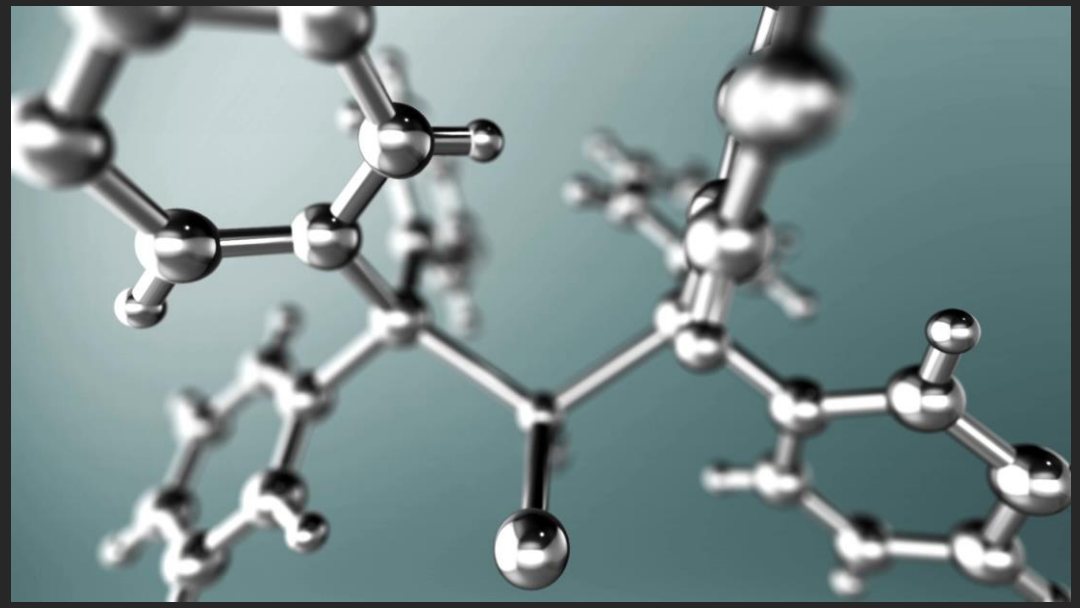


Clinical and analytical interferences with HbA1c assays 10-05-2022

Critically Appraised Topic

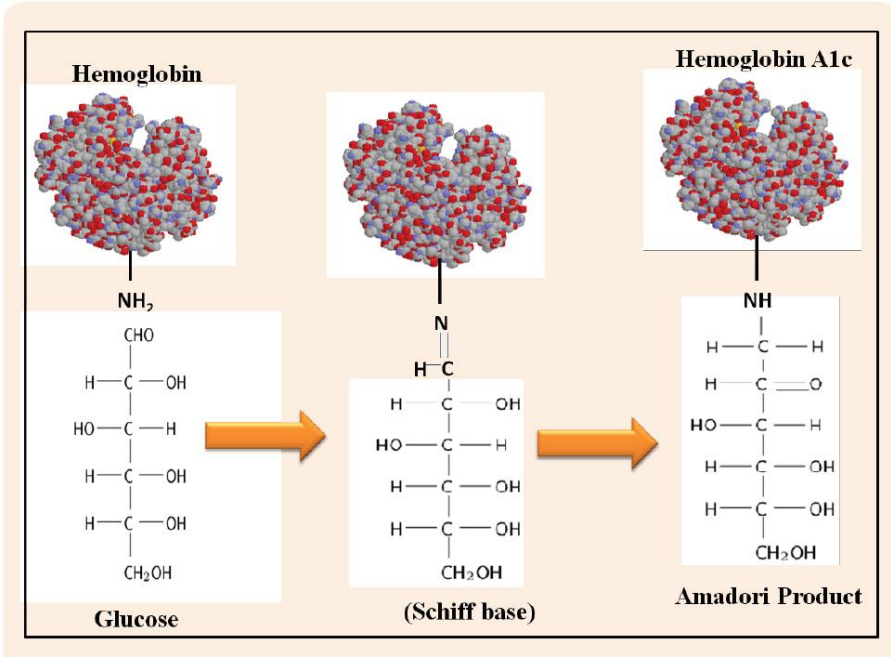
Alexander Ockerman
Supervisor: Christine Van Laer

Diabetes is a major global health threat

- Hundreds of millions affected worldwide and rising
  > 400 million, projected >640 million by 2040
- Chronic long term complications -> macro- and microvascular: myocardial infarction, stroke, impotence, nephropathy, retinopathy, neuropathy, amputations, infections,..
- Enormous impact on morbidity, mortality and healthcare costs (estimated 10% of healthcare costs)

Importance of correct HbA1c measurements and interpretation in managment and diagnosis!

What is HbA1C?



Terminal N-Valine on the beta chain + glucose ->

Unstable Schiff base -> Stable Amadori Product

Chauhan, N. Laboratory Diagnosis of HbA1c: A Review. *Journal of Nanomedicine Research* **5,** (2017).

Standard interpretation norm*			IFCC (mmol/mol)	NGSP (%)
Normal reference range			20-42	4-6
Decision limits	Monitoring therapy	Target treatment	53	7
		Limit change therapy	64	8
	Diagnosis	Low risk	<40	<5.8
		Increasing risk future diabetes	40-46	5.8-6.4
		Diabetes	>46	>6.4

Weykamp, C. HbA1c: A review of analytical and clinical aspects. *Annals of Laboratory Medicine* **33,** 393–400 (2013).

Standardization of HbA1c measurements

NGSP/DCCT

- %
- Directly linked to clinical outcomes

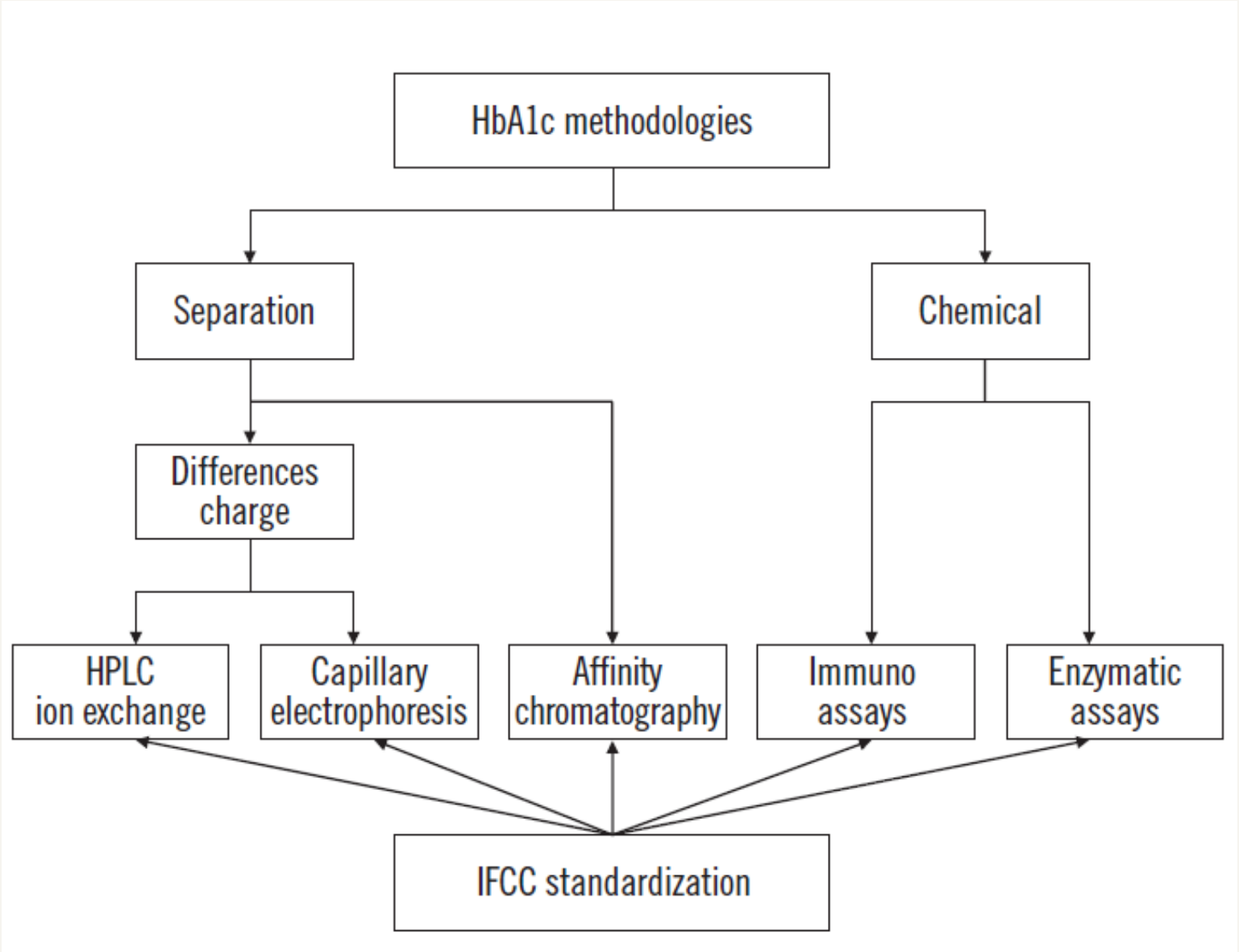
IFCC

- mmol/mol
- Traceability to a higher order reference method

Master equation for conversion:

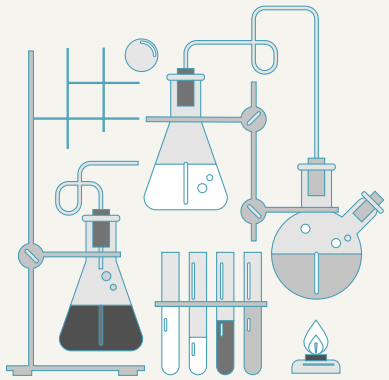
NGSP = [0.09148 * IFCC] + 2.152
IFCC = [10.93 * NGSP] - 23.50

Different methods of HbA1c measurements



Weykamp, C. HbA1c: A review of analytical and clinical aspects. *Annals of Laboratory Medicine* **33,** 393–400 (2013).

Separation methods



- HPLC

  difference in polarity to separate the different fractions over a column ->ratio of the HbA1c peak area to the total hemoglobin peak areas

- Capillary electrophoresis

  difference in charges to separate the Hb fractions using a high-voltage electrical field and electroosmotic flow

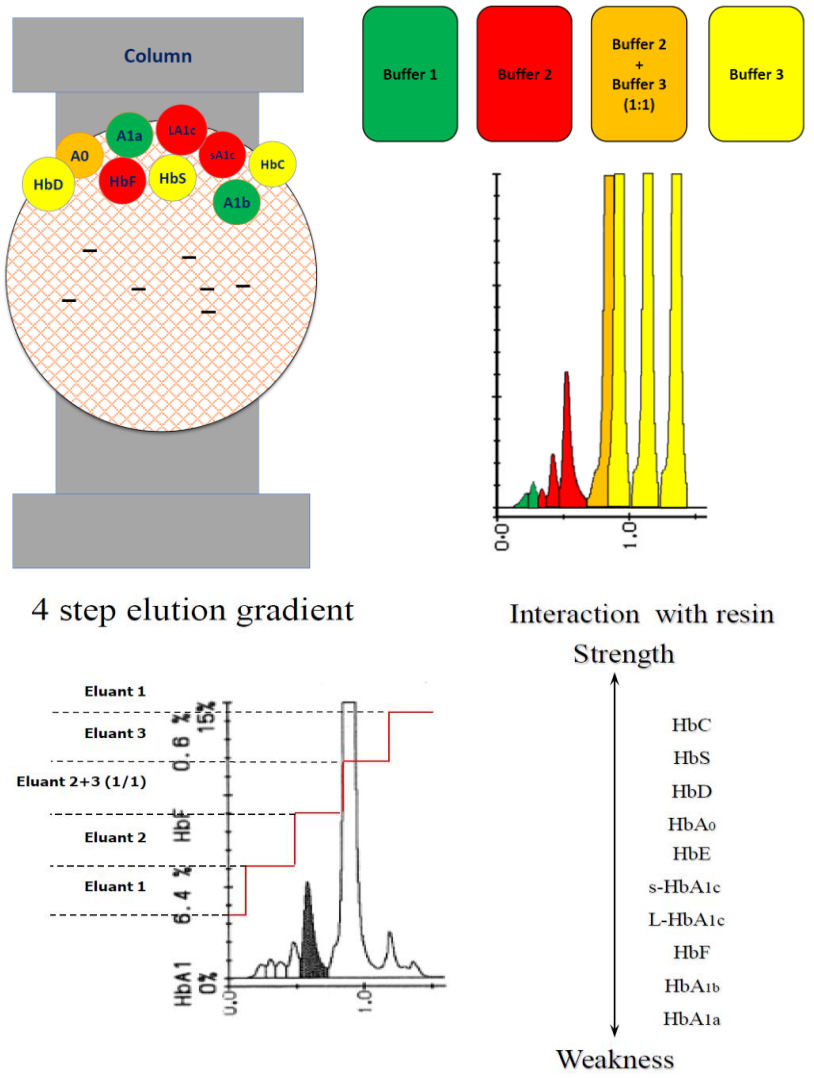
- Boronate affinity chromatography

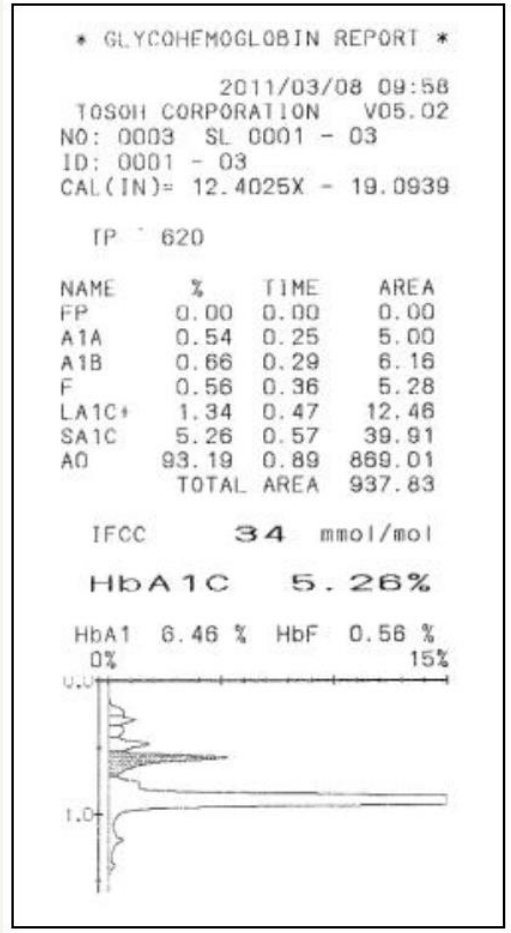
  column packed with particles coated with boronic acid -> glycated Hb affinity to cis-diol binding

  measures total glycated Hb over non-glycated Hb

  least interference from variant Hb

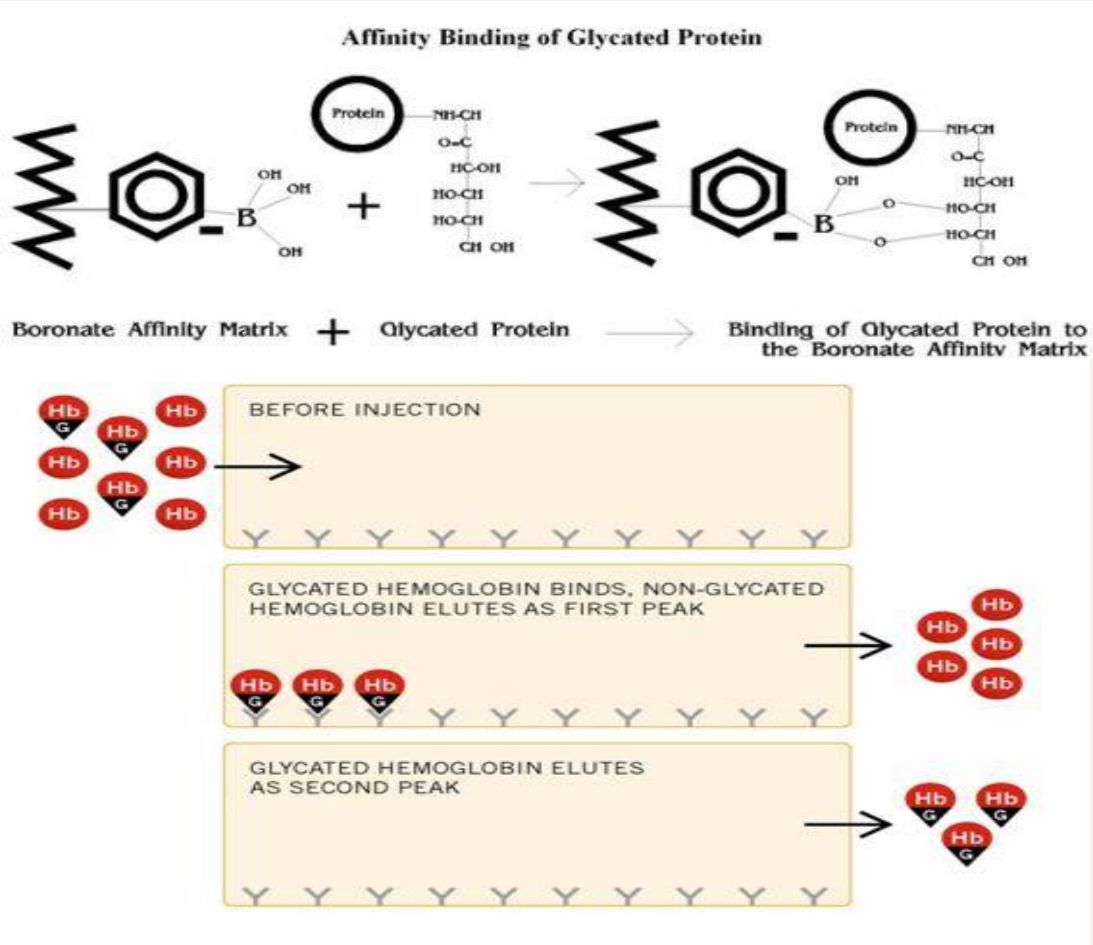
Tosoh G8 HPLC

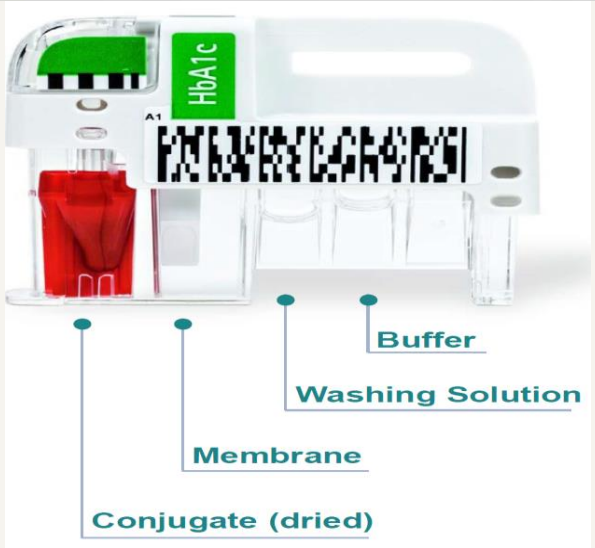




```
* GLYCOHEMOGLOBIN REPORT *

              2011/03/08 09:58
 TOSOH CORPORATION     V05.02
NO: 0003   SL 0001 - 03
ID: 0001 - 03
CAL(IN)= 12.4025X - 19.0939

  TP  620

NAME        %      TIME      AREA
FP       0.00      0.00      0.00
A1A      0.54      0.25      5.00
A1B      0.66      0.29      6.16
F        0.56      0.36      5.28
LA1C     1.34      0.47     12.46
SA1C     5.26      0.57     39.91
A0      93.19      0.89    869.01
        TOTAL AREA        937.83

  IFCC      34   mmol/mol

  HbA1C    5.26%

HbA1  6.46 %  HbF  0.56 %
0%                    15%
```

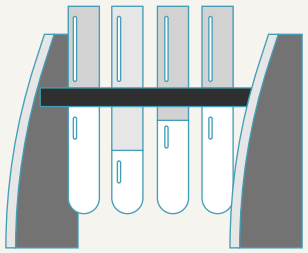
Alere Afinion Boronate Affinity





https://www.trinitybiotech.com/haemoglobins/boronate-affinity-chromatography/
https://www.globalpointofcare.abbott/en/product-details/afinion-hba1c.html

Chemical methods



- Immuno-assays

  antibodies recognize the structure of the N-terminal glycosylated valine on the β-chain of the Hb

  turbidity of the resulting immunocomplexes is measured photometrically using a turbidimeter or nephelometer

- Enzymatic assays

  enzyme that specifically cleaves the N-terminal valine this protease cleaves the beta-chain to liberate peptides -> react with fructosyl peptide oxidase, and the resulting hydrogen peroxide is used to quantify HbA1c

Alternative markers of long-term glucose control

- Fructosamine and Glycated albumin (GA)

  Fructosamine =All ketoamine linkages that result from glycation of serum proteins
  Includes glycated albumin, lipoproteins and glycated globulins

  Glycated albumin (GA) = serum albumin that undergoes glycation

  Reflects glycemic control of a short to intermediate time frame (2 - 3 weeks)
  Rate of glycation is 9- to 10-fold higher than in hemoglobin

- Estimated HbA1c (eA1c)

  An approximate HbA1c level based on the average CGM-measured glucose levels is calculated
  Discrepancies with measured HbA1c may signal analytical/clinical interferences

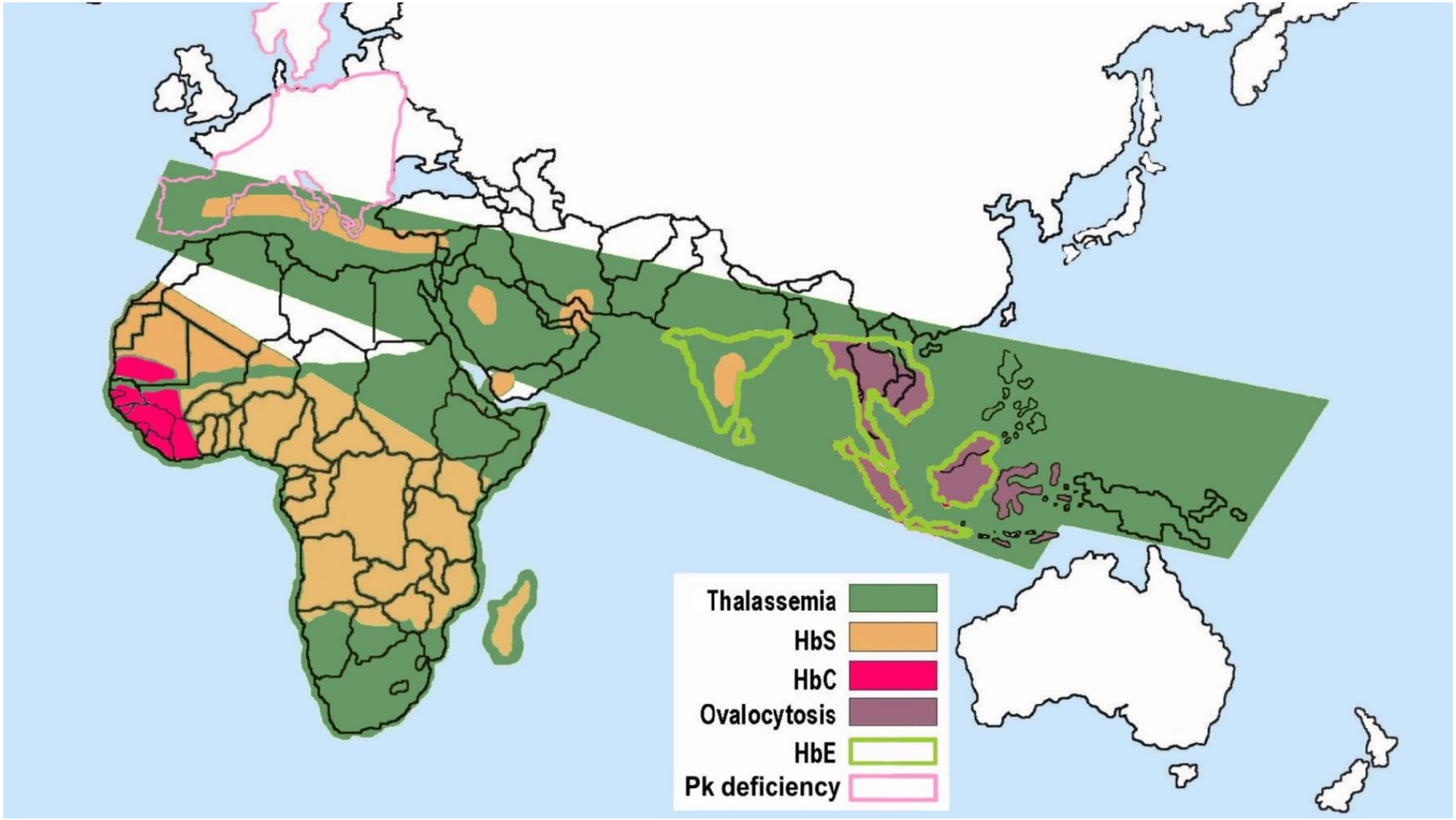
Analytical interferences

Analytical Interferences							
Method	Manufacturer/ Analyzer	HbAC	HbAS	HbAE	HbAD	HbF	CarbHb
Ion-exchange HPLC	Tosoh G8 Bio-Rad Variant II Menarini ADAMS A1c	No	No	No*	No	No <30%	No
Boronate Affinity	Alere Afinion Trinity Hb9210	No	No	No	No	No <15%	No
Immuno-assays	Roche Cobas c513	No	No	No	No	No<10-15%	-
Immuno-assays	Siemens DCA 2000	No	Yes↑	Yes↑	No	No<10%	No
Enzymatic-assays	Abbot Architect c Diazyme Direct enzymatic	No	No	No	No	-	-
Electrophoresis	Sebia Capillarys 2	No	No	No	No	No<15%	No

Based on NGSP.org

Hemoglobin variants

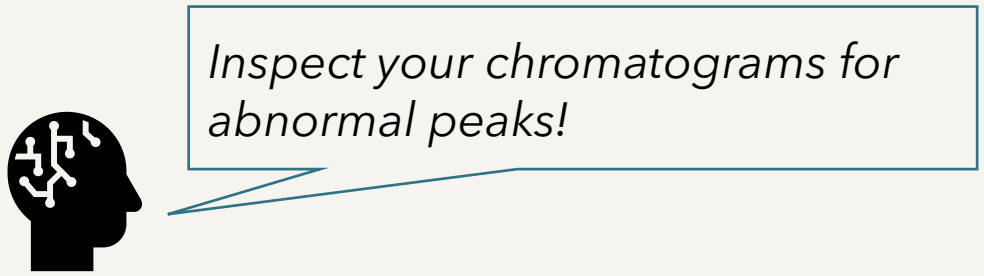
- Most common Hb variants worldwide : HbS > HbE > HbC > HbD
- Single amino acid substitution in beta chain of HbA -> change in chemical properties
- Change in ionic charge of the Hb molecule -> possible interferences with ion exchange HPLC
- HbAE remains difficult for HPLC methods with falsely lowering results, depends on software version used, presumably no problems with Tosoh G8 as of 5.24 and 5.28
- Generally no interference for Boronate Affinity: separates total glycated Hb from nonglycated hemoglobin, regardless of the Hb species
- HbA1c can be used in heterozygotes as RBC lifespan is normal
- Can't be used in homozygotes (HbEE, HbSS, HbSC) as RBC lifespan is reduced



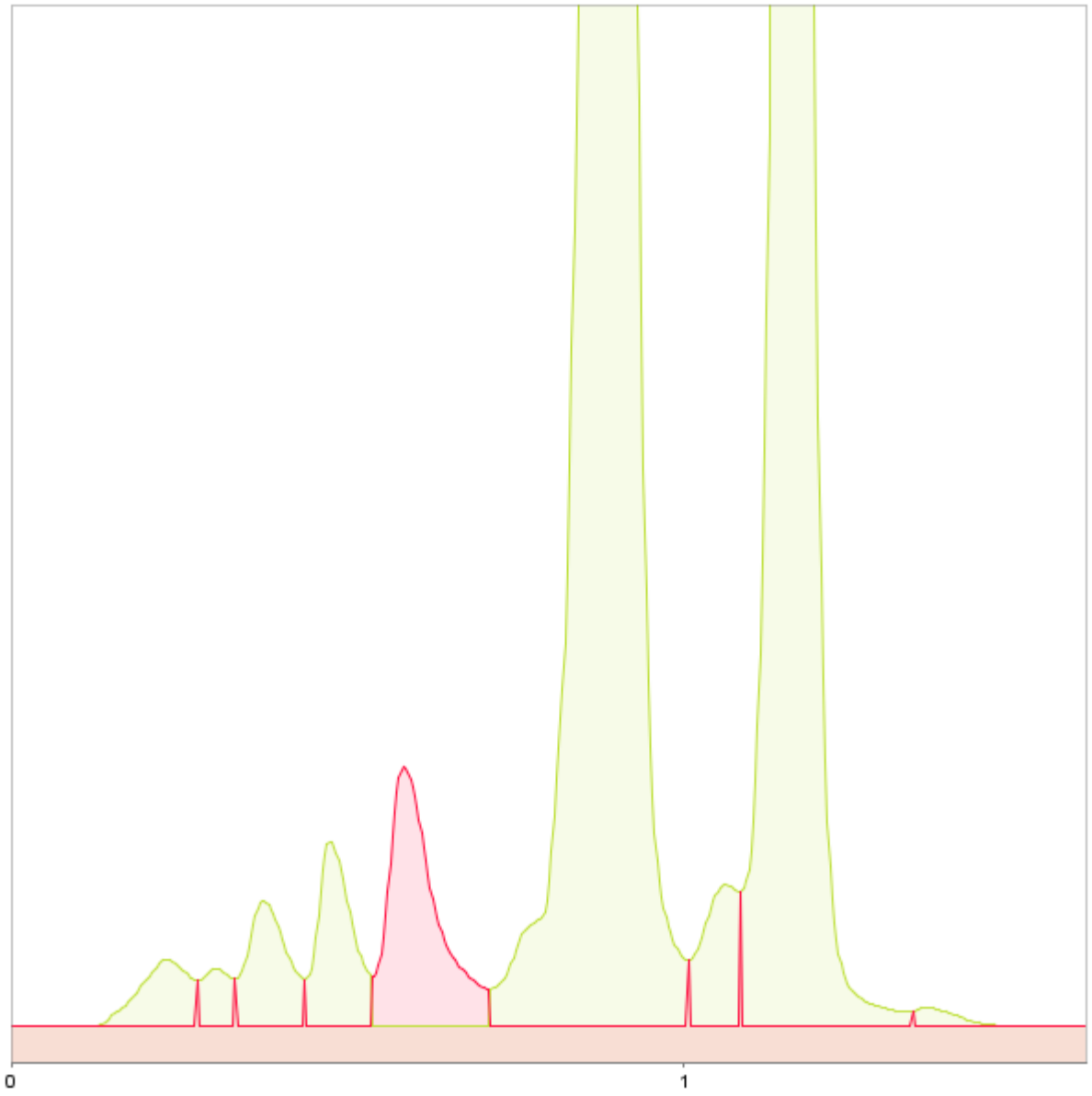
Wikipedia - Armando Moreno Vranich

HPLC advantage vs immuno-assay

- HPLC assays allow the detection of Hemoglobin variants through visual inspection of the chromatogram
- Especially important in patient groups where Hb variant prevalance is high (increasing globalization)
- Immuno-assays: you do not know if value is impacted by variant/HbF: black box



HbAS



Grafiek data

	%	Time (in minuten)	Area
A1A	1.02	0.23	8.79
A1B	0.53	0.31	4.53
F	1.04	0.38	13.41
LA1C+	1.98	0.47	17.02
SA1C	4.99	0.58	32.34
A0	92.70	0.88	795.74
H-V0	0.00		
H-V1	31.28	1.15	403.92
P00	1.02	1.06	13.22
P01	0.17	1.36	2.21

Tekst

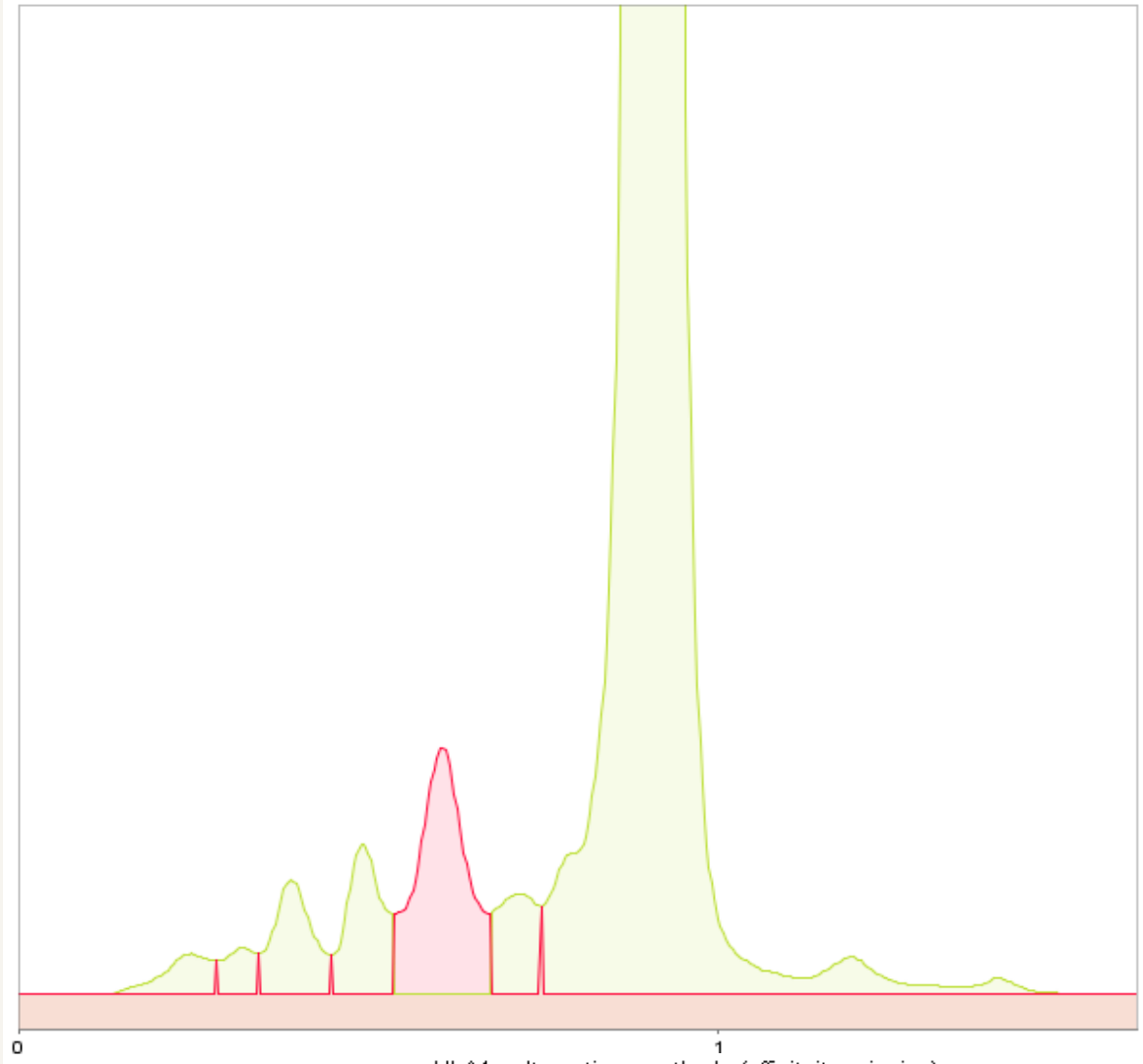
Unknown Peak /

HbA1c alternatieve methode (affiniteits principe)

Mogelijks abnormaal Hb? Te correleren met aanvraag hemoglobinopathie.

HbA1c (IFCC)	30	mmol/mol	20 - 42
HbA1c (DCCT)	5.0	%	4.0 - 6.0

HbAE



Grafiek data

	%	Time (in minuten)	Area
A1A	0.55	0.25	8.17
A1B	0.42	0.32	6.28
F	1.28	0.39	19.43
LA1C+	1.54	0.49	23.11
SA1C	5.77	0.61	52.36
A0	92.82	0.90	1390.67
H-V0	0.00		
H-V1	0.00		
H-V3	1.17	0.72	17.70

Tekst

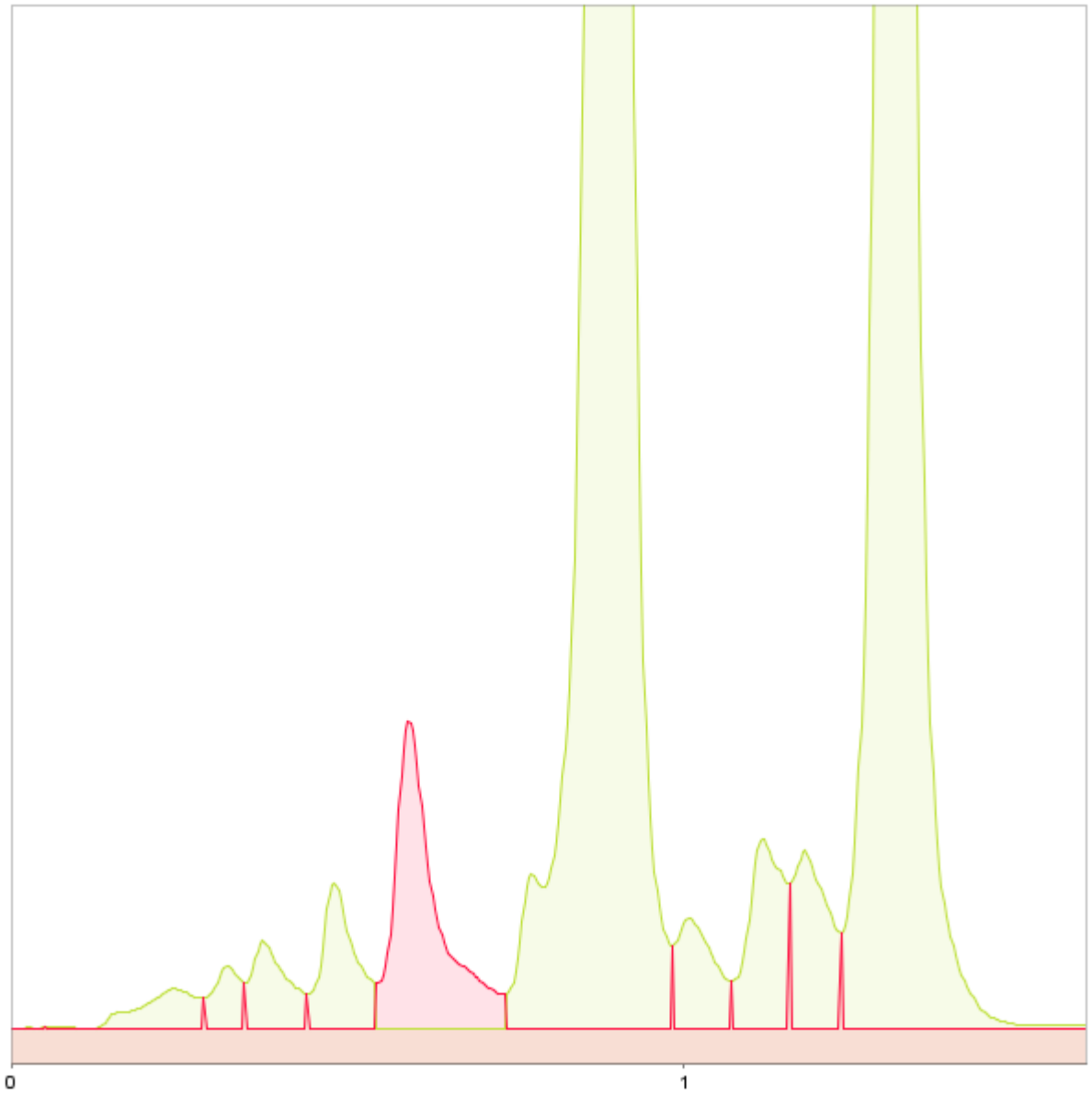
HBE Suspected /

HbA1c alternatieve methode (affiniteits principe)

Mogelijks abnormaal Hb? Te correleren met aanvraag hemoglobinopathie.

HbA1c (IFCC)	34	mmol/mol	20 - 42
HbA1c (DCCT)	5.3	%	4.0 - 6.0

HbAC



Grafiek data

	%	Time (in minuten)	Area
A1A	0.79	0.24	3.89
A1B	0.60	0.32	2.97
F	0.74	0.37	5.68
LA1C+	1.77	0.48	8.70
SA1C	5.99	0.59	22.97
A0	92.18	0.88	454.26
H-V2	30.84	1.31	236.06
P00	0.99	1.01	7.56
P01	1.55	1.12	11.89
P02	1.50	1.18	11.50

Total area 765.48

Toestel vlag

HB-var detect

Tekst

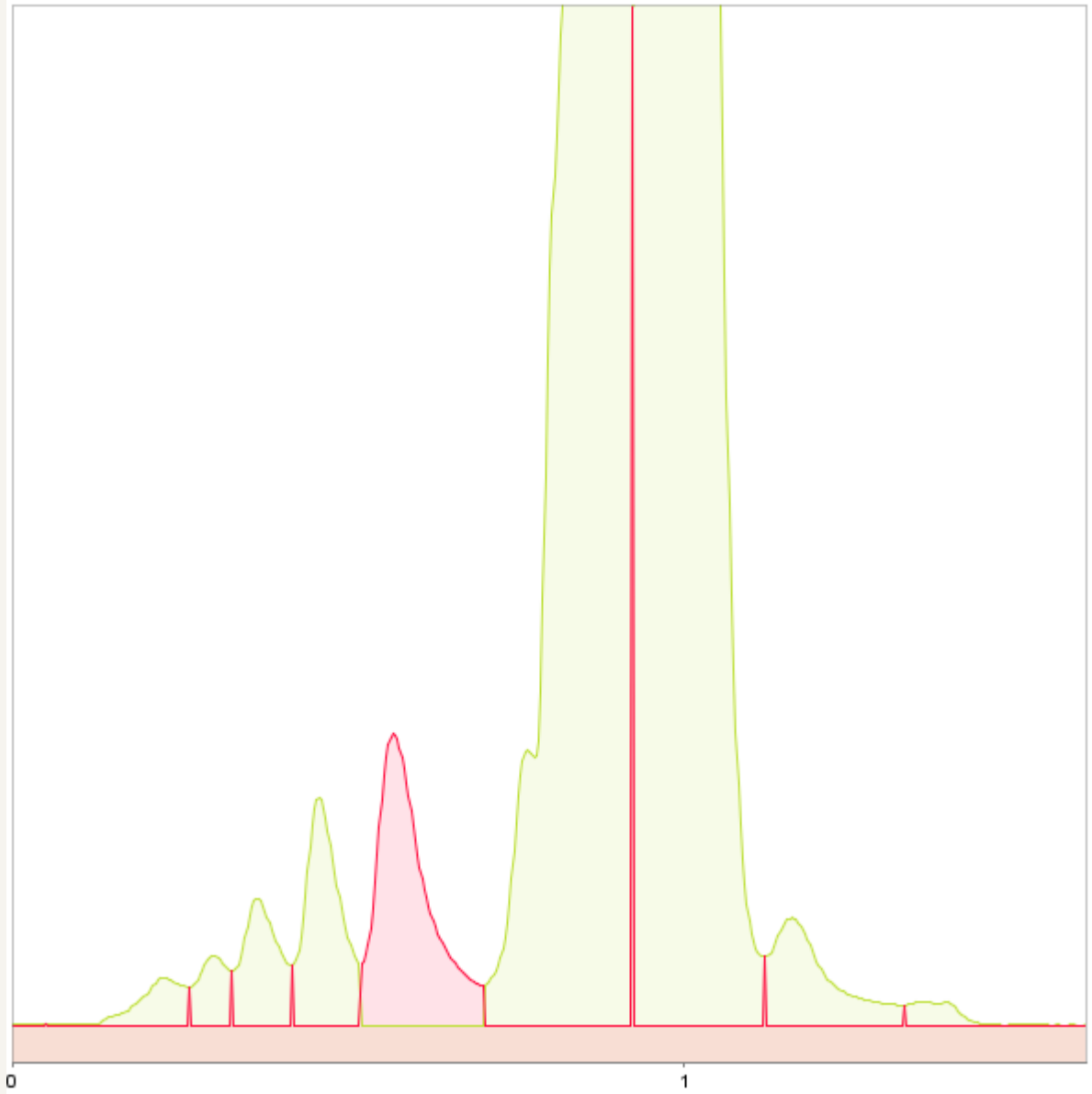
Unknown Peak /

HbA1c alternatieve methode (affiniteits principe)

Mogelijks abnormaal Hb? Te correleren met aanvraag hemoglobinopathie.

HbA1c (IFCC)	40	mmol/mol	20 - 42
HbA1c (DCCT)	5.9	%	4.0 - 6.0

HbAD



Grafiek data

	%	Time (in minuten)	Area
A1A	0.63	0.23	5.46
A1B	0.63	0.30	5.51
F	0.87	0.36	12.37
LA1C+	2.45	0.46	21.42
SA1C	5.68	0.57	38.32
A0	91.90	0.86	802.47
H-V0	36.61	1.00	522.89
H-V1	0.00		
P00	1.18	1.16	16.86
P01	0.21	1.36	2.97

Tekst

Unknown Peak /

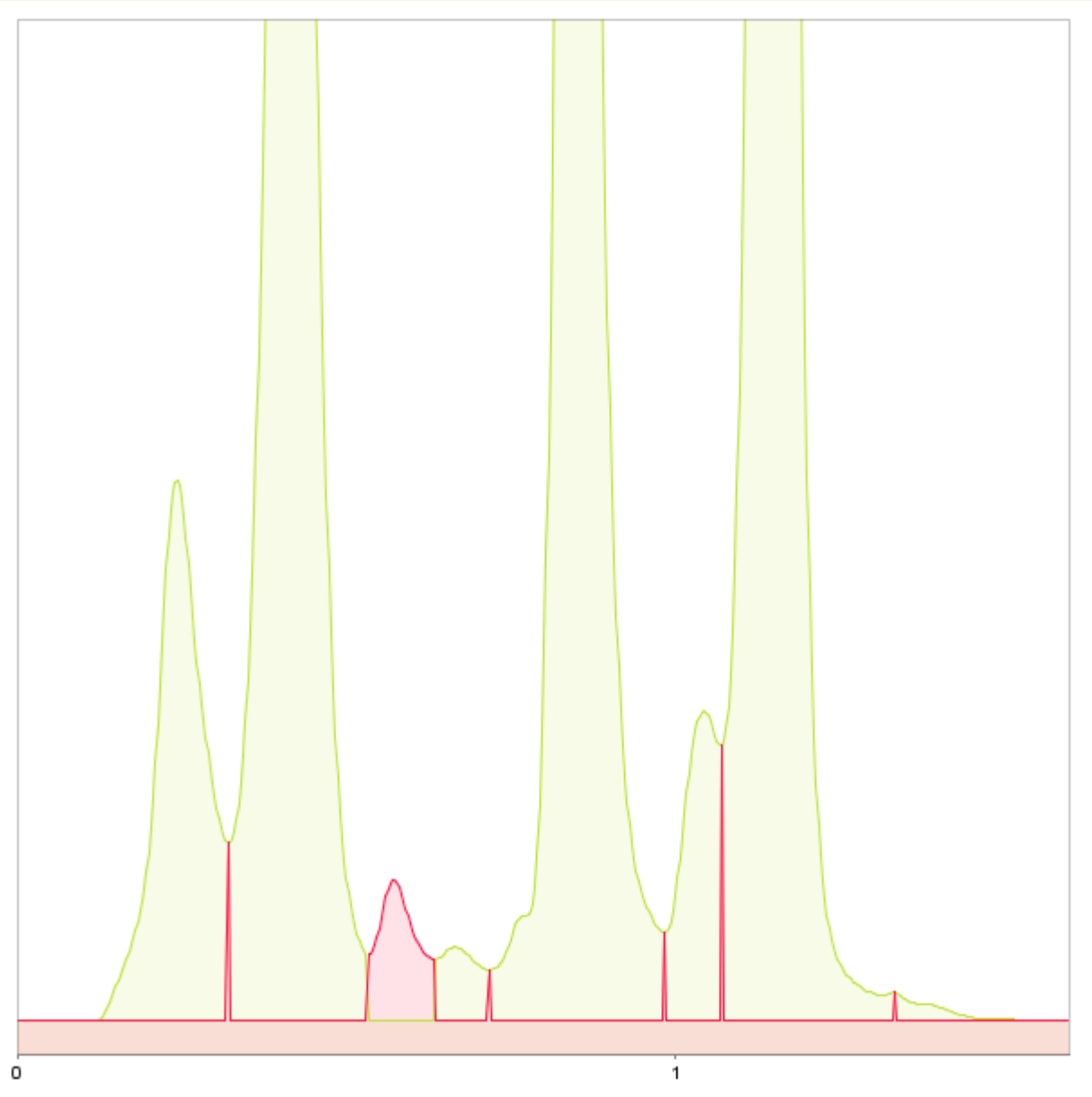
HbA1c alternatieve methode (affiniteits principe)

Mogelijks abnormaal Hb? Te correleren met aanvraag hemoglobinopathie.

HbA1c (IFCC)	38	mmol/mol	20 - 42
HbA1c (DCCT)	5.7	%	4.0 - 6.0

Elevated fetal hemoglobin (HbF)

- Most of the current ion-exchange HPLC methods separate normal levels of HbF into a separate peak
- In the past HbF fraction co-eluted with sA1c fraction and caused falsely low value
- Most methods are impacted when HbF levels are too high, generally above 15-30% as seen in Hereditary persistence of fetal hemoglobin (HPFH), very young patients or patients treated with hydroxyurea



HbF

Grafiek data

	%	Time (in minuten)	Area
A1A	18.91	0.24	75.51
F	22.61	0.42	320.19
SA1C	5.57	0.57	15.70
A0	74.99	0.85	299.36
H-V1	46.75	1.14	662.13
H-V3	0.61	0.66	8.65
P01	2.25	1.04	31.89
P02	0.20	1.34	2.80

Total area 1416.23

Toestel vlag

HBE suspected

Tekst

HbF > 22% / Unknown Peak / Peak missing / HBE Suspected /

Carbamylated hemoglobin (carbHb)

- Occurs in CKD through uremia – urea dissociates into cyanate ions
- Non-enzymatic condensation of cyanate with the N-terminal valine of hemoglobin
- In older separation techniques gave rise to falsely high HbA1c
- Newer HPLC analyzers separate carbHb better and see no interference

Lipemia and icteria

- Severe hypertriglyceridemia (>1,750 mg/dL) and severe hyperbilirubinemia (>20 mg/dL) may falsely increase HbA1c values with immuno-assays
- Falsely low HbA1c concentrations on Roche c501 immune assay with triglycerides >1300mg/dL
- Separation methods unaffected

Clinical interferences

Clinical Interferences						
	Iron deficiency	Shortened Erythrocyte Survival	Increased Erythrocyte Survival	Chronic Kidney Disease	Age	Liver Disease
Influence on HbA1c	Increase	Decrease	Increase	Variable	Increase	Decrease

Increased RBC turnover

- Any clinical situation that enriches the red blood cell pool with younger erythrocytes
- Hemolytic anemia, acute blood loss, recent transfusion, hypersplenism
- Will lower HbA1c for any given level of blood sugar, regardless of method
- Glycated albumin or fructosamine should be used in these patients or wait till normalization/treated (if possible)

Decreased RBC turnover

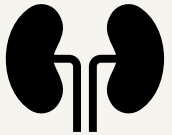
Technical correct measurement but no true reflection of glycemic control!!

- Splenectomy or hyposplenism
- Increases mean age of RBC's – longer exposition to plasma glucose
- Higher HbA1c for any given level of blood sugar, regardless of method

Iron Deficiency Anemia (IDA)

- Associated with higher HbA1c and Fructosamine levels
- Son et al. higher mean absolute HbA1c of 0,3% - can be important in diagnosis
- Iron therapy lowers HbA1c levels in these patients – importance of substitution
- In late pregnancy HbA1c is often increased due to IDA
- Suggested mechanism through malondialdehyde – enhances Hb glycation rate

Chronic Kidney Disease (CKD)



- Renal disease develops in many diabetic patients – diabetic nephropathy
- Value of HbA1c in CKD remains controversial
- Difficult to evaluate the interplay of different factors: carbHb, renal anemia, erythropoietin intake, dialysis,..
- Recent studies propose use of Glycated Albumin – better correlation with clinical outcomes and mean plasma glucose

Liver disease

- Chronic liver disease may cause false low HbA1c – especially in decompensated cirrhosis
- Lower HbA1c possible even in absence of overt cirrhosis
- Possible mechanisms are macrocytic/hemolytic anemia – hypersplenism
- In known patients, HbA1c only to be used in context of full liver set and RBC parameters
- Further studies necessary to clarify exact mechanisms

Age and race

- Some studies suggest increase of 0,1% per decade increase in age
- Possibly higher HbA1c in African Americans and Hispanic populations (0,1 tot 0,4%)
- Clinical impact is currently under discussion

Other factors

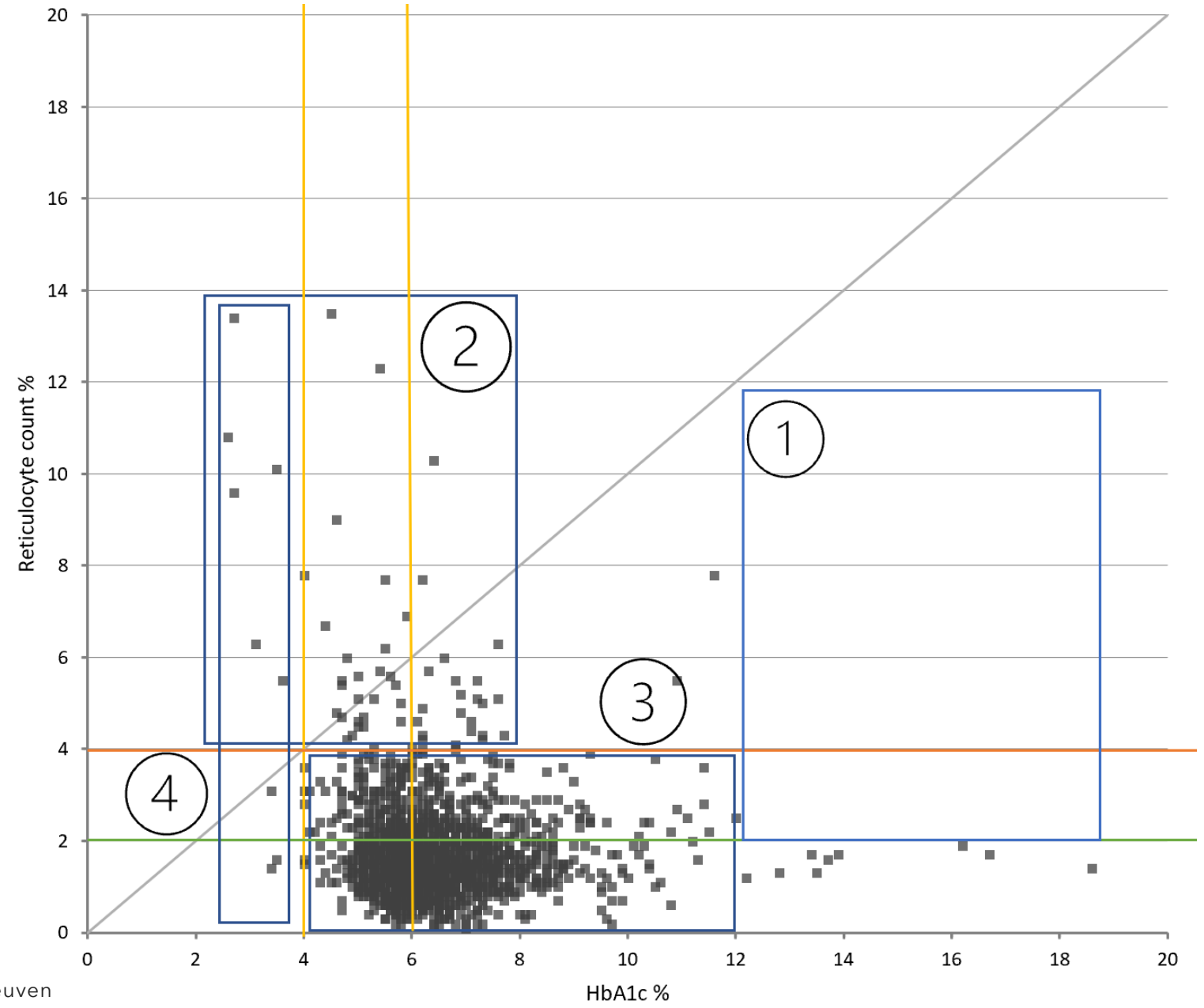
- High Vitamine C & E intake >1g/day decreases HbA1c in non-diabetic subjects – through glycation inhibition? Relevance unclear
- Lead poisoning, chronic alcohol intake (acetaldehyde-HbA1c compound), salicylates, opioids have been reported to falsely increase HbA1c – mechanisms unclear

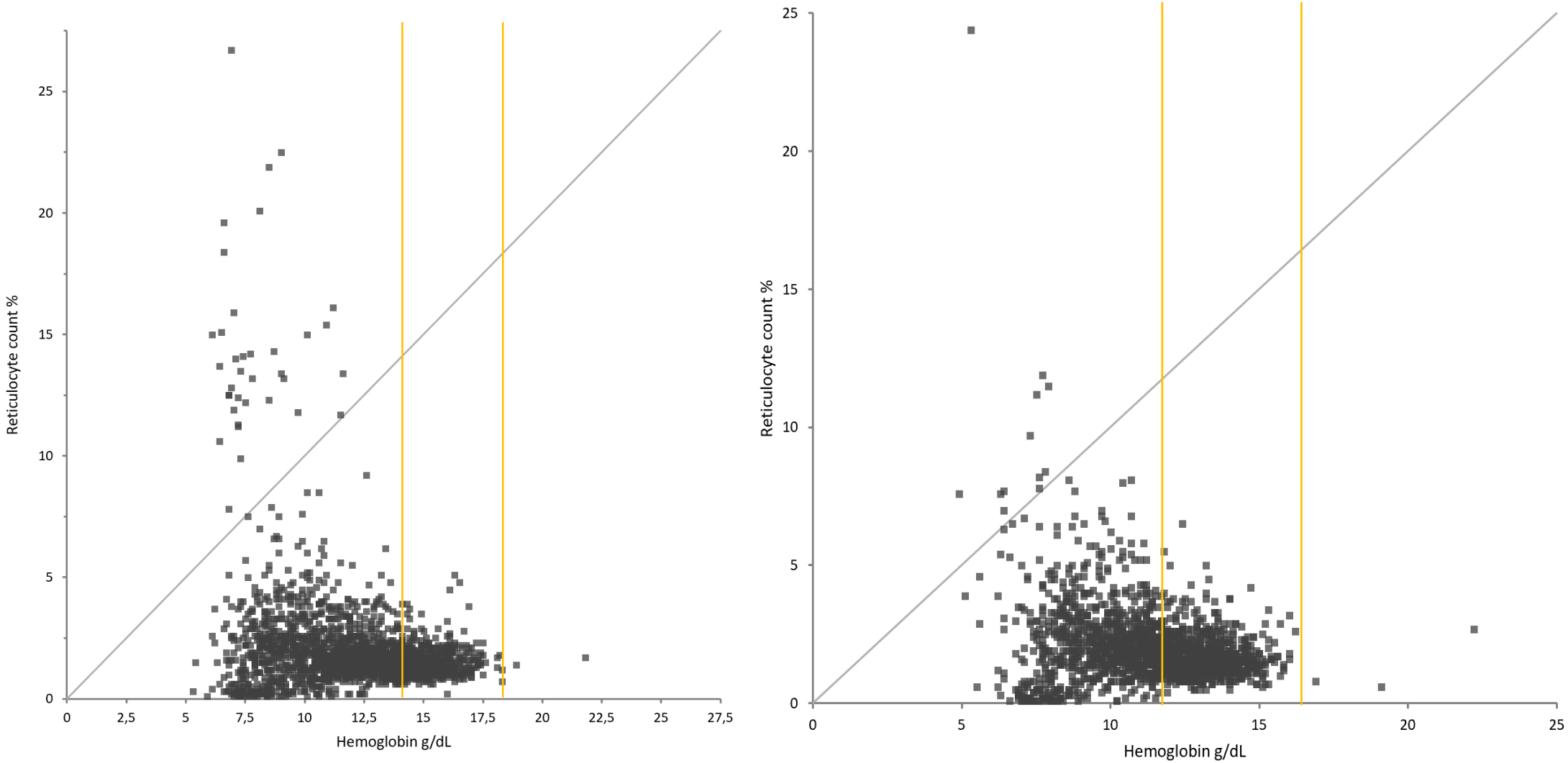
Can reticulocytes help interpret HbA1c values and should they routinely be determined?

Currently only 2,9% of all HbA1c requests are accompanied by a reticulocyte count in UZ Leuven, while 14% of all HbA1c requests are accompanied by a Hb measurement

Could help clinicians pick up increased red blood cell turnover and thus be aware of falsely lowered HbA1c

Reticulocytes correlation with HbA1c (n=1782)





Hb correlation with reticulocyte count – males- females n= 2106 and 1893

Three propositions in our lab

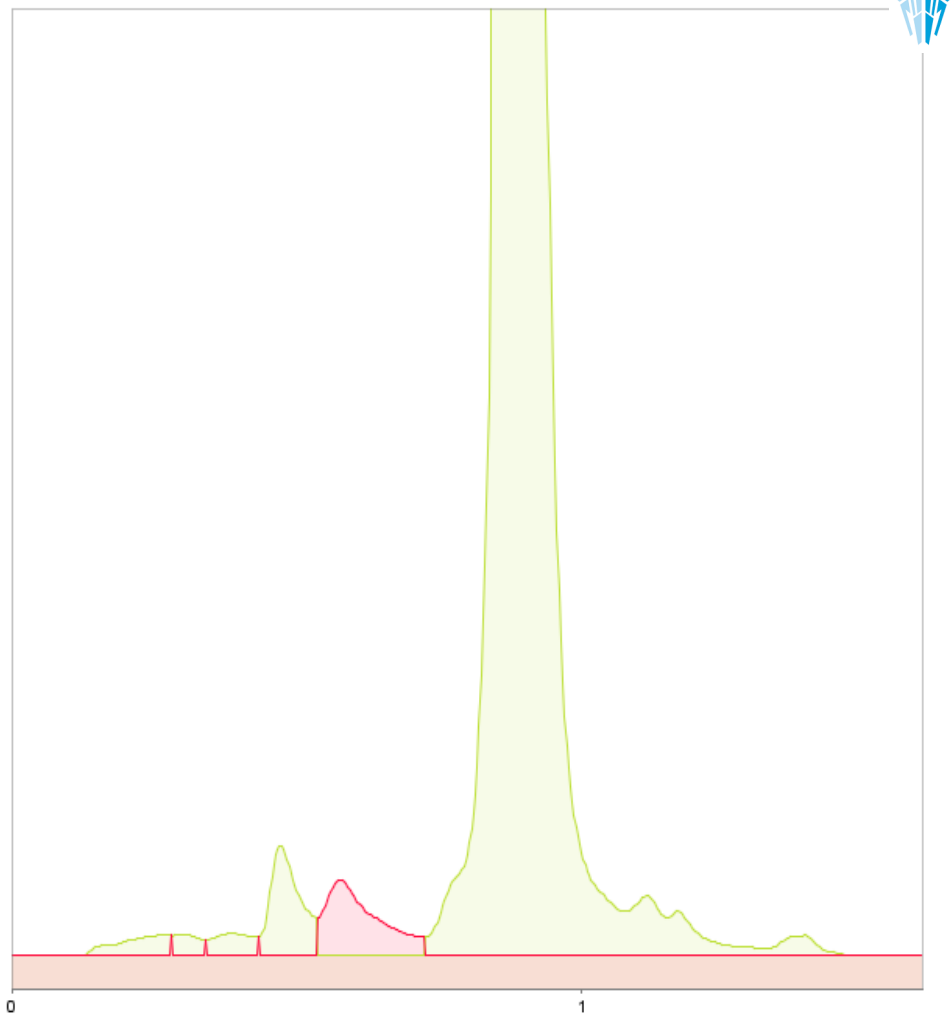
1. A comment on the laboratory report when reticulocytes are elevated (>2%): 'correlate with glycemic profile/estimated HbA1c'.

2. Suggest that all HbA1c measurements should be accompanied by a hemoglobin measurement. When hemoglobin levels are decreased (below 14 g/dL in males and below 12 g/dL in females), reflex order a reticulocyte count. Inform the clinician that they should also order a reticulocyte count when there is clinical suspicion of increased red blood cell turnover (including other hemolysis parameters as haptoglobin, bilirubin, LDH).

3. For HbA1c values lower than 4%, reflex order a reticulocyte count.

Grafiek data

	%	Time (in minuten)	Area
A1A	0.00	0.23	3.64
A1B	0.00	0.27	2.01
F	0.00	0.39	3.18
LA1C+	0.00	0.47	11.40
SA1C	2.41	0.58	13.68
A0	0.00	0.87	804.73
H-V0	0.00		
H-V1	0.00		



HbA1c niet uitvoerbaar o.w.v. mogelijke analytische interferentie

Resultaat HbA1c niet betrouwbaar gezien sterk verhoogde RBC turnover of jonge RBC (cfr. verhoogd aantal reticulocyten). Graag controlestaal na normalisatie hemoglobine en reticulocytose.

Hemoglobine	9.6	g/dL	14.0 - 18.0
Hematocriet	0.309		0.400 - 0.540
RBC telling	2.61	10**12/L	4.50 - 6.00
MCV	118.4	fL	76.0 - 96.0
MCH	36.8	pg	27.0 - 32.0
MCHC	31.1	g/dL	30.0 - 35.0
RDW	15.9	%	11.7 - 14.5
Reticulocyten telling	283	10**9/L	20 - 100
Reticulocyten telling	10.9	%	0.5 - 2.0
Glucose	89	mg/dL	55 - 100

HbA1c niet uitvoerbaar o.w.v. mogelijke analytische interferentie

Resultaat HbA1c niet betrouwbaar gezien sterk verhoogde RBC turnover of jonge RBC (cfr. verhoogd aantal reticulocyten). Graag controlestaal na normalisatie hemoglobine en reticulocytose.

What is the analytic influence of some common hemoglobin variants with tosoh G8 in UZ Leuven?

VS

Tosoh G8 HPLC

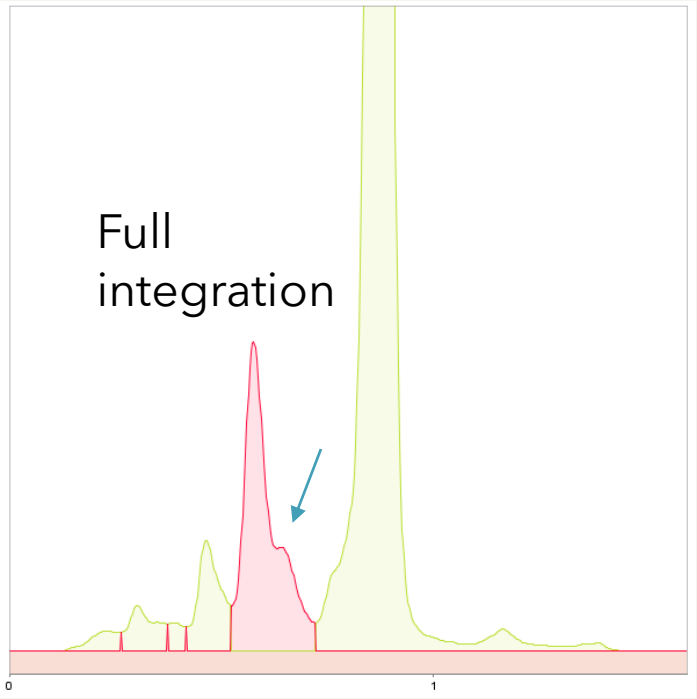
Alere Afinion Boronate Affinity

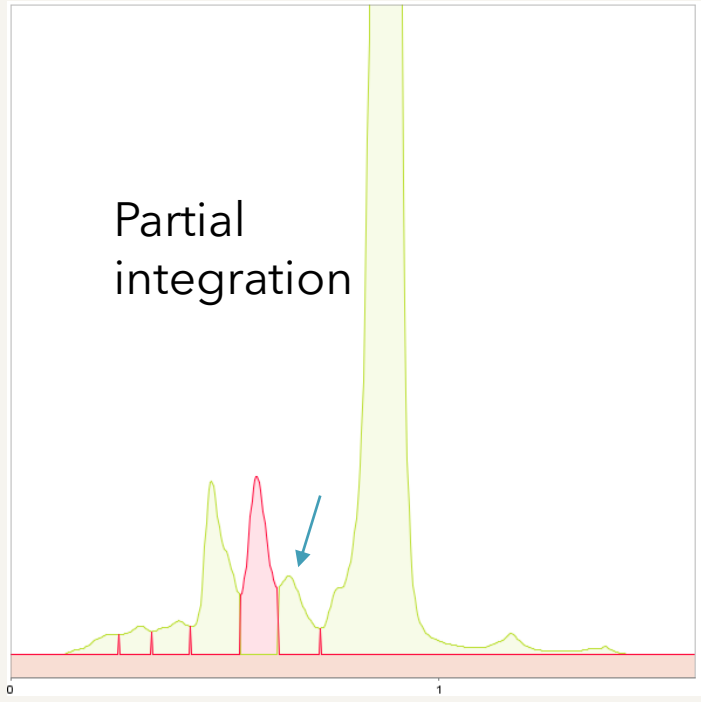
Hemoglobin variant	n	mean abs difference % (NGSP)	95% CI % (NGSP)	correlation coefficient	Intercept 95% CI	Slope 95% CI
Hb S	27	0,164	-0,240 to 0,569	0,961	-0,98 to 0,82	0,89 to 1,20
Hb C	7	-0,113	-0,490 to 0,264	0,975	-6,84 to 1,79	0,63 to 2,00
Hb D	12	-0,19	-0,067 to 0,447	0,993	-1,45 to 0,25	1 to 1,29
Hb E	21	0	-1,071 to 1,072	0,619	0,40 to 2,84	0,45 to 0,90
Hb Riccarton*	20	0,2	0,264 to 0,667	0,988	-0,47 to 1,26	0,80 to 1,09
Hb J-Baltimore	5	0,764	-1,532 to 3,060	0,184	-1,23 to 8,69	-0,56 to 1,63
Hb G-Siriraj	3	-1,063	-2,035 to -0,092	0,789	-1,48 to 2,13	0,47 to 1,11

Parameter	Excellent	Goed	Acceptabel	Slecht	Onacceptabel
Afwijking Doelwaarde	<0.2%	0.2 - 0.29%	0.30 - 0.39%	0.40 - 0.49%	≥ 0.50%
Reproduceerbaar-heid (CV)*	<1.4%	1.4 – 1.99%	2.0 – 2.99%	3.0 – 3.99%	≥ 4%
Lineariteit (r)	>0.9970	0.9950-0.9970	0.9900- 0.9949	0.9800-0.9899	<0.9800

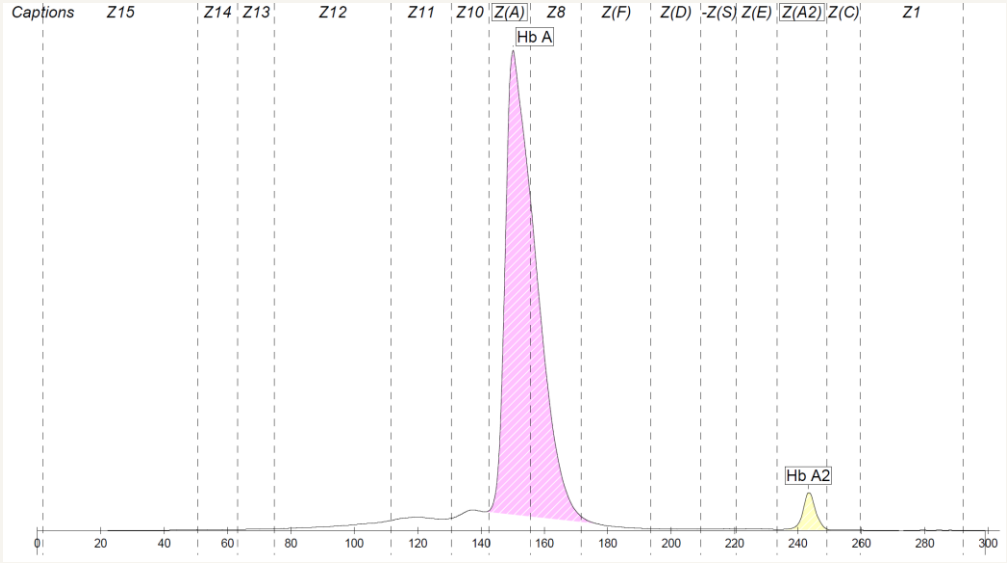
Sciensano criteria for EKE evaluation

Hb Riccarton





Hemoglobin variant	n	Mean abs difference % (NGSP)	95% CI % (NGSP)	correlation coefficient
Hb Riccarton (all)	30	0,03	-0,6 to 0,7	0.967
Full integration	20	0,20	-0,26 to 0,66	0.988
Partial integration	10	-0,31	-0,78 to 0,16	0.948



Follow up HbA1c in common Hb variants UZ Leuven

- HbAS: Tosoh G8 for follow up
- HbAC: Tosoh G8 for follow up
- HbAD: Tosoh G8 for follow up
- HbAE: Alere Afinion, until more experience with new software version 5.28
- Hb Riccarton: Tosoh G8 when fully integrated, Alere Afinion when partially integrated
- Hb Raleigh: interference from both HPLC and Boronate Affinity-> fructosamine/GA

Always compare measurements of both methods the first time you encounter a new variant in a patient (if you have boronate affinity)!

To Do's

1) Add a comment to the lab rapport when reticulocytes are above 2% 'correlate with glycemic profile/estimated HbA1c'.

2) Discuss these findings with the endocrinology department : what is their clinical assessment when estimated A1c values from GCM show discrepancies with lab measured HbA1c and how do they feel about adding Hb or reticulocytes to their HbA1c lab request.

Thanks for your attention!

Special thanks to Christine Van Laer and Mercedeh Tajdar