variability of miR-16 and miR-223 compared to the other matrices	[85]

Review: G. Lombardi et al. / Clinical Biochemistry 49 (2016) 1331–1339

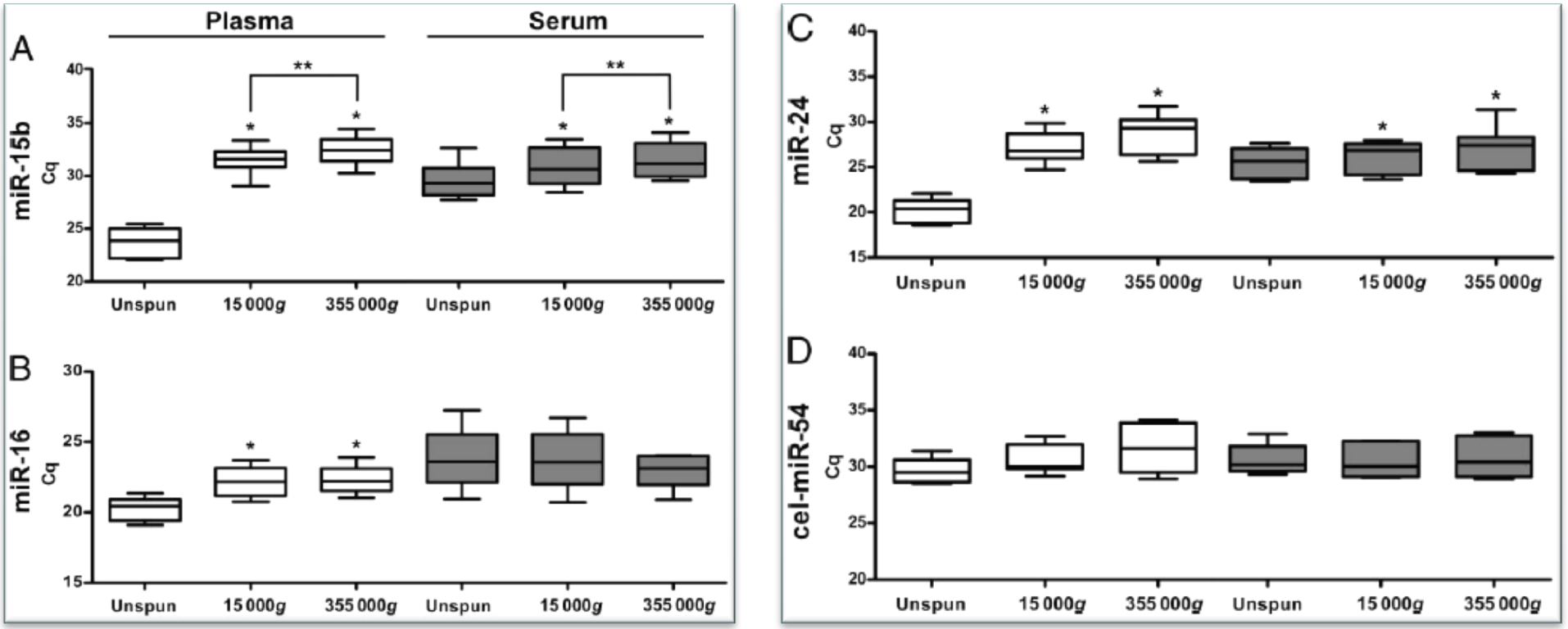
Relationship between free **haemoglobin** (Hb) and miRNA content of plasma samples



A dilution series of lysed RBCs in plasma (top) was prepared and Hb content measured by absorbance at 414 nm [33]. RNA was isolated from the samples indicated by the box and levels of miR-16 and miR-451 were quantified using a standard curve. While a change in plasma colour is only clearly visible from a RBC concentration of 0.125% (v/v) the amount of free Hb as well as this of both miR-451 and miR-16 already substantially increased at a RBC concentration of 0.031% (v/v).

Kirschner MB, et al. PLoS ONE 6: e24145, 2011

miRNA concentrations in serum and plasma after centrifugation



Paired plasma (left 3 columns) and serum (right 3 columns) samples were obtained from 10 healthy individuals. We spiked 400 L of the uncentrifuged (Unspun) and 2 supernatant aliquots with C. elegans cel-miR-54, extracted the RNA, and analyzed the samples for miR-15b (A), miR-16 (B), miR-24 (C), and cel-miR-54 (D). The resulting miRNA concentrations are reported as raw Cq values. The boxes represent the 25th and 75th percentiles; the horizontal line in each box represents the mean; the error bars indicate the range. Significant differences (P< 0.05, Wilcoxon signed rank test) from the unspun control (*) and significant differences between the 15 000g and 355 000g centrifugation steps (**) are indicated.

Analysis of Circulating MicroRNA: Preanalytical and Analytical Challenges. Clinical Chemistry 57:6 (2011)

MicroRNA Expression in Stepwise Processed Plasma Samples Using qRT-PCR Profiling

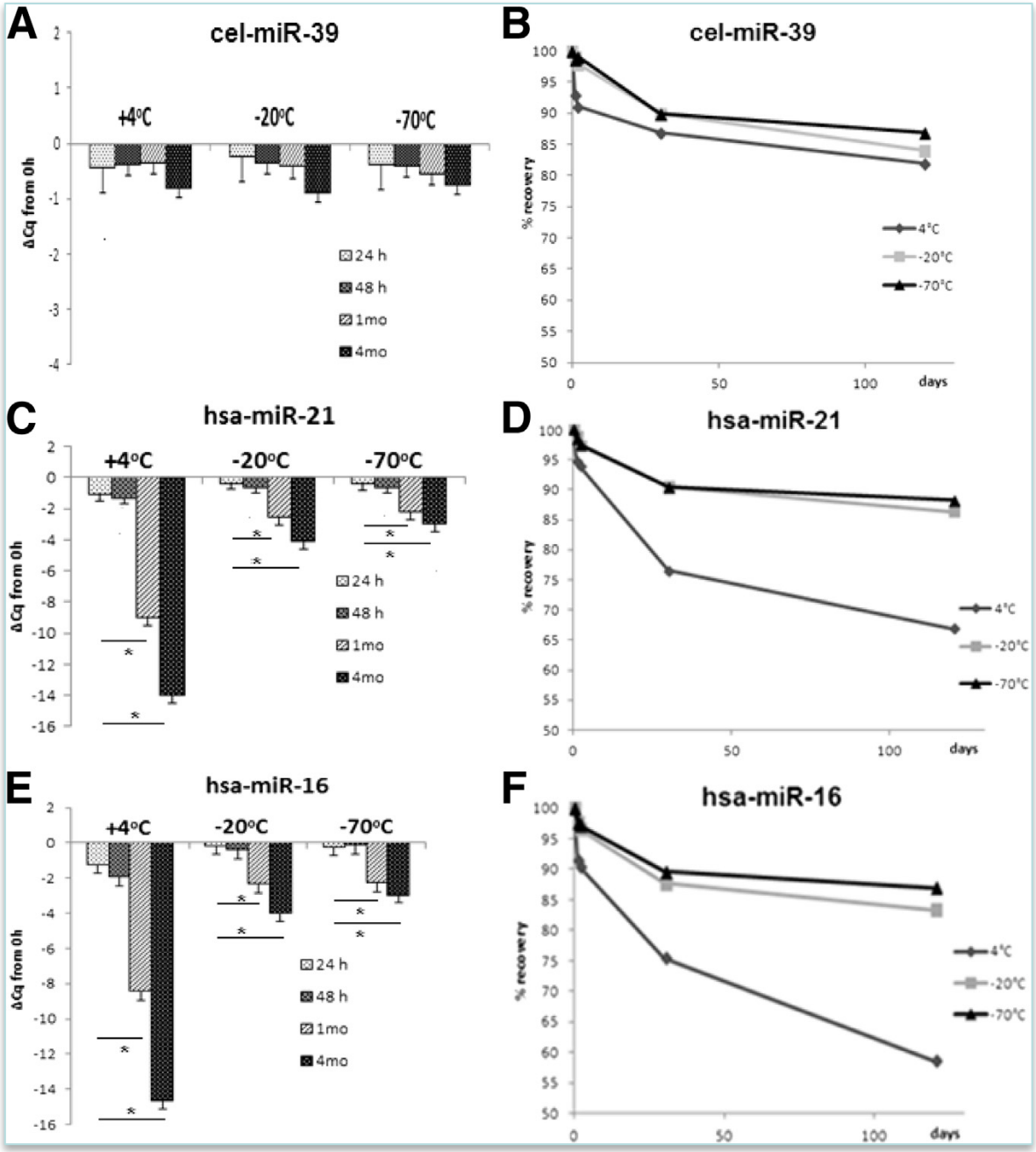
B

	sample	donor	miR-142-3p	let-7a	miR-223	miR-16	miR-451	miR-122
Normalized CT Difference from Mean	$Platelet_{CONC}$	1-1	-5.3	-4.1	-4.3	-2.7	0.1	1.4
		1-2	-5.7	-3.9	-3.9	-2.5	-0.1	1.7
		2-1	-5.0	-3.8	-3.8	-1.6	0.6	0.5
		2-2	-5.4	-4.1	-4.1	-2.3	-0.3	0.4
		3-1	-5.7	-5.6	-5.6	-2.4	0.5	0.2
		3-2	-6.3	-5.3	-5.3	-2.6	-0.3	0.0
	$Plasma_{RICH}$	1-1	-3.5	-3.2	-3.2	-1.3	0.5	0.9
		1-2	-3.4	-3.2	-3.2	-1.3	0.5	0.8
		2-1	-3.1	-3.5	-3.5	-1.2	-0.6	0.2
		2-2	-3.9	-3.6	-3.6	-1.1	0.2	0.3
		3-1	-4.6	-4.3	-4.3	-1.0	-0.2	0.2
		3-2	-4.3	-4.2	-4.2	-0.9	-0.3	0.3
	$Plasma_{STD}$	1-1	-0.2	-0.8	-0.8	0.5	-0.8	-1.3
		1-2	0.1	-0.5	-0.5	0.7	-0.4	-0.6
		2-1	0.7	0.4	0.4	0.5	-0.8	-0.5
		2-2	1.3	0.5	0.5	1.1	0.0	-0.2
		3-1	2.1	1.7	1.7	1.0	-0.5	0.0
		3-2	2.5	1.9	1.9	0.8	0.0	0.0
	$Plasma_{POOR}$	1-1	3.0	2.8	2.8	1.5	-0.2	-0.7
		1-2	2.7	2.3	2.3	1.1	-0.6	-1.1
		2-1	2.0	2.3	2.3	1.0	0.4	0.2
		2-2	2.9	2.5	2.5	1.2	0.1	-0.1
		3-1	2.9	3.7	3.7	1.0	0.1	0.0
		3-2	3.7	3.5	3.5	1.1	-0.3	-0.2
	$Plasma_{FILT}$	1-1	6.2	5.5	5.5	1.9	0.2	-0.5
		1-2	6.4	5.5	5.5	2.0	0.4	-0.6
		2-1	5.3	4.7	4.7	1.4	0.2	-0.2
		2-2	5.2	4.6	4.6	1.1	0.0	-0.5
		3-1	5.1	4.5	4.5	1.5	0.0	-0.2
		3-2	4.6	4.0	4.0	1.8	0.8	0.0
Average Fold Difference	$Plasma_{RICH}$ to $Plasma_{POOR}$		102	92	92	5	1	1
	$Plasma_{STD}$ to $Plasma_{FILT}$		21	19	19	2	2	1
	$Plasma_{POOR}$ to $Plasma_{FILT}$		6	4	4	1	1	1

Plasma Processing Conditions Substantially Influence Circulating microRNA Biomarker Levels.
PLoS ONE 2013; 8(6): e64795.

Stability of miRNAs in plasma at different storage time and temperature

Stability study of circulating miRNAs in **plasma** at different storage time and temperature conditions. A and B: cel-miR-39. C and D: hsa-miR-21. E and F: hsa-miR-16. The results are presented as differences in raw Cq values from the 0-hour control (0h) (positive differences represent decreases and negative differences represent increases in miRNA concentrations) and as percentage recoveries from the 0h for each miRNA. The data represent the means SD of three independent experiments. Statistical evaluation was based on the U-test. *P<0.05.

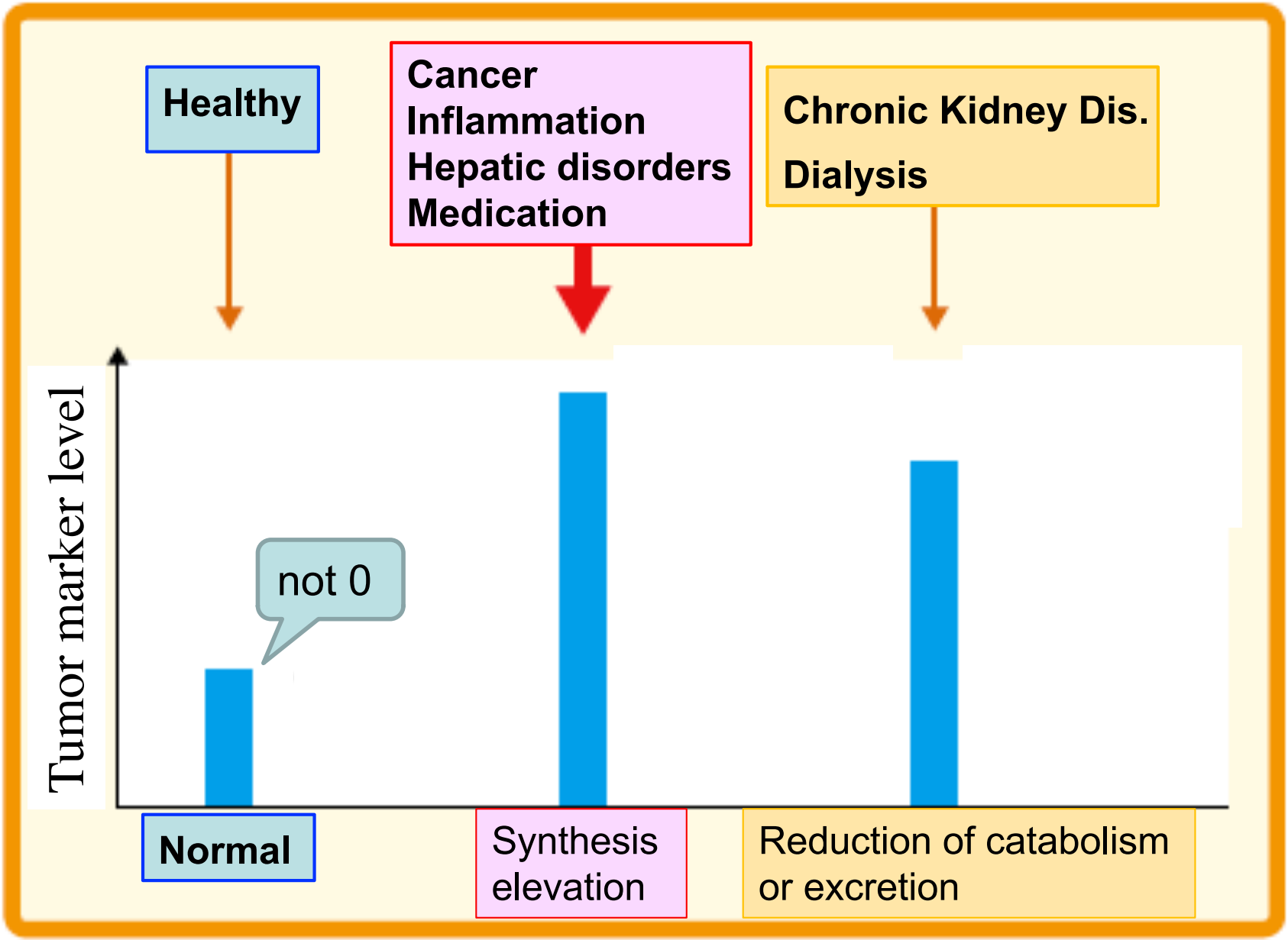


Quantification of Circulating miRNAs in Plasma Effect of Preanalytical and Analytical Parameters on Their Isolation and Stability. J Mol Diag 15(6), 2013

3． Patients' conditions

- Pathological states
- Dehydration and dilution
  - Malnutrition is often associated with dehydration.
  - Large molecules might be concentrated.
- Anabolism and catabolism, imput and output
  - Serum albumin concentration decreases not only by synthesis reduction due to hepatic dysfunction, but also excretion elevation from kidney, digestive tract and skin.

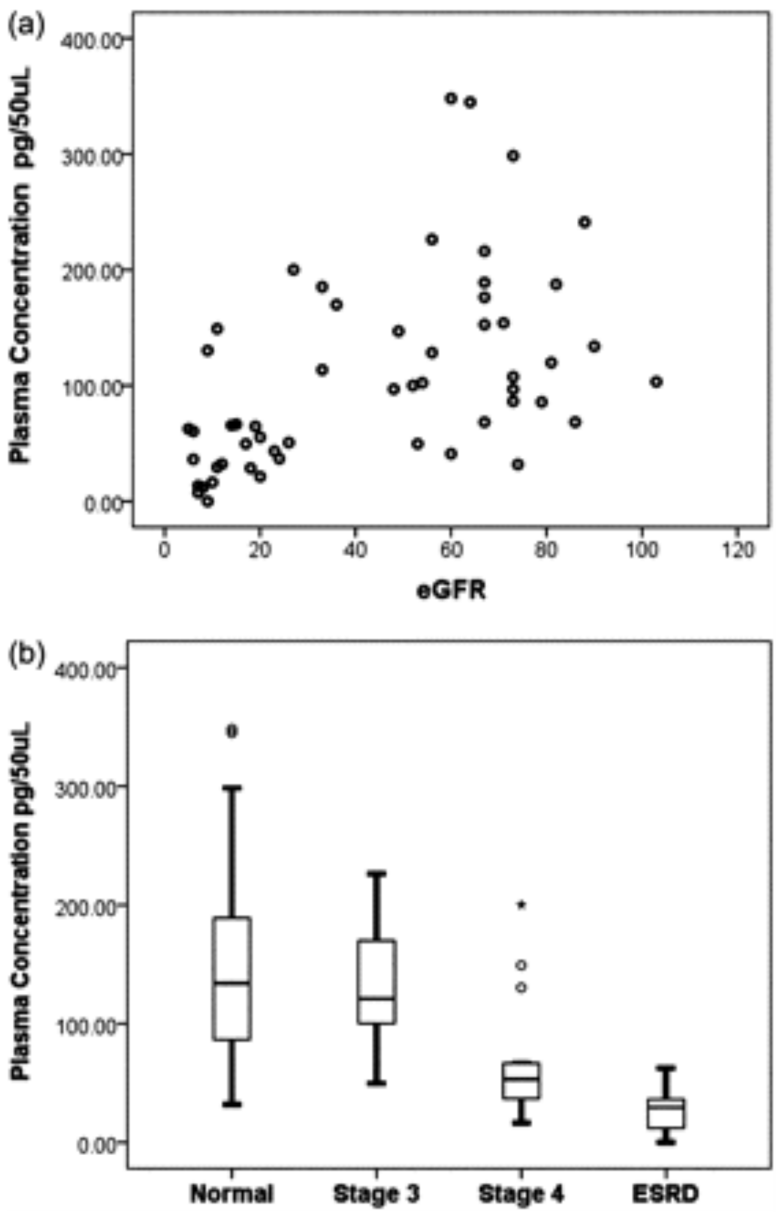
Possible mechanism of elevation of tumor marker levels in sera

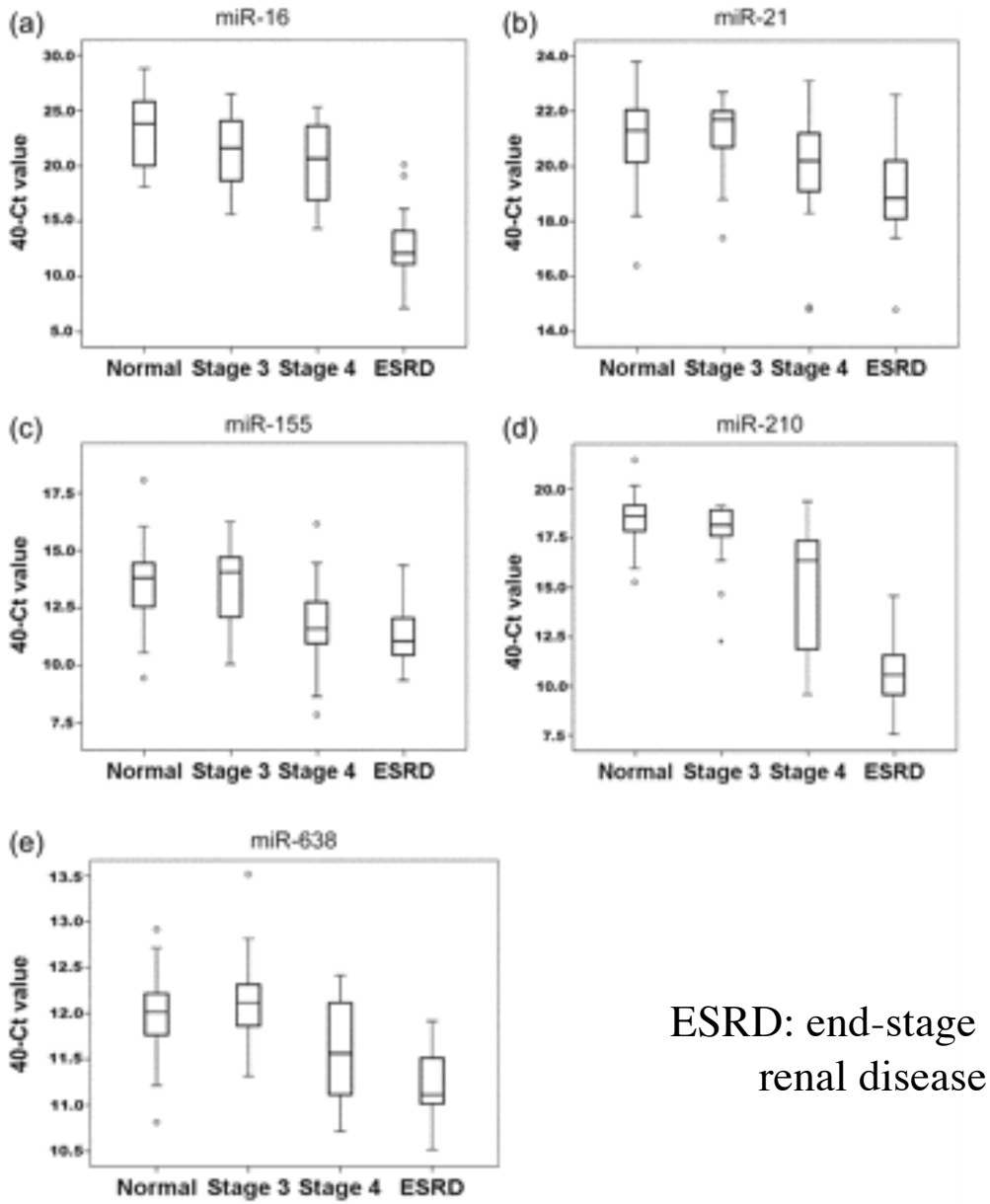


Cut-off values of tumor marker in dialysis patients

Test	MW	Correction of cut-off value	Related cancers
CEA	180,000	twice	colorectal, gastric, lung, breast
CA19-9	> 3,000,000 (cancer)	twice	pancreas, colorectal
ProGRP	10,000	2 - 3 fold	lung small cell
SCC	46,000	2.5 – 3 fold	uterine cervix, lung
PSA	34,000	-	prostate
AFP	65,000	-	HCC, germ cell
CA15-3	90,000	-	breast

Total and some miRNAs are decreased in CKD





ESRD: end-stage renal disease

Circulating microRNA expression is reduced in chronic kidney disease. Nephrol Dial Transplant. 2011;26(11):3794-3802.

4. Measurement procedure

✓ *Not* standardized

✓ *Not* harmonized

Each immunoassay reagent is different from each other (antibody, labeling, buffer, time, calibrator, standard material).

Harmonization and standardization

- Harmonization
  - Equivalent results among different measurement procedures for the same laboratory test
- Standardization
  - Equivalent results are achieved by metrological traceability to a fit-for-purpose higher order reference system

- Equivalent
  - Equivalent does not mean identical
  - Equivalent means within a total allowable error consistent with an acceptable risk of harm from decisions based on a lab test result

Greg Miller, IFCC-GC in 2018

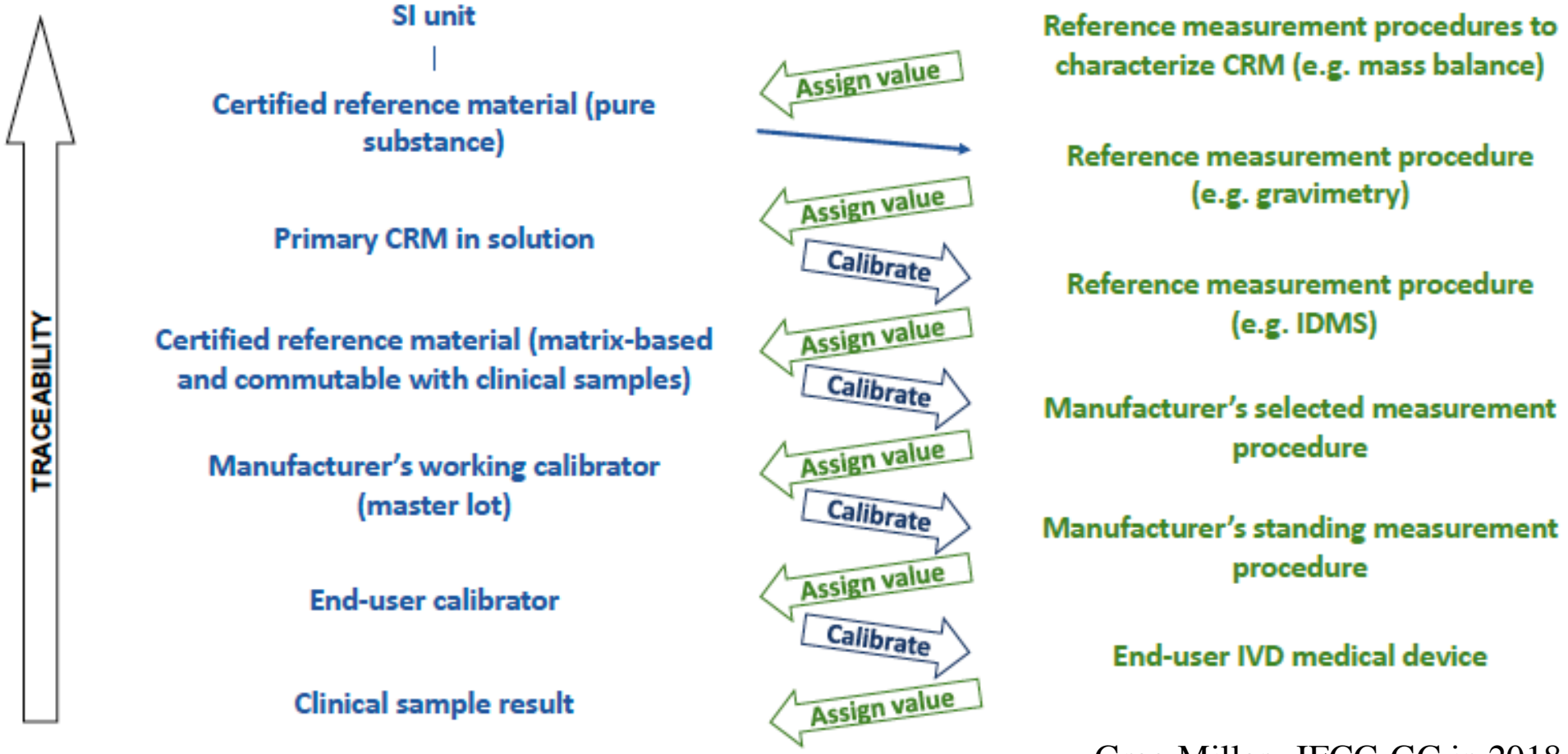
Traceability class by ISO 17511

Class	RMP	Primary RM (pure substance)	Secondary RM (valued substance)	Examples
1	+	+ traceable to SI unit	+	Electrolyte, Glu, cortisol
2	+	-	+	Enzyme
3	+	-	-	Coagulation factor
4	-	-	+	Protein, TM, HIV
5	-	-	-	Protein, EBV, VZV

Standardization

Harmonization

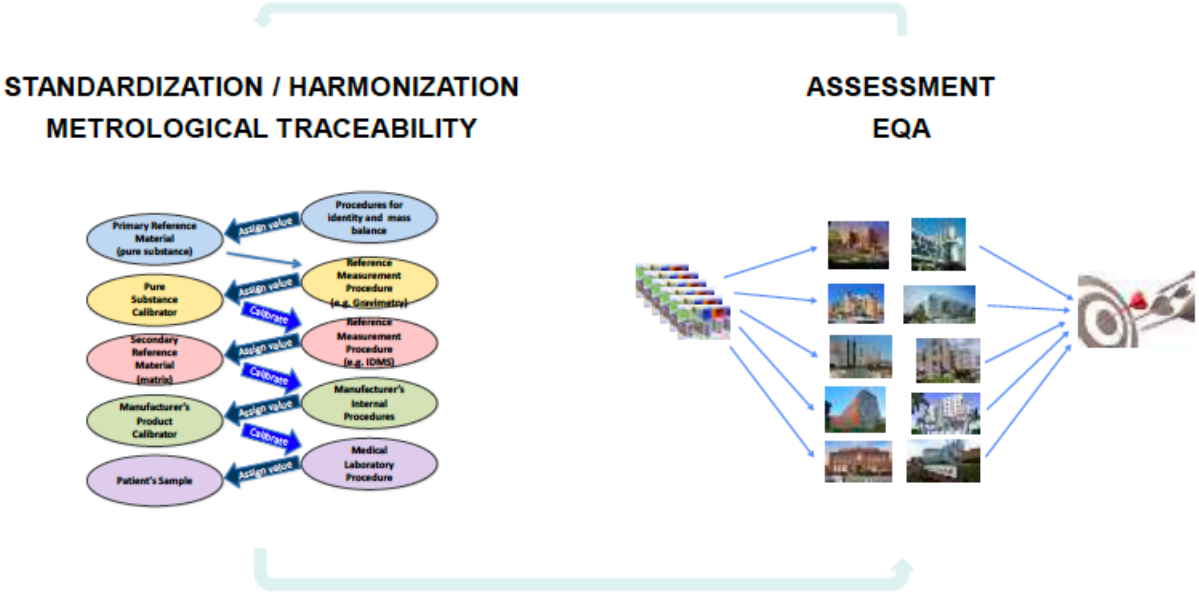
Metrological traceability: an unbroken chain of calibrations from a clinical sample result to a higher order reference system component (ISO 17511)



Greg Miller, IFCC-GC in 2018

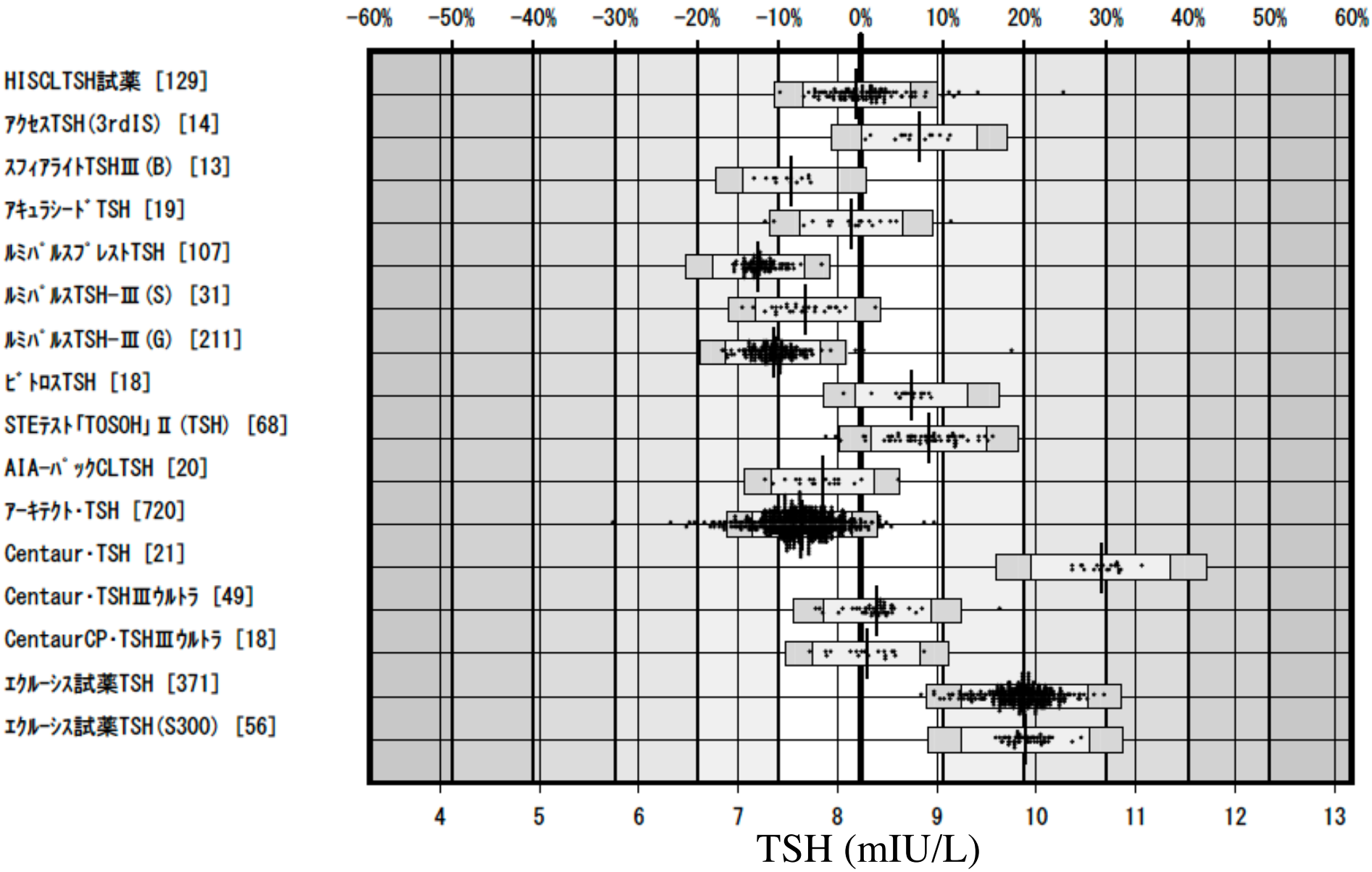
Harmonization needs EQA feedback to the IVD industry

- We need a mechanism for EQA providers to cooperate to:
  1. Cover measurands on an annual or biennial cycle
  2. Prepare aggregated data summaries among schemes



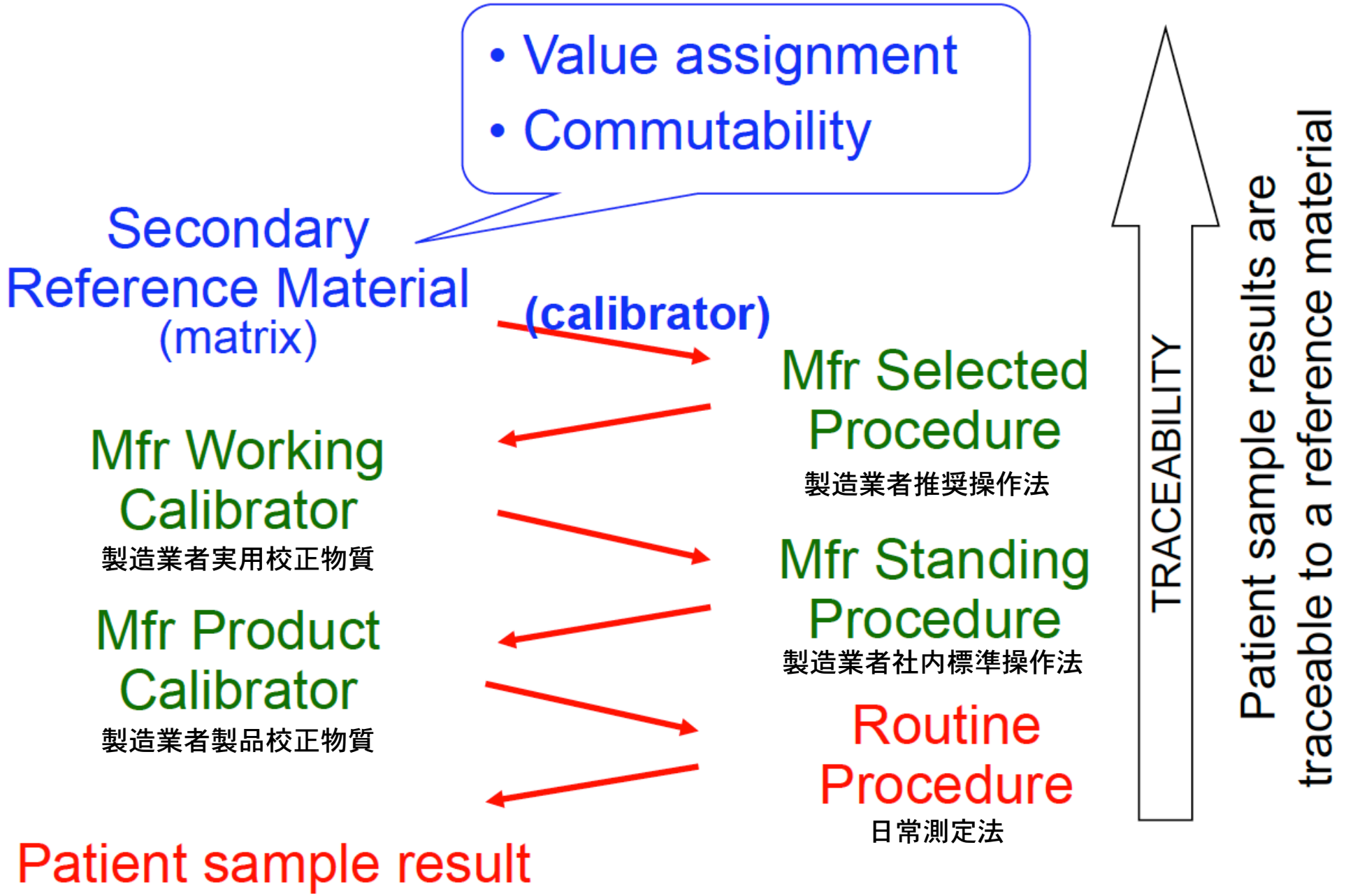
Greg Miller, IFCC-GC in 2018

EQA result of TSH (middle conc.)



2018 EQA program of Japan Medical Association

TSH belongs to class 4 of ISO 17511



Clinical Chemistry July 2017

Papers in Press. Published May 18, 2017 as doi:10.1373/clinchem.2016.269456
The latest version is at http://hwmaint.clinchem.aaccjnls.org/cgi/doi/10.1373/clinchem.2016.269456

Clinical Chemistry 63:7
000–000 (2017)

Endocrinology and Metabolism

Harmonization of Serum Thyroid-Stimulating Hormone Measurements Paves the Way for the Adoption of a More Uniform Reference Interval

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Reference measurement system for TSH

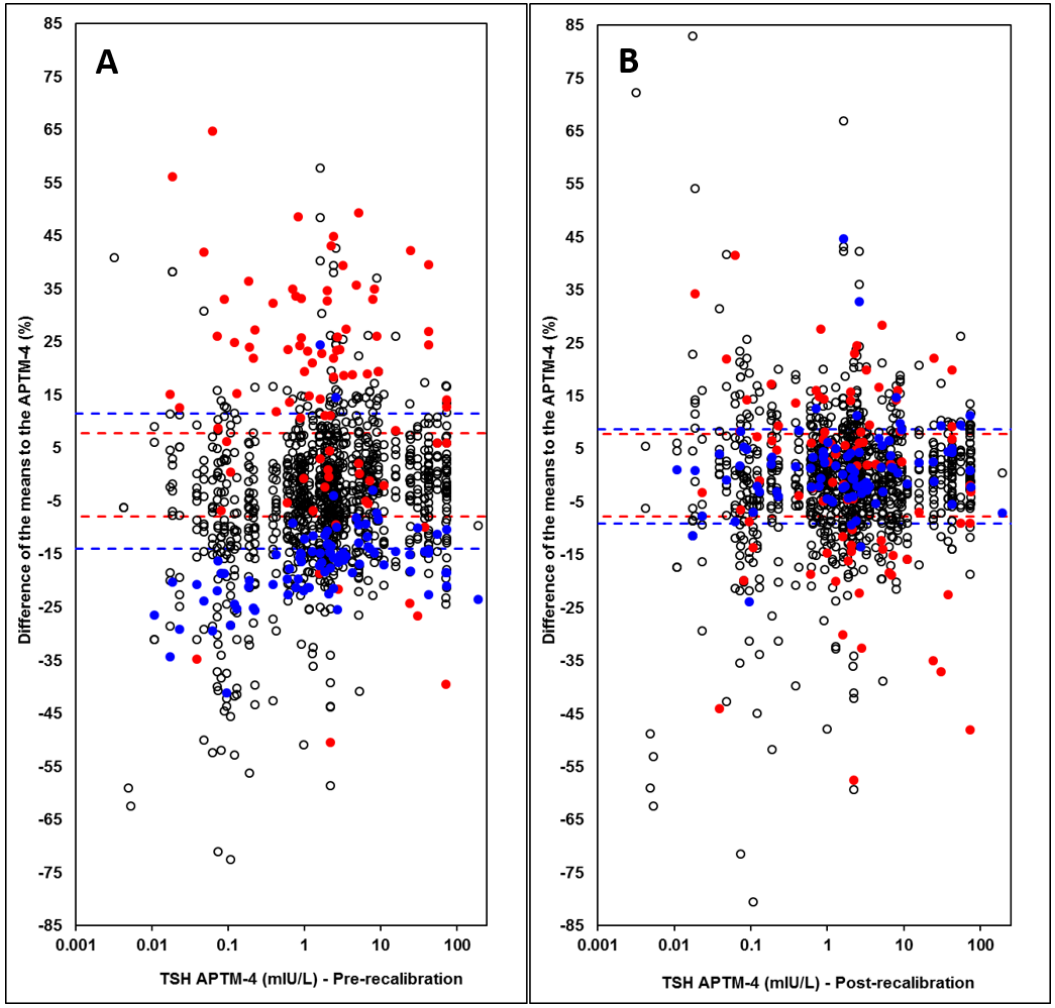
- TSH analysis is “mixture” analysis
  - Serum TSH – intact, total, with glycosylation pattern encountered in specified diagnostic applications
  - Results in mIU/L defined by WHO IRP 80/558 & 81/565

- “The” problem
  - WHO IRP’s not commutable with TSH assays
  - Reference measurement procedure technically not to expect in the short- to midterm

Figure 1. Combined difference (%) plots to the APTM-4 before (A) and after recalibration (B).

- For each assay and sample, the difference of the mean from duplicate measurements is plotted
- Filled and colored circles: differences of the assays that were most discrepant before recalibration
- Open black circles: all other assays
- Red broken lines: 7.8% bias limits
- Blue broken lines: 15th and 85th centiles of the differences

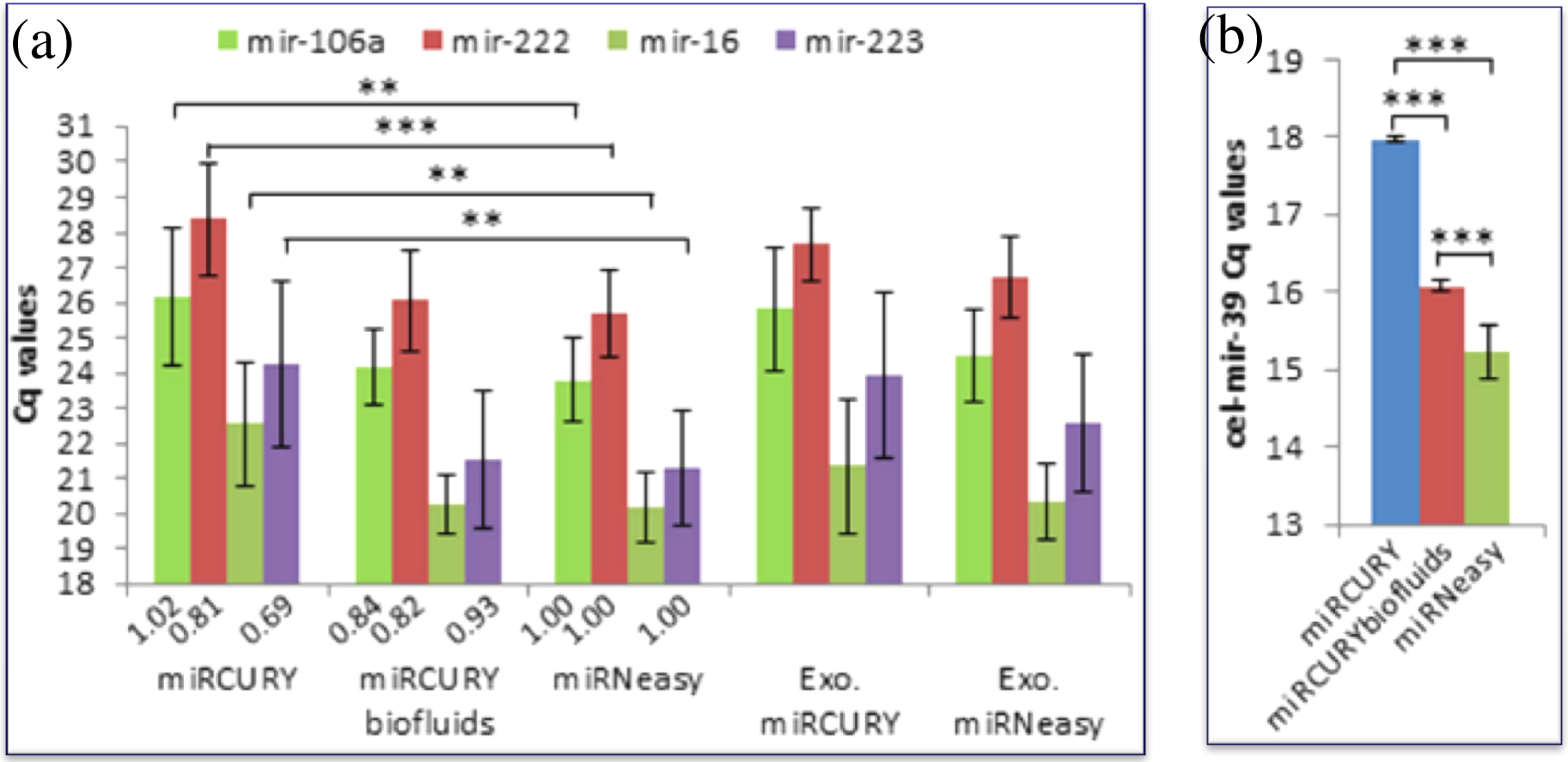
Recalibration eliminates the calibration differences between the assays



APTM: all procedure trimmed mean

Thienpont LM, et al. Clin Chem 2017 July

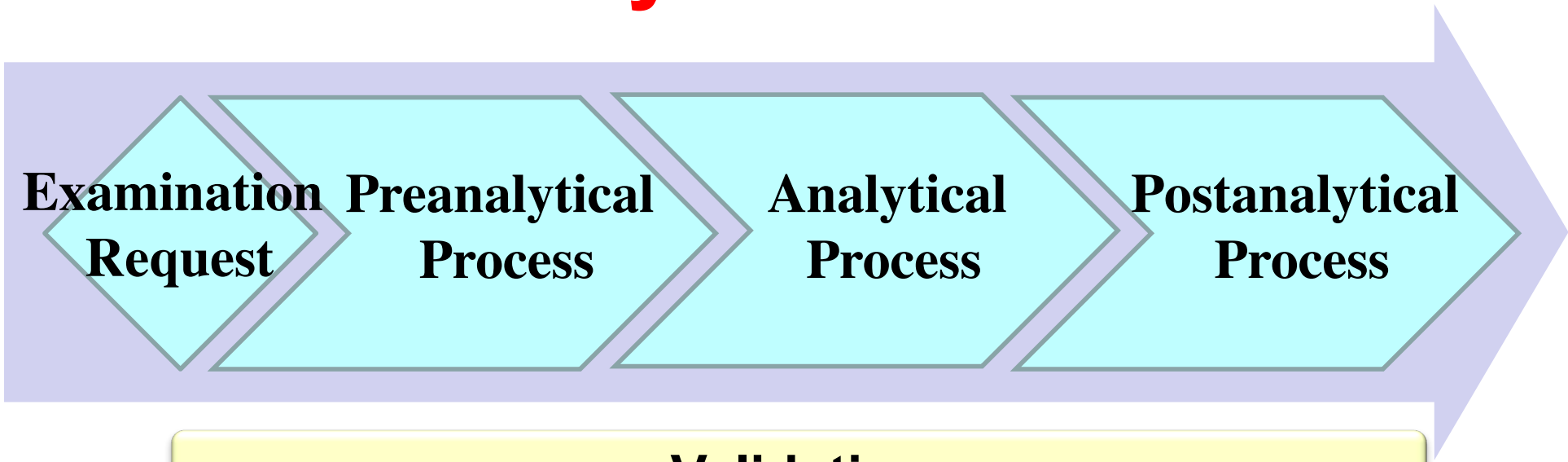
Detection of miRNAs in plasma by RT-qPCR, using different RNA extraction methods and fixed RNA volumes



(a) RNA was extracted from 200 µL of plasma (n = 6). Exosomes were isolated from 2 x 2 mL of plasma (n = 3), then subjected to RNA isolation. The results represent mean Cq values ± SD, using 2.5 µL of RNA/RT reaction. Expression levels of plasma mir-106a, mir-222 and mir-223 were normalized to mir-16 levels and expressed as fold change relative to miRNeasy® condition under the histograms. (b) RNA was isolated from 200 µL of plasma (n = 3) containing 25 fmol of cel-mir-39-3p as a spike-in control directly added into the lysis solution before mixing it with the plasma sample. The results represent mean Cq values ± SD, using 2.5 µL of RNA/RT reaction. **P < 0.01 ***P < 0.001.

Assessing cellular and circulating miRNA recovery: the impact of the RNA isolation method and the quantity of input material. *Scientific Reports* **6**: 19529 (2016)

Quality Assurance



Validation

Internal Quality Control (IQC)

External Quality Assessment (EQA)

ISO 15189
Lab. director, Quality manager
Document (SOP, Worksheet, etc.)
Learning, Education
Equipment, Reagent, other tools

Laboratory **Total** Testing Procedure that enables **HARMONIZATION**