



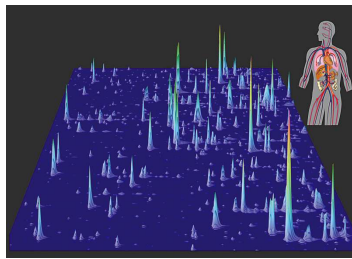
university of
 groningen

faculty of science
 and engineering

analytical biochemistry

Validation of LC-MS/MS methods for the quantification of protein biomarkers: the example of the soluble receptor for advanced glycation endproducts (sRAGE)

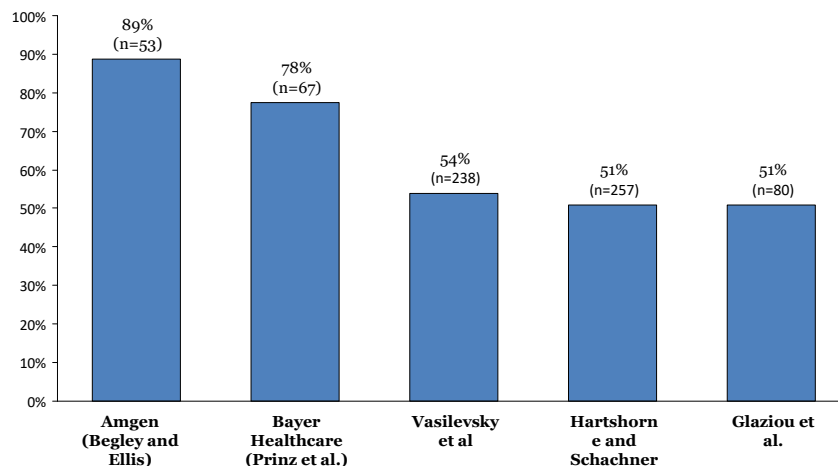
Rainer Bischoff
 r.p.h.bischoff@rug.nl



Spetses Summer School, Sept. 25, 2019

Why analytical method validation?

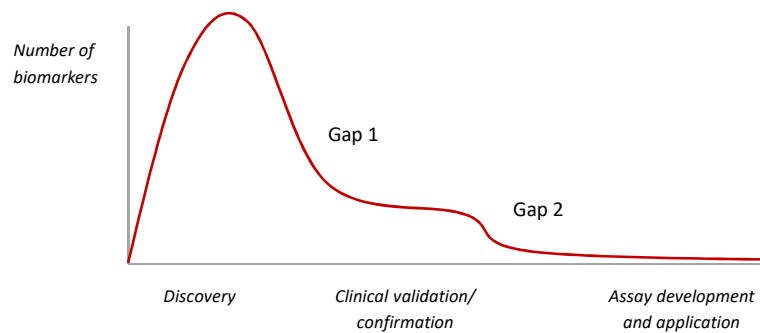
Because there is a high level of irreproducibility in life science studies, estimated to exceed 50%



Freedman et al., PLoS Biol 2015, 13, e1002165.

Why analytical method validation?

Because there is a large gap between the number of discovered biomarker candidates and the number of validated/confirmed biomarkers.



What is analytical method validation?

VALIDATION

thorough demonstration of the reliability of a bioanalytical method for its intended purpose



is the reported concentration result indeed what was originally present in the sample.

Validation takes place **before** application of the method

Quality Control

QUALITY CONTROL

limited demonstration of the (continued) reliability of a bioanalytical method

Quality control takes place **during** application of the method

Reference Substance

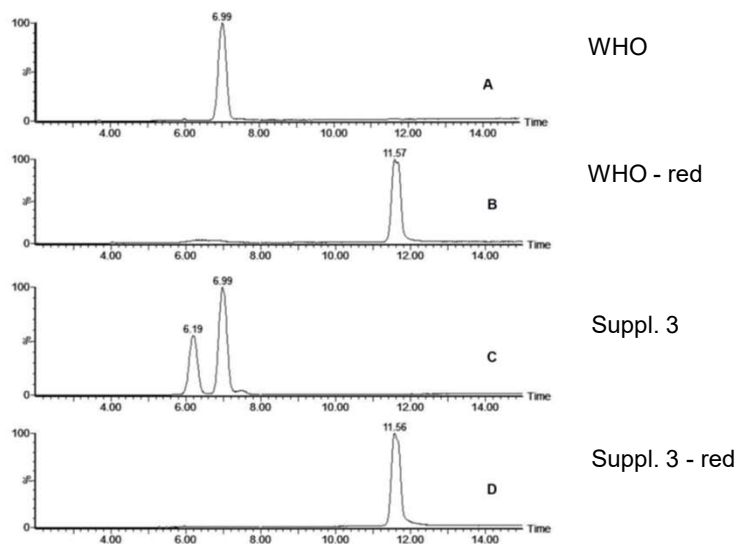
REFERENCE SUBSTANCE

*a substance used to prepare calibrators and quality control samples, preferably of known purity, which is indicated on a **certificate of analysis***

Certificate of Analysis

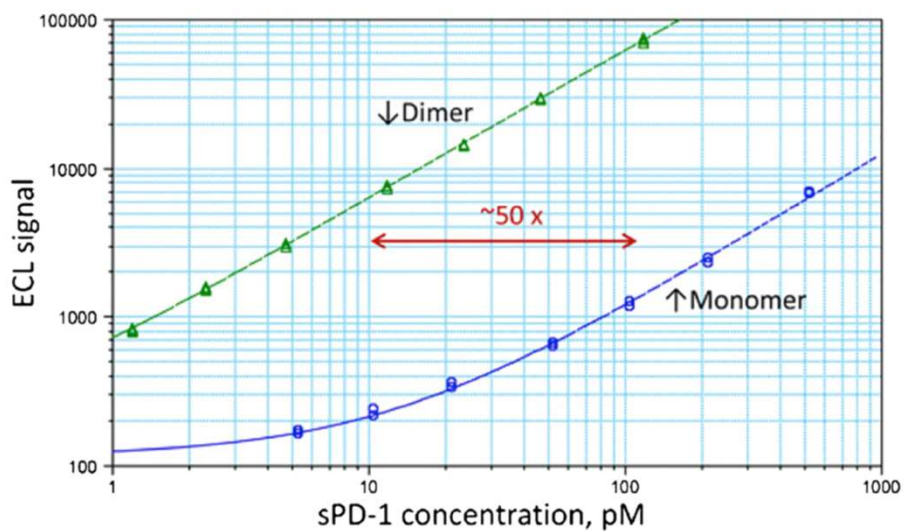
Product Name	Digoxin analytical standard	
Product Number	06003	
Product Brand	FLUKA	
CAS Number	20630-75-5	
Molecular Formula	$C_{41}H_{64}O_{14}$	
Molecular Weight	780.94	
TEST	SPECIFICATION	LOT 110K132TV RESULTS
Appearance (Color)	White to Off-White	White
Appearance (Form)	Powder	Powder
Solubility (Color)	Colorless to Faint Yellow	Very Faint Yellow
Solubility (Turbidity)	Clear to Very Slightly Hazy	Clear
Infrared spectrum	80 mg/mL in Pyridine	Conforms
EMM	14.5 - 16.6 in EtOH	15.3
Wavelength	218 - 220 nm	219 nm

Reference Substances: IGF-1



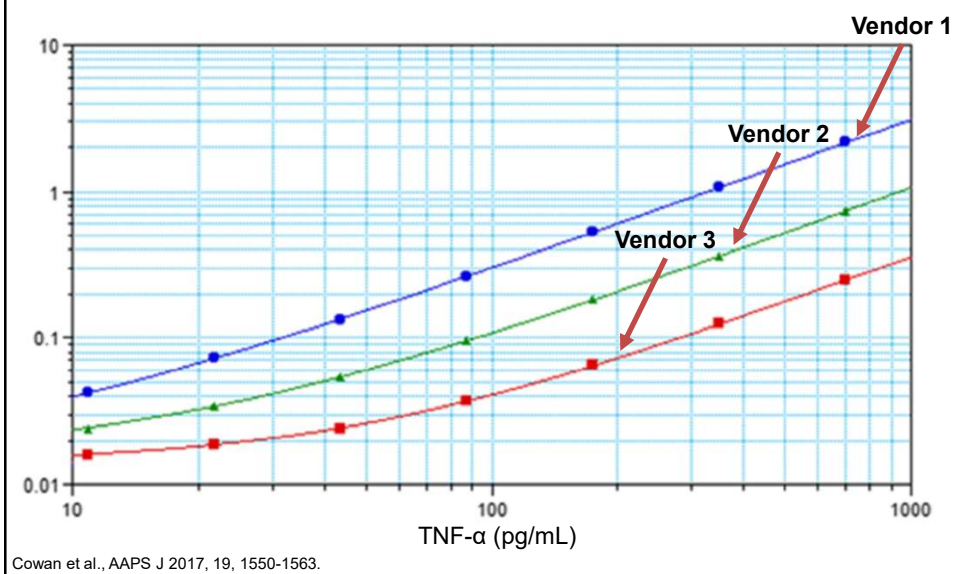
Bronsema et al., Clinical Chemistry and Laboratory Medicine, 2018; 56;1905-1912.

Calibration and Proteoforms: sPD-1



Cowan et al., AAPS J 2017, 19, 1550-1563.

Calibration and Vendors: TNF- α



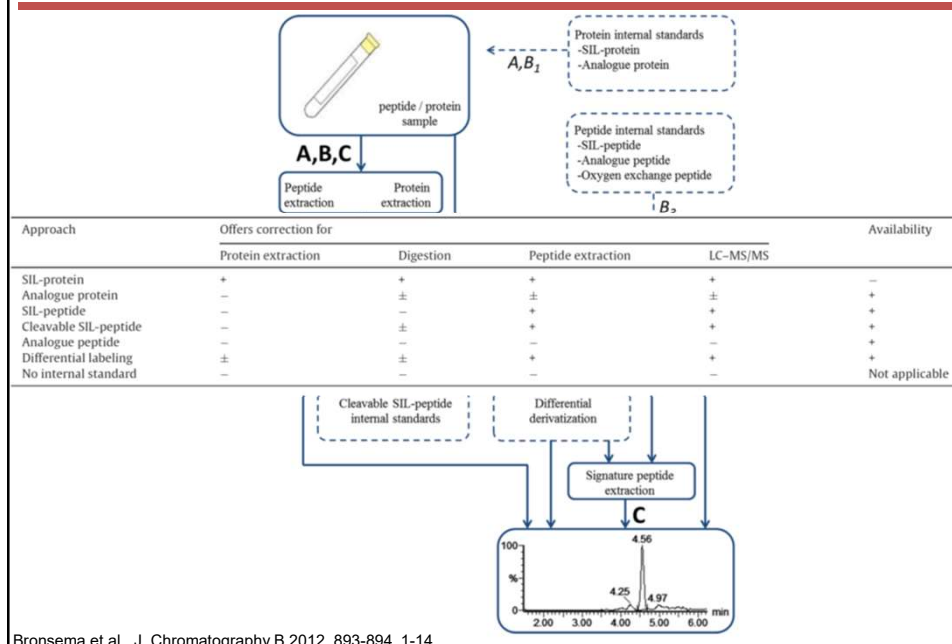
Internal Standard

For LC-MS methods, responses are often normalized by using an **internal standard**

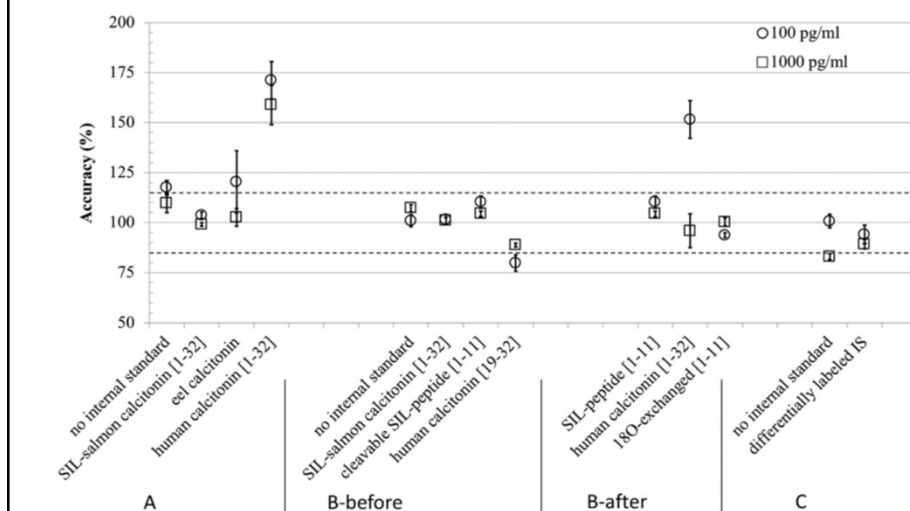
a chemical substance that is added in a constant amount to all samples, to compensate for variability in the analytical procedure

it should behave similarly to the analyte but generate a response that can be distinguished from that of the analyte

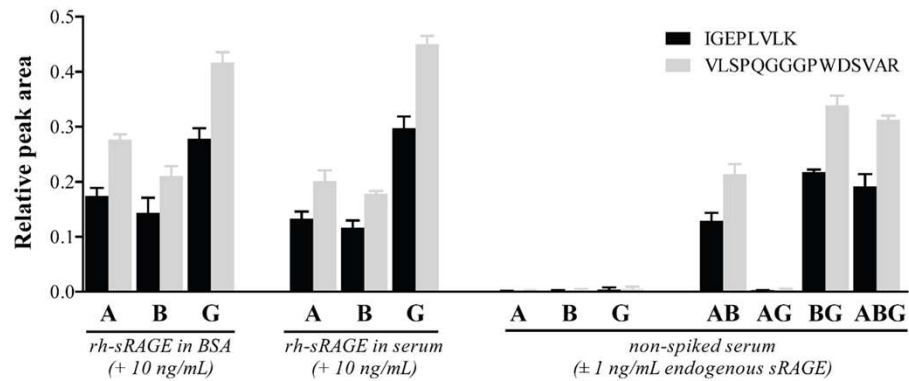
Internal Standards in Protein Analysis



Internal Standards: Accuracy & Precision



Recombinant \neq Endogenous Protein

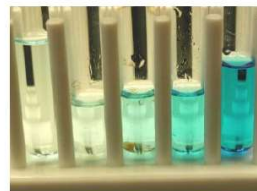


Klont et al., J. Proteome Res., 8, 2018, 2892-2899

Calibrators

CALIBRATOR

a sample with a known analyte concentration, prepared by adding reference solution to a suitable matrix, which is used to calculate the unknown concentration in a study sample
a set of calibrators is called a **calibration curve**



Biological Matrix

BLANK MATRIX

an aliquot of the relevant matrix, which does not contain the analytes of interest

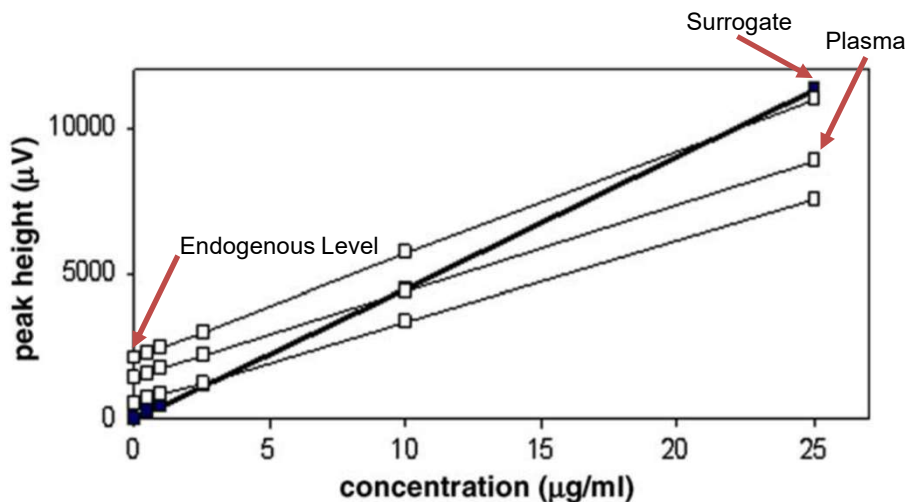
authentic matrix

the actual matrix itself, for xenobiotics: obtained from undosed subjects

proxy or surrogate matrix

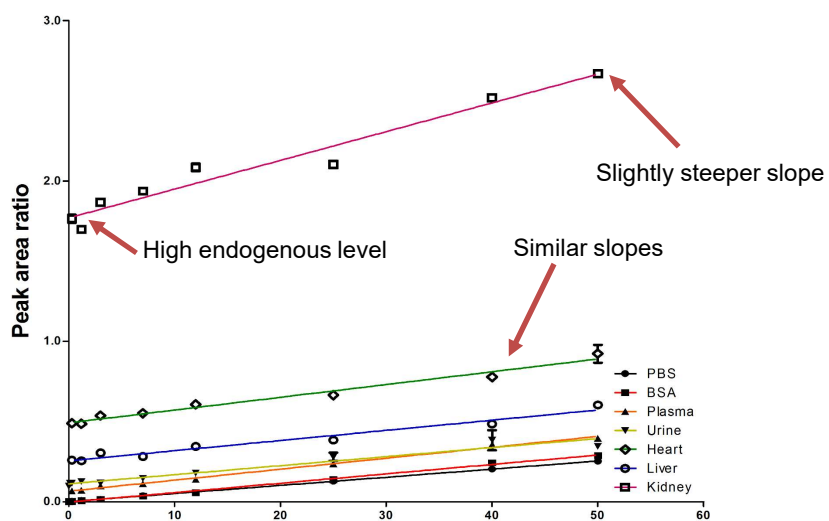
an alternative matrix, preferably with identical properties, for endogenous compounds

Suitability of Surrogate Matrix (1)



v.d. Merbel, TrAC - Trends in Analytical Chemistry 2008, 27, 924-933.

Suitability of Surrogate Matrix (2)



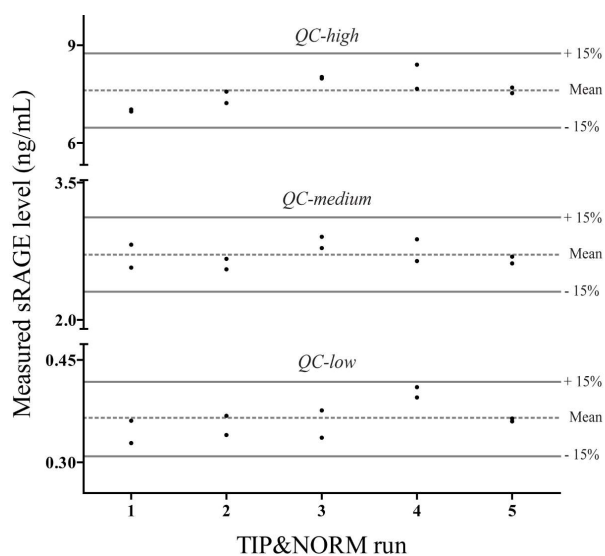
Gil, et al. J. Pharmaceutical and Biomedical Analysis 2018, 160, 289-296.

Quality Control Sample

QUALITY CONTROL SAMPLE

a sample with a known analyte concentration, prepared by adding reference solution to a suitable matrix, which is analysed against a calibration to monitor the quality of the method

Quality Control: sRAGE

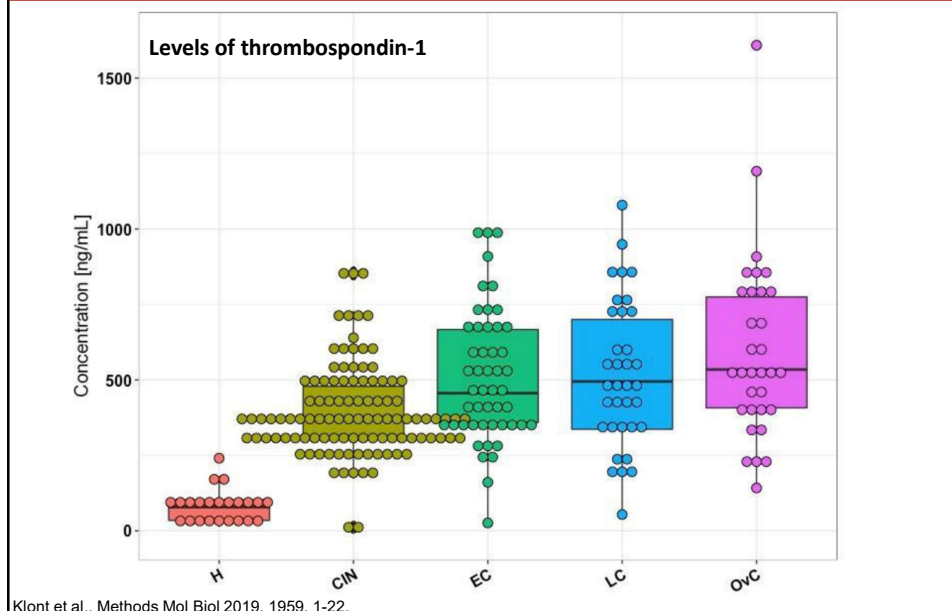


Klont et al., Talanta 2018, 182, 414-421.

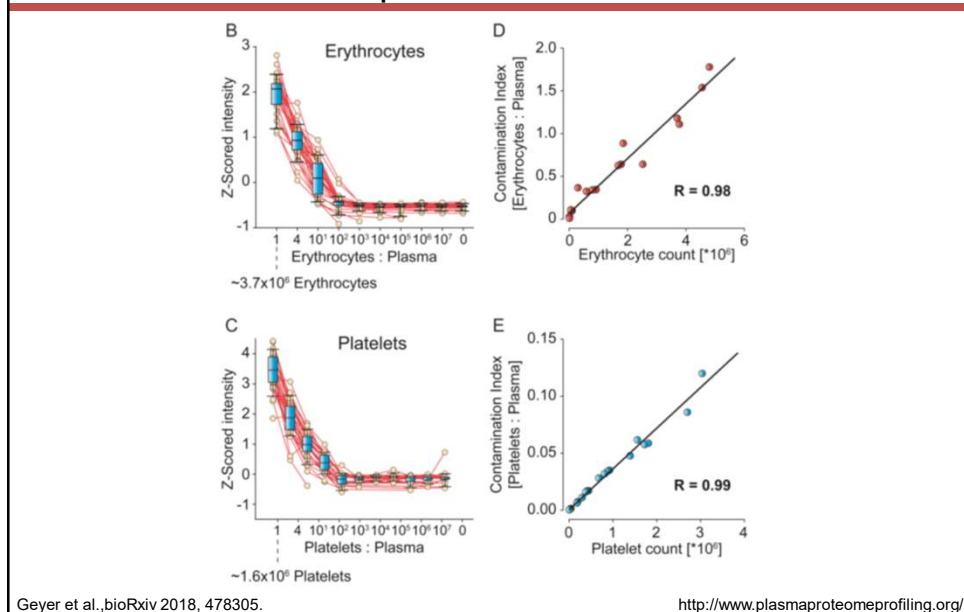
| 20

Preanalytics

Preanalytical Artefacts: Serum



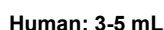
How to find Preanalytical Artefacts? Spike-In Studies



Design of Experiments



Taking a sample means modifying a sample



Handling a sample means modifying a sample

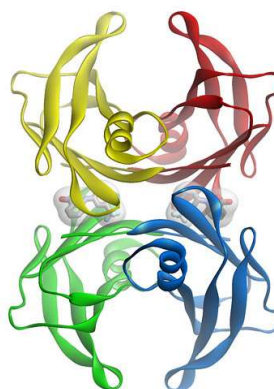
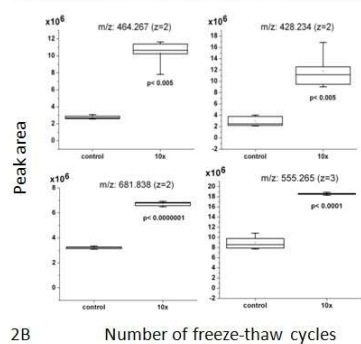
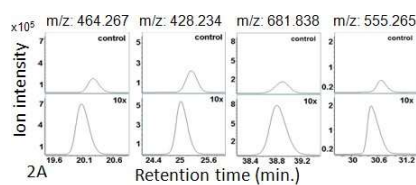
Freeze/thaw cycles

Snap freeze in liq. N₂ and thaw on ice 1-10 times

Autosampler stability

Leave in autosampler for extended time periods

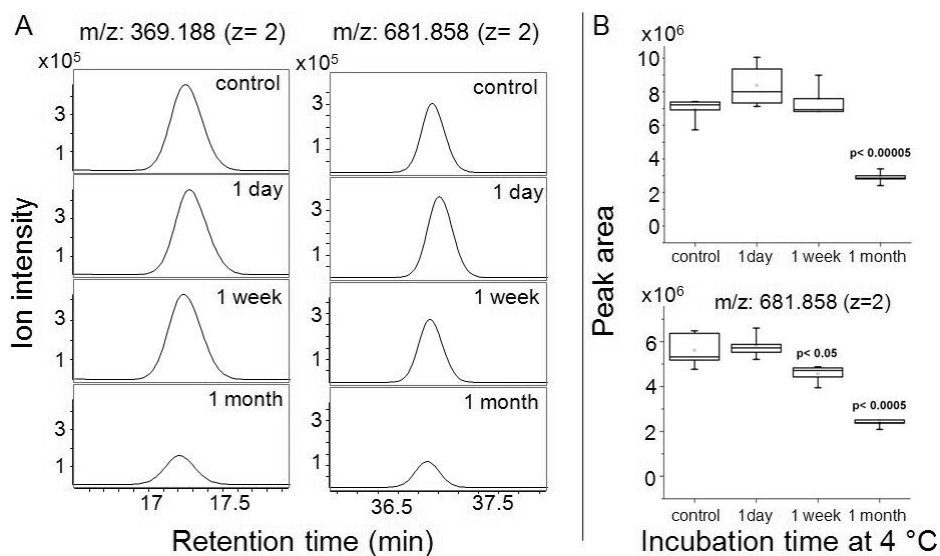
Freeze-thaw cycles of CSF



Transthyretin

Rosenberg et al., J. Proteome Res., 8: 5511-5522, 2009.

Storage in Autosampler at 4°C



Rosenling et al., J. Proteome Res., 8: 5511-5522, 2009.

International guidelines and working groups

Clinical Chemistry 62:1
24-29 (2016)

Perspectives

CLSI C62-A:
A New Standard for Clinical Chemistry

Mission / About ICH /

Harmonisation

Collaboration and developing best practices
is the way forward

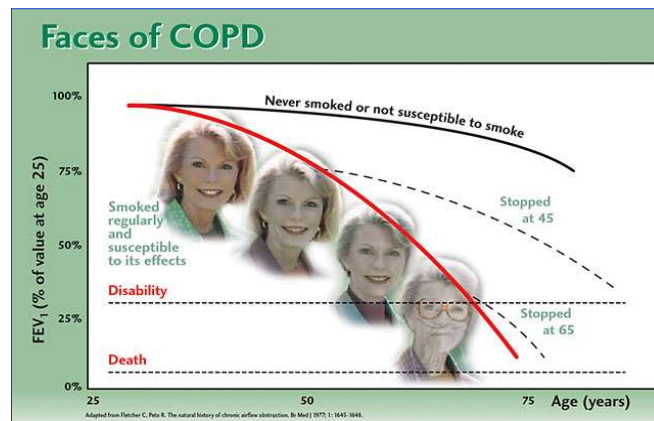
and Human Services
Administration
Evaluation and Research (CDER)
Center for Veterinary Medicine (CVM)

Agreed by Pharmacokinetics Working Party (PKWP)	June 2011
Adoption by CHMP	21 July 2011
Date for coming into effect	1 February 2012

Comments should be provided using this [template](#). The completed comments form should be sent to PKWPsecretariat@ema.europa.eu.

Keywords CHMP, EMEA, Guideline, validation, bioanalytical method, analyses

COPD and lung function decline



- 20% of the smokers develop COPD, more than 200 million people have COPD
- Progressive loss of lung function with a large impact on the quality of life
- Insufficient insight in the molecular mechanisms of COPD
- Limited therapeutic options

sRAGE and COPD

Systematic search for biomarkers that have pointed out to be promising in large-scale clinical studies

Table 4. Prioritized proteins able to discriminate between COPD patients and healthy smokers

Protein name	Abbreviation	Protein ID	Sample	Concentration in smoker controls	Concentration in COPD patients	Significance (p-value)	Reference
Club cell protein 16	CC-16	P11684	serum	5.6 (3.1) ng/ml	4.9 (3.4) ng/ml	<0.001	[78]
C-C motif chemokine 18	PARC/CCL-18	P55774	serum	81 (21) ng/ml	105 (26) ng/ml	<0.0001	[73]
C-reactive protein	CRP	P02741	serum	1.6 (0.8–3.3) µg/ml	3.2 (1.5–7.1) µg/ml	<0.001	[27]
Fibrinogen		Q08830, P02671, P02675	EDTA plasma	391 (348–436) mg/dl	448 (388–517) mg/dl	<0.001	[27]
Interleukin-6	IL-6	P05231	serum	0.6 (0.3–1.3) pg/ml	1.5 (0.8–3.1) pg/ml	<0.001	[27]
Soluble receptor for advanced glycation endproducts	sRAGE	Q15109	serum	1.7 (0.7) ng/ml	1.4 (0.6) ng/ml	<0.001	[65]
Surfactant protein D	SPD	P35247	Serum	114(76–162) ng/ml	121 (85–174) ng/ml	0.021	[80]

Concentrations are presented as mean (standard deviation) or median (interquartile range).

Biomarkers, most remained at the level of the initial discovery, and only fibrinogen has been approved by the Food and Drug Administration (FDA) as predictive for all-cause mortality and COPD exacerbations. There is thus a need for future investigations of these biomarkers in large-scale and well-characterized studies in order to prove their usefulness as surrogate endpoints. Based on this, the aim of the present review is to advance COPD biomarker development by providing a comprehensive overview of protein biomarker candidates which have been evaluated in clinical studies and prioritize them according to their potential of becoming valid, clinically useful COPD biomarkers.

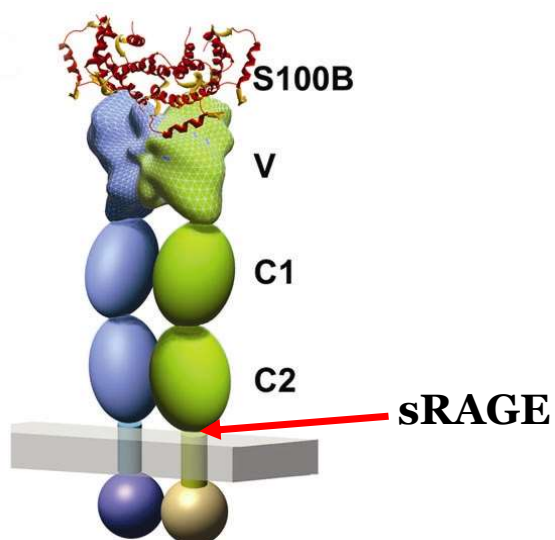
Keywords: COPD, biomarker, surrogate marker, review

*Correspondence to: Nick ten Hacken, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9713 GZ, Groningen, The Netherlands. Email: n.tenhacken@azg.umcg.nl

Received: December 19, 2015; Accepted: February 18, 2016; Published Online: March 18, 2016

Citation: Orús S, Klotz F, Horvathich P, et al. 2016. Prioritization of COPD protein biomarkers, based on a systematic study of the literature. *Advances in Precision Medicine*, vol. 1(1): 12–24. <http://dx.doi.org/10.1080/24741275.2016.1151105>

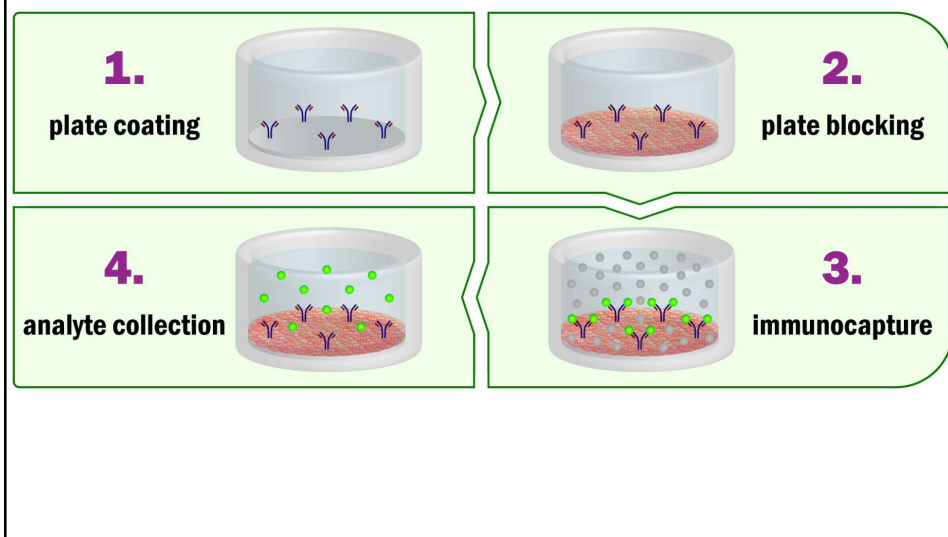
Quantification of sRAGE as an example



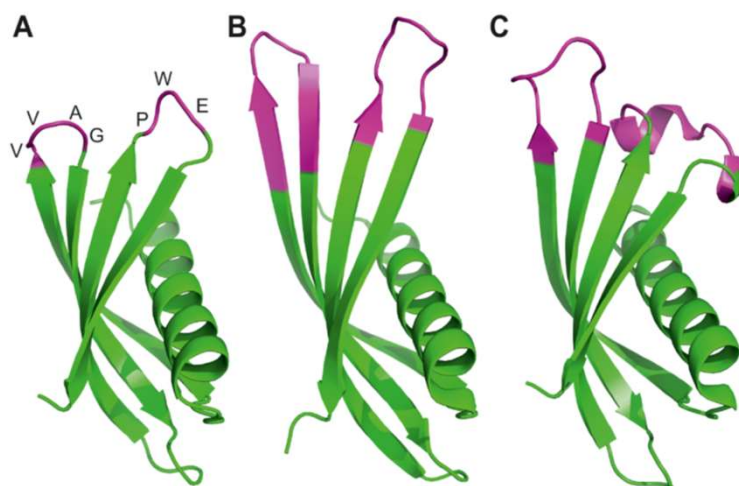
Workflow

Affinity Enrichment	
Antibody	Affimer
Reduction/Alkylation/Digestion	
LC-MS/MS (SRM)	

Affinity Enrichment



Affimers for Affinity Enrichment



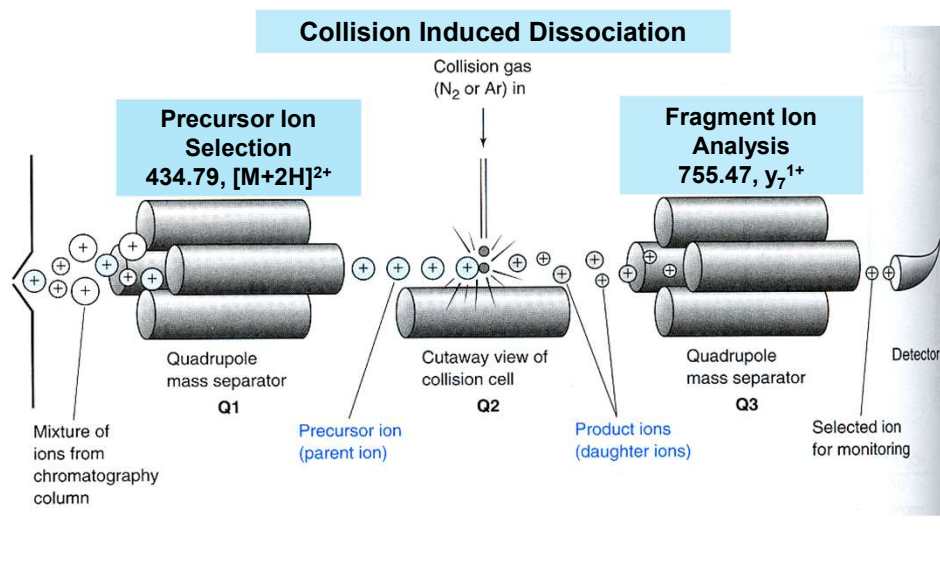
Tiede et al., Elife, 6, 2017, e24903.

LC-MS Method

LC-MS parameters

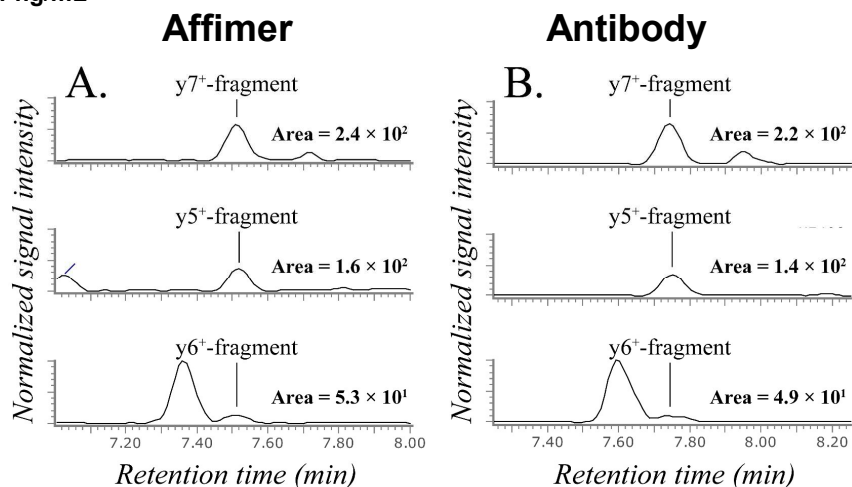
- › Waters Acquity UPLC M-class
+ Waters Xevo TQ-S with ionKey
- › iKey HSS T3 Analytical column
(100Å, 1.8 µm, 0.15 x 50 mm)
- › + trap column (Dionex PepMap100 (100 Å, 5 µm, 0.3 x 5 mm))
- › 10 minute linear gradient (3 µL/min)
3 to 33% eluent B (0.1% FA in ACN)
in eluent A (0.1% FA in H₂O)

Selected Reaction Monitoring (SRM)



Affimer- vs. Antibody-Based Enrichment

0.1 ng/mL



Klont et al., J. Proteome Res., 8, 2018, 2892-2899

Method Validation (1)

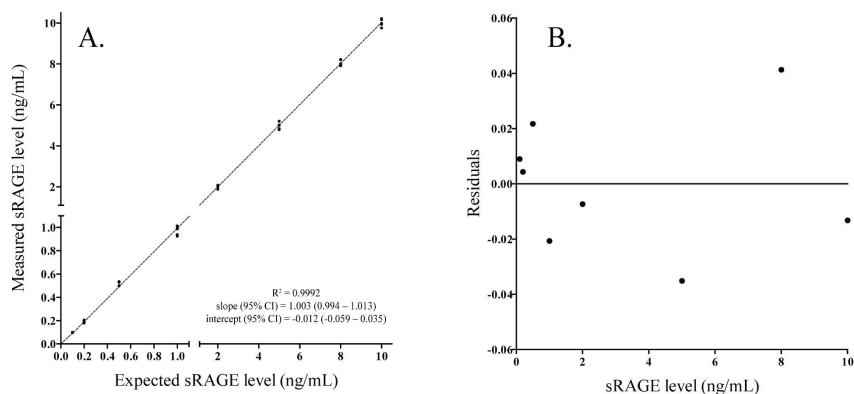
Method validation:

- › Calibration curve (in 1% BSA / PBS, pH 7.4)
 - Linear calibration curve with 1/x-weighting
 - 0.1-10 ng/mL
- › Accuracy & Precision (3 days, 6 replicates)

• LLOQ (0.1 ng/mL)	CV = 9%
• QC-L (0.4 ng/mL; -5% to 4%)	CV = 10%
• QC-M (2.7 ng/mL; -8% to 7%)	CV = 12%
• QC-H (8.1 ng/mL; -5% to 4%)	CV = 8%

Klont et al., Talanta 2018, 182, 414-421.

Calibration



Klont et al., Talanta 2018, 182, 414-421.

Method Validation (2)

Method validation:

- › Stability (3 replicates, QC-low & QC-high)
 - 28 days autosampler (CV & bias < 15%)
 - 13 days benchtop (CV & bias < 15%)
 - 10 freeze-thaw cycles (CV & bias < 15%)
 - 4 months storage
 - -20 ° C (CV & bias < 15%)
 - -80 ° C (CV & bias < 15%)
- › Stock stability (5 replicates, highest calibrant)
 - 443 days Bias = 1%, CV = 3%

Klont et al., Talanta 2018, 182, 414-421.

Method Validation (3)

Method validation:

- › Recovery (duplicate analysis)
 - 6 different serum samples Rec. = 83%
CV = 6%
- › Spike recovery
 - 6 different serum samples Bias = -12%
 - Hemolytic serum sample Bias = -13%
 - Lipemic serum sample Bias = -8%

Klont et al., Talanta 2018, 182, 414-421.

Method Validation (4)

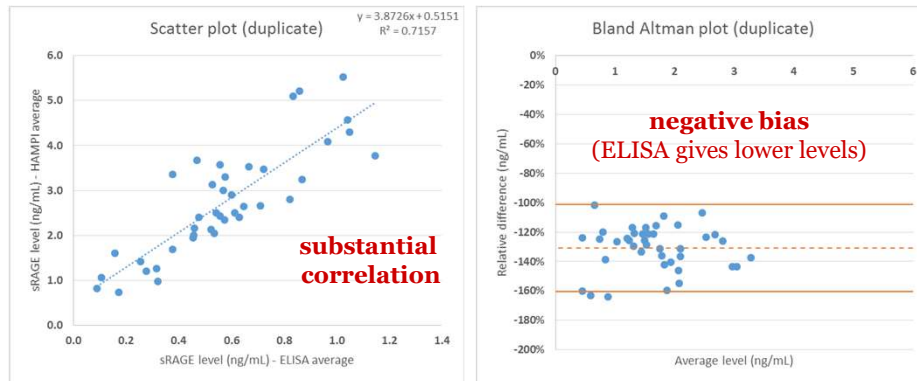
Method validation:

- › Interference test (5 replicates, 0.2 ng/mL)
 - HMGB1 (4 µg/mL) Bias = 10%
 - S100A12 (4 µg/mL) Bias = 11%
 - SAA1 (4 µg/mL) Bias = 7%
 - CML-BSA (4 µg/mL) Bias = 4%
 - Cigarette Smoke Extract(5%) Bias = -8%
 - Lysed A549 human alveolar epithelial cells
(5%≡500,000 cells/mL) Bias = -1%

Klont et al., Talanta 2018, 182, 414-421.

Comparison ELISA-LC-MS

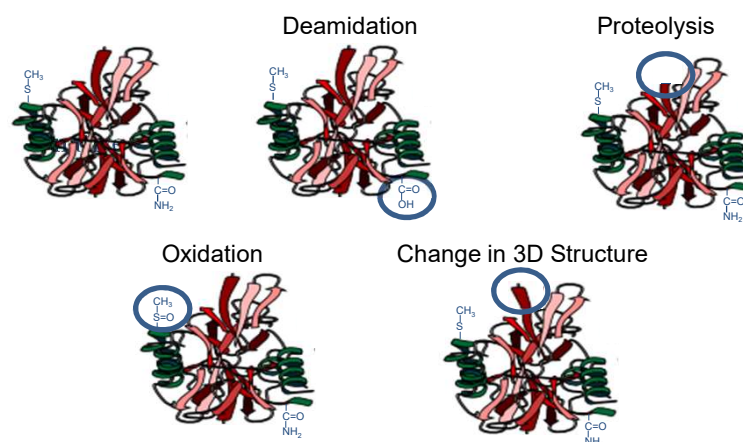
LC-MS versus R&D Systems Human RAGE DuoSet ELISA:



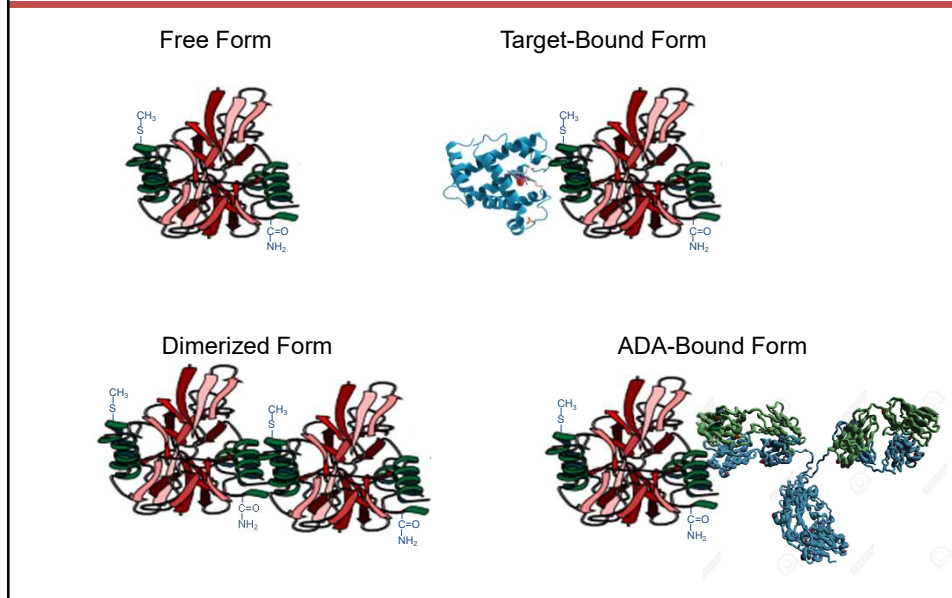
→ Lower ELISA levels due to insufficient amount of antibody

Klont et al., Talanta 2018, 182, 414-421.

No 'One-Size-Fits-All' Protein Assay (1)



No 'One-Size-Fits-ALL' Protein Assay (2)

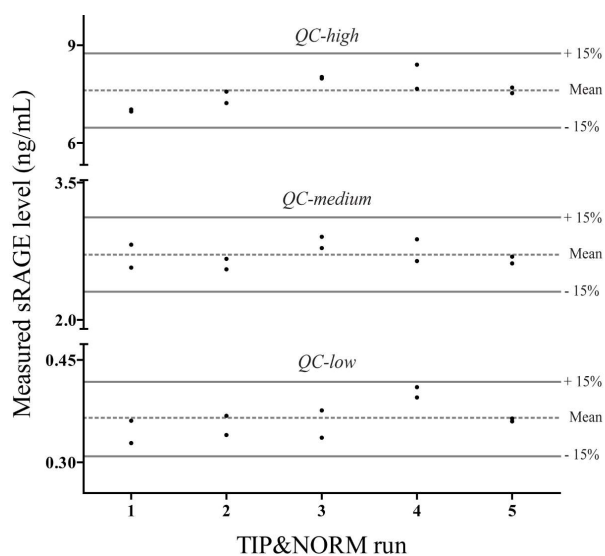


Test with Clinical Material (1)

Clinical studies:

- › NCT00807469 (→ TIP study)
 - “Responses Induced by Smoking in Individuals Being Susceptible and Non-Susceptible for Development of COPD”
- › NCT00848406 (→ NORM study)
 - “A Study to Obtain Normal Values of Inflammatory Variables From Healthy Subjects (NORM)”
- **6 analytical runs**
- **377 clinical samples**

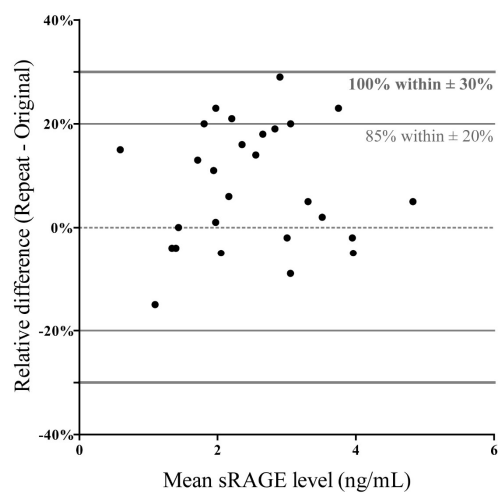
Quality Control (1)



Klont et al., Talanta 2018, 182, 414-421.

Quality Control (2)

Repeat Analyses



Klont et al., Talanta 2018, 182, 414-421.

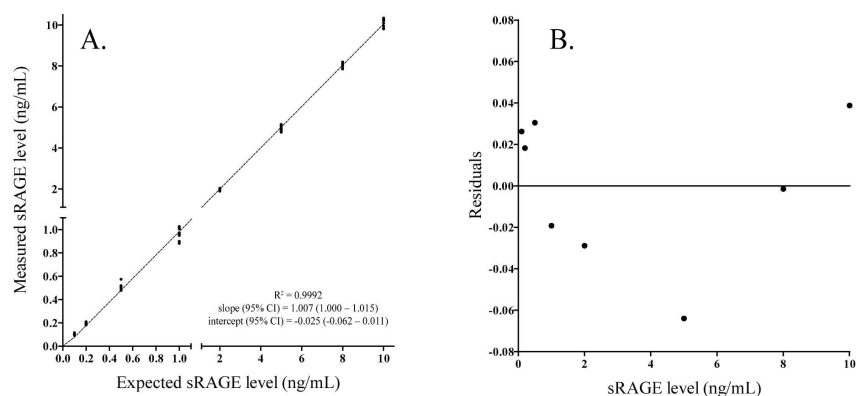
Test with Clinical Material (2)

Clinical study:

- NCT00608764 (COPDgene)
 - “Examining the Genetic Factors That May Cause Chronic Obstructive Pulmonary Disease (COPD) (COPDGene)”

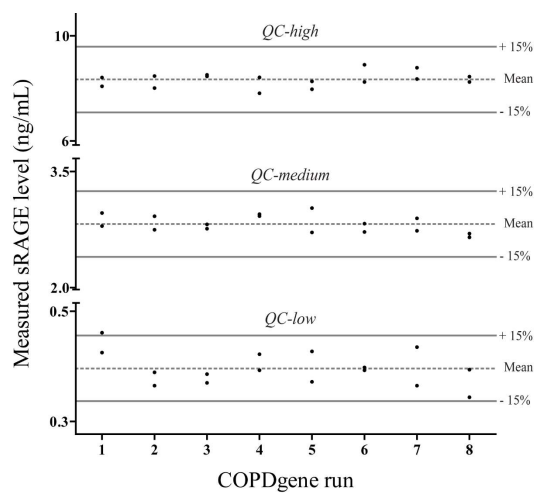
- **8 analytical runs**
- **510 clinical samples**

Calibration



Klont et al., Talanta 2018, 182, 414-421.

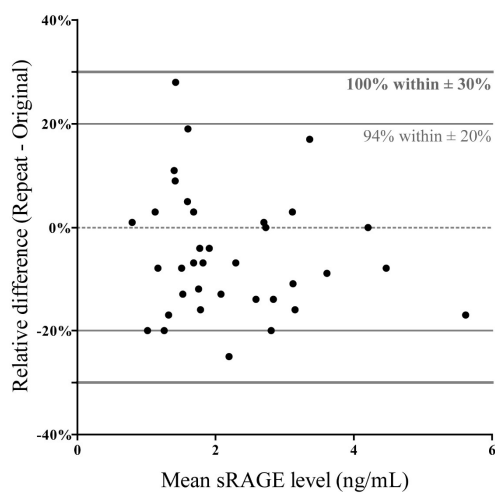
Quality Control (1)



Klont et al., Talanta 2018, 182, 414-421.

Quality Control (2)

Repeat Analyses



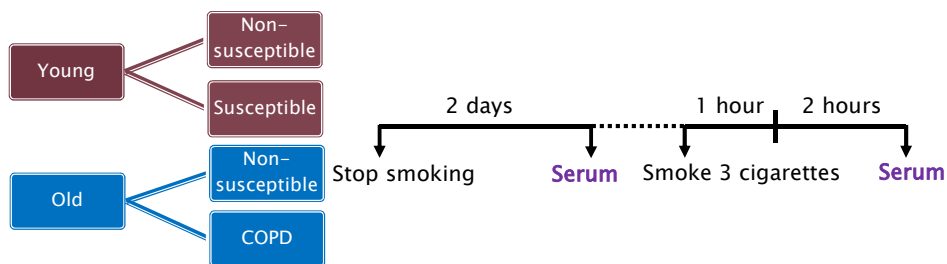
Klont et al., Talanta 2018, 182, 414-421.

Confounding Factors

sRAGE and smoking (1)

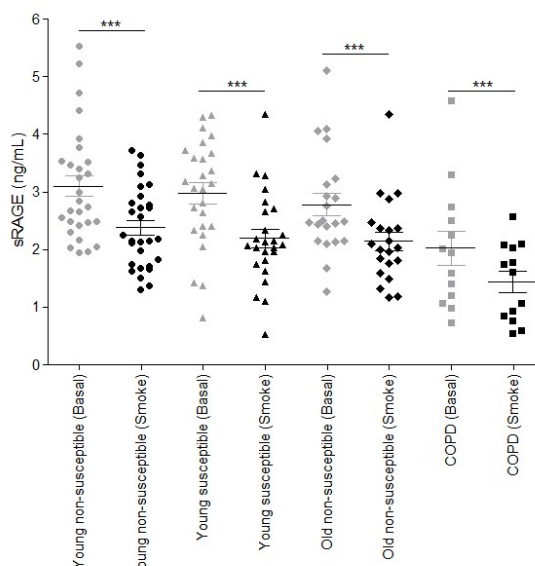
Serum samples

- ▶ COPD patients GOLD I–IV
- ▶ Healthy controls young (20–40 y) or old (40–80 y) that are either smokers or non-smokers.



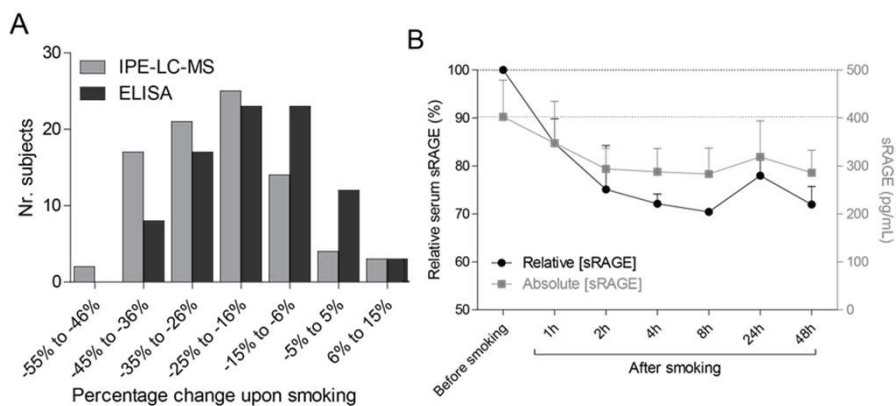
Lo Tam Loi, et al., BMJ Open 2013, 3, pii: e002178, 002110.001136/bmjopen-002012-002178.

sRAGE and smoking (2)



Pouwels et al., Am. J. Respir. Dis. Crit. Care Med., 2018, 198: 1456-1458.

sRAGE and acute smoke exposure



Pouwels et al., Am. J. Respir. Dis. Crit. Care Med., 2018, 198: 1456-1458.

Conclusions

- Analytical method validation is a critical prerequisite for biomarker qualification, as it allows multi-centric studies.
- The translation of biomarkers from discovery to clinical utility suffers from inadequately controlled **preanalytical factors**, a disregard for the effects of **data processing and statistical data analysis** on the results as well as from an inappropriate **study design** not addressing the ultimate **context of use** as well as from the effect of **confounding factors**.
- There is no 'one-size-fits-all' quantitative protein assay, since each assay measures only a small part of a protein. Systematic bias is more often the norm than the exception.

Acknowledgements

sRAGE

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Nico van de Merbel
Nick ten Hacken

Other Work

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Vikram Mitra
Therese Rosenling
Kees Bronsema

 Applied and
Engineering Sciences



<http://biomarkerdevelopmentcenter.nl/>

University of Groningen



umcg



PRAXIS



university of
groningen

faculty of science
and engineering

analytical biochemistry

Thank you – questions?

