Astana, 19.9.2018

Flow cytometry for the diagnosis of human immunodeficiency

Ulrich Sack, Leipzig University

I thank Michael Borte, Andreas Boldt, Stephan Borte, and Aydan İkincioğulları for slides and input

Disclosure of Conflicts of Interest

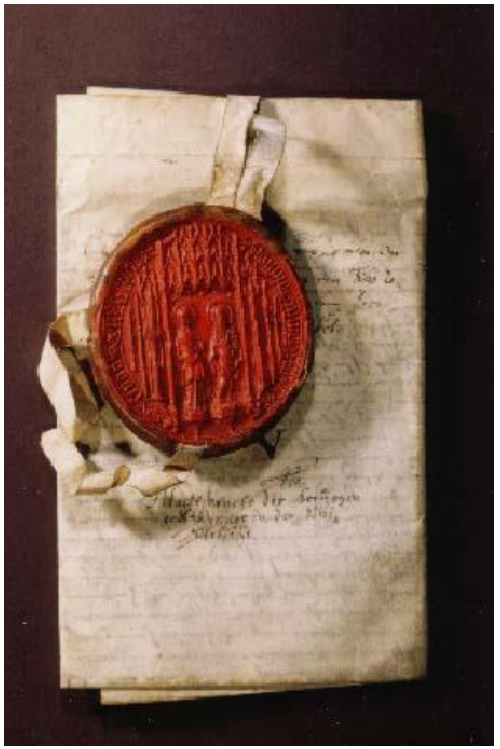
Membership in INSTAND e.V. and DGKL e.V.
Technical Assessor for INAB and DAkkS
IFCC WG-FC supported by Beckman-Coulter

A Tradition of Crossing Boundaries

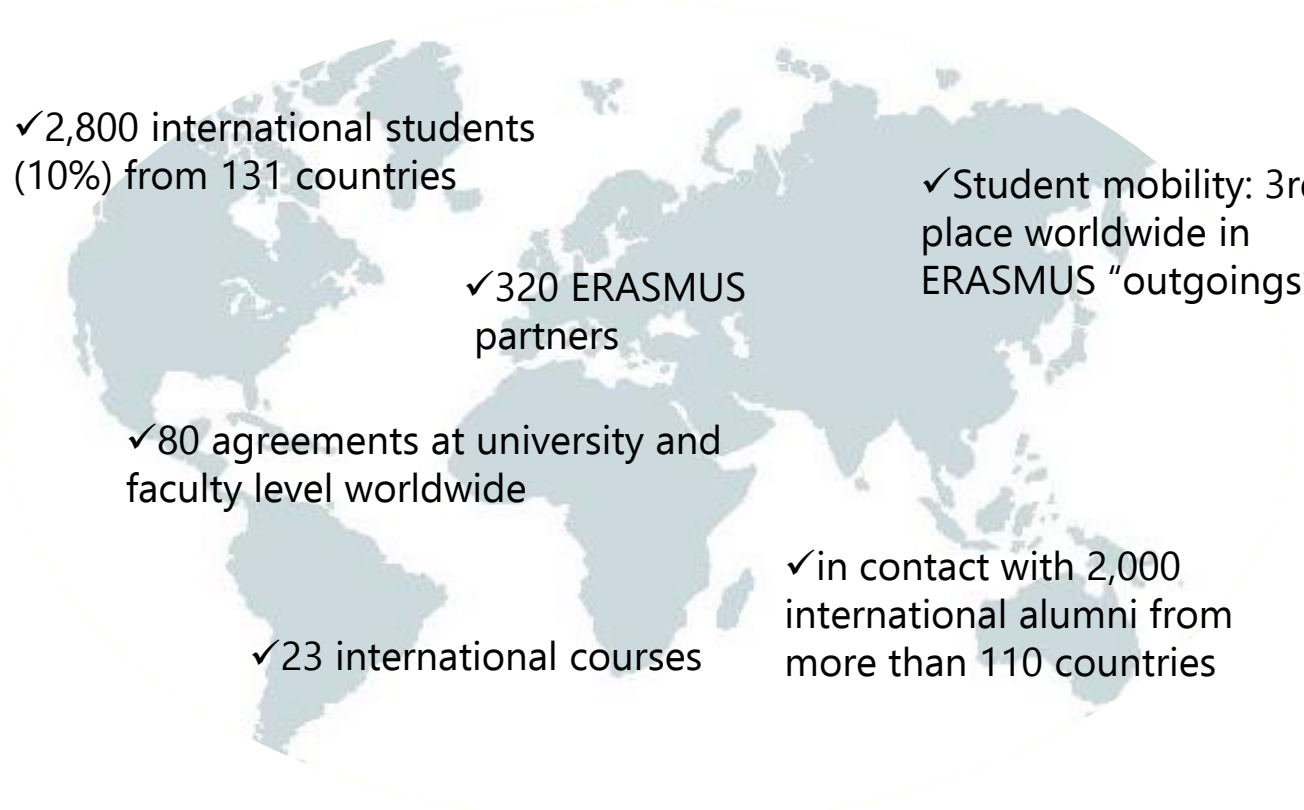
Campus on Augustusplatz at the start of the 20th century and the 21st century: "...in Leipzig something new is evolving which at the same time clearly brings our own history to mind." (Prof. Dr. Frank Zöllner)

The second oldest

- 1409: founding of Universität Leipzig by masters graduates and professors from Prague



International – open to the whole world



✓2,800 international students (10%) from 131 countries

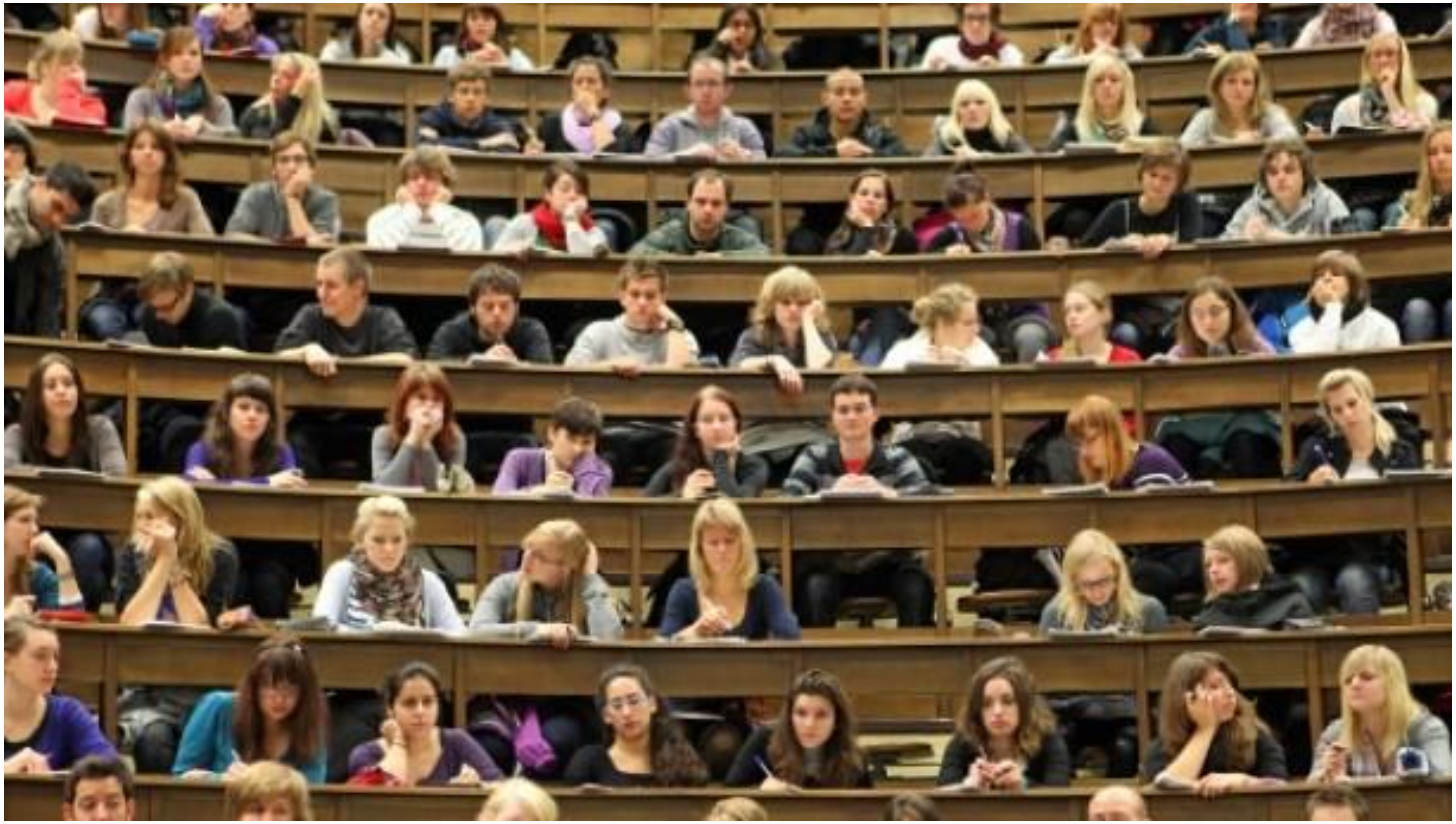
✓320 ERASMUS partners

✓80 agreements at university and faculty level worldwide

✓23 international courses

✓Student mobility: 3rd place worldwide in ERASMUS "outgoings"

✓in contact with 2,000 international alumni from more than 110 countries



Institut für Klinische Immunologie, Medizinische Fakultät der Universität

Significant alumni from Universität Leipzig

Wundt – Heisenberg – Ostwald – Hertz – Litt – Leibniz – Müntzer – Lessing – Goethe – Wagner – Nietzsche – Kästner ...

Today more than 150,000 alumni from Universität Leipzig live all over the world, including:

- *Hans-Dietrich Genscher*, 1946-1949, studied Law
- *Angela Merkel*, 1973-1978, studied Physics

A flavour of Leipzig

Trading centre since 1165 – “the mother of all trade fairs”

New potential in the automotive and supply industry, biotechnology, and media and life sciences

Leipzig is the cradle of the 1989 Peaceful Revolution

Diverse cultural and student life: 40,000 students among 500,000 inhabitants

Very reasonable accommodation and good quality of life

Content

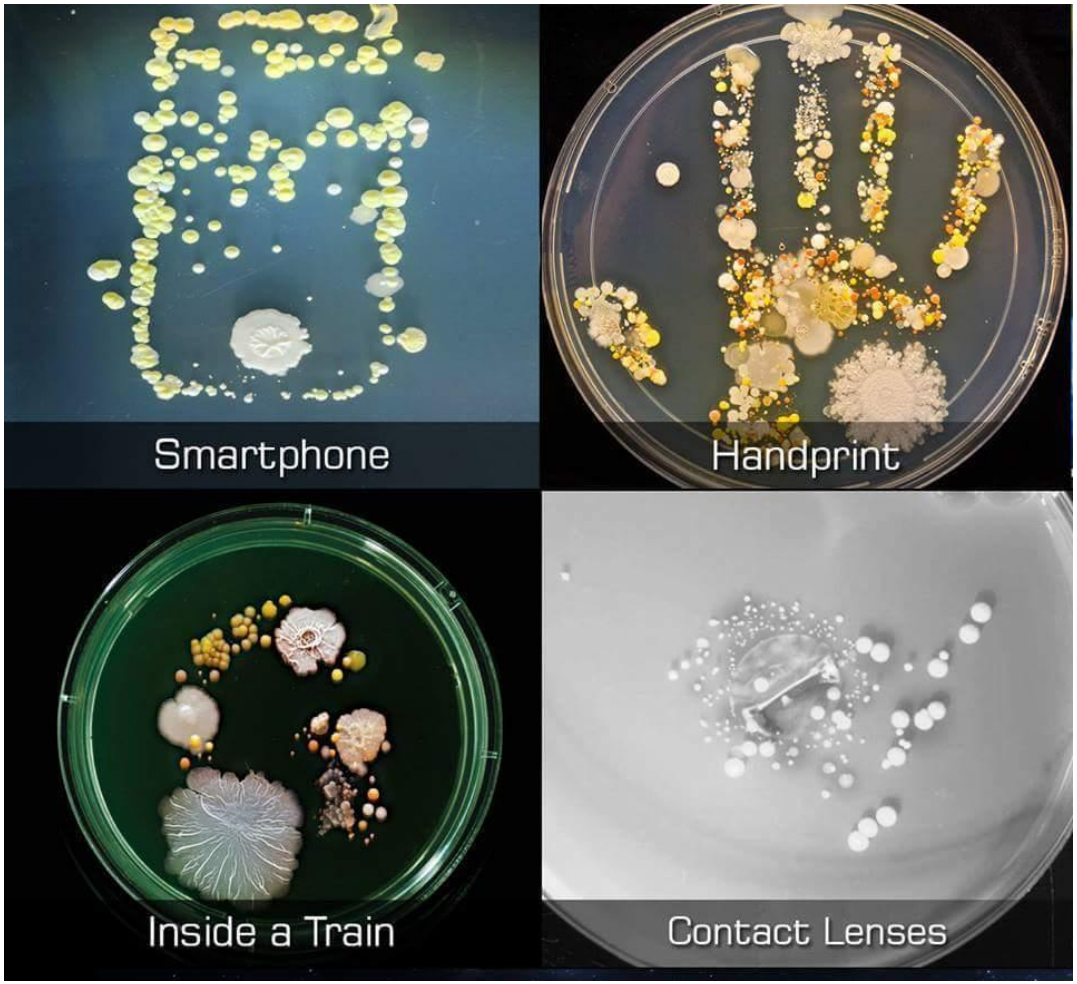
- Immunodeficiencies
- PID – rare diseases?
- Newborn screening for PID
- PID flow cytometry
- PID flow cytometric screening
- Functional testing

Immunodeficiencies

- Secondary immunodeficiencies
  - Infectious?
  - Induced by malnutrition, chronic diseases, stress
  - Local focus on ID induced by HSCT, immunotherapies, irradiation, …
- PID
  - Not only in children
  - Main topic in this presentation

Introduction: PID classification and relevance

Primary immunodeficiency diseases (PIDs) are a genetically heterogeneous group of disorders affecting distinct components of the innate and adaptive immune system. Clinical representation is dominated by infectious diseases.

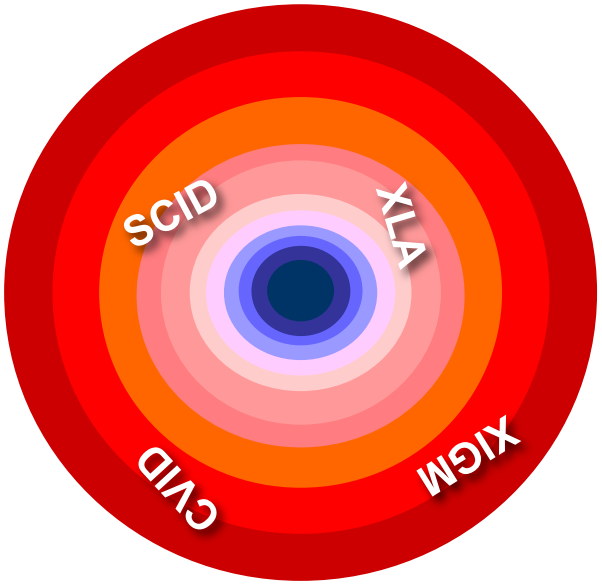


- more than 280 primary immunodeficiency diseases defined
- immunodeficiency signs part of countless inherited diseases

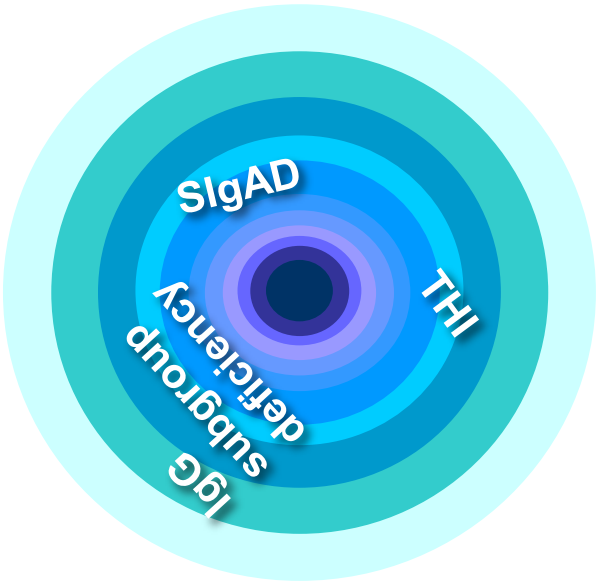
Disease Fact file OMIM
C1r deficiency 51 OMIM:216950
C1s deficiency 52 OMIM:120580
C2 deficiency 90 OMIM:217000
C3 deficiency 61 OMIM:120700
C4 deficiency
Disease Fact file OMIM
C4A deficiency 53 OMIM:120810
C4B deficiency 54 OMIM:120820
C5 deficiency 91 OMIM:120900
C6 deficiency 92 OMIM:217050
C7 deficiency 93 OMIM:217070
C8 deficiency
Disease Fact file OMIM
C8 α-polypeptide deficiency 55 OMIM:120950
C8 β-polypeptide deficiency 56 OMIM:120960
C8 γ-polypeptide deficiency 57 OMIM:120930
C9 deficiency 94 OMIM:120940
Defects of the alternative complement pathway
Factor B deficiency 95 OMIM:138470
Factor D deficiency 98 OMIM:134350
Factor H1 deficiency 101 OMIM:134370
Properdin factor C deficiency 100 OMIM:312060, OMIM:300383
Defects of complement regulatory proteins
Hereditary angioedema 97 OMIM:106100, OMIM:606860
C4-binding protein deficiency
Disease Fact file OMIM
C4 binding protein α deficiency 58 OMIM:120830
C4 binding protein β deficiency 59 OMIM:120831
Decay-accelerating factor (CD55) deficiency 102 OMIM:125240
Factor I deficiency 99 OMIM:217030
CD59 deficiency 103 OMIM:107271
Mannose-binding lectin deficiency
Disease Fact file OMIM
Mannose-binding lectin deficiency 96 OMIM:154545
Mannan-binding lectin - associated serine protease 2 deficiency 119 OMIM:605102
Periodic fever syndromes
Familial mediterranean fever 140 OMIM:608107
Hyperimmunoglobulinemia D with periodic fever syndrome 141 OMIM:260920, OMIM:251170
Tumor necrosis factor receptor-associated periodic syndrome 142 OMIM:142680, OMIM:191190
Cold autoinflammatory syndrome
Disease Fact file OMIM
Familial cold urticaria and Muckle-Wells syndrome 143 OMIM:120100, OMIM:191900, OMIM:606416
Chronic infantile neurological cutaneous and articular syndrome 144 OMIM:607115, OMIM:606416
Granulomatous sinovitis with uveitis and cranial neuropathies 145 OMIM:186580, OMIM:605956

Clinical Presentation/Natural Course

Severe



Mild/asymptomatic

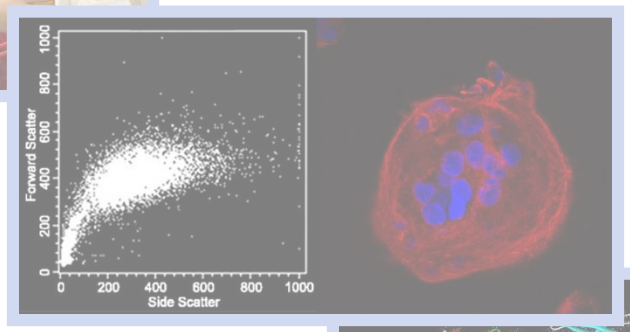


Classification of Primary Immunodeficiencies

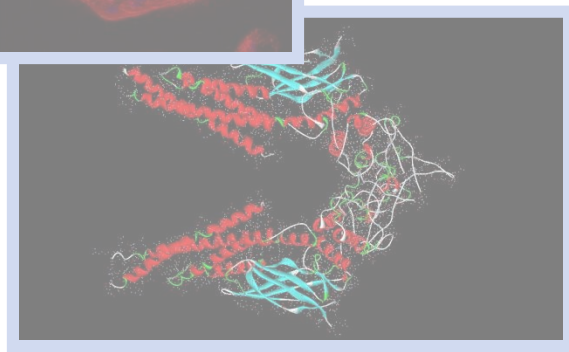
- Combined B and T cell immunodeficiencies
- Predominantly antibody deficiencies
- Autoinflammatory disorders
- Diseases of immune regulation
- Defects of the innate immune system
- Defects of phagocyte number, function, or both
- Complement deficiencies
- Other well-defined immunodeficiencies
- Syndromes with PID

Screening levels for primary immunodeficiencies

Symptomatology and
clinical diagnostics

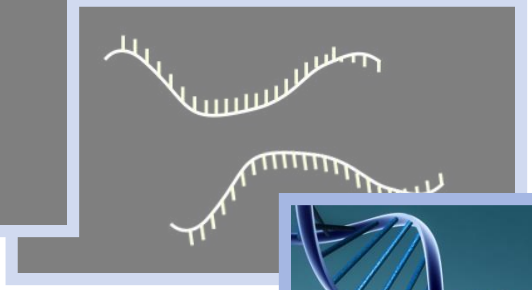


Single-cell
function & phenotype



Proteins and
cellular intermediates

RNA species



Genomic DNA
and excision products

Are PID really „rare“ diseases ?

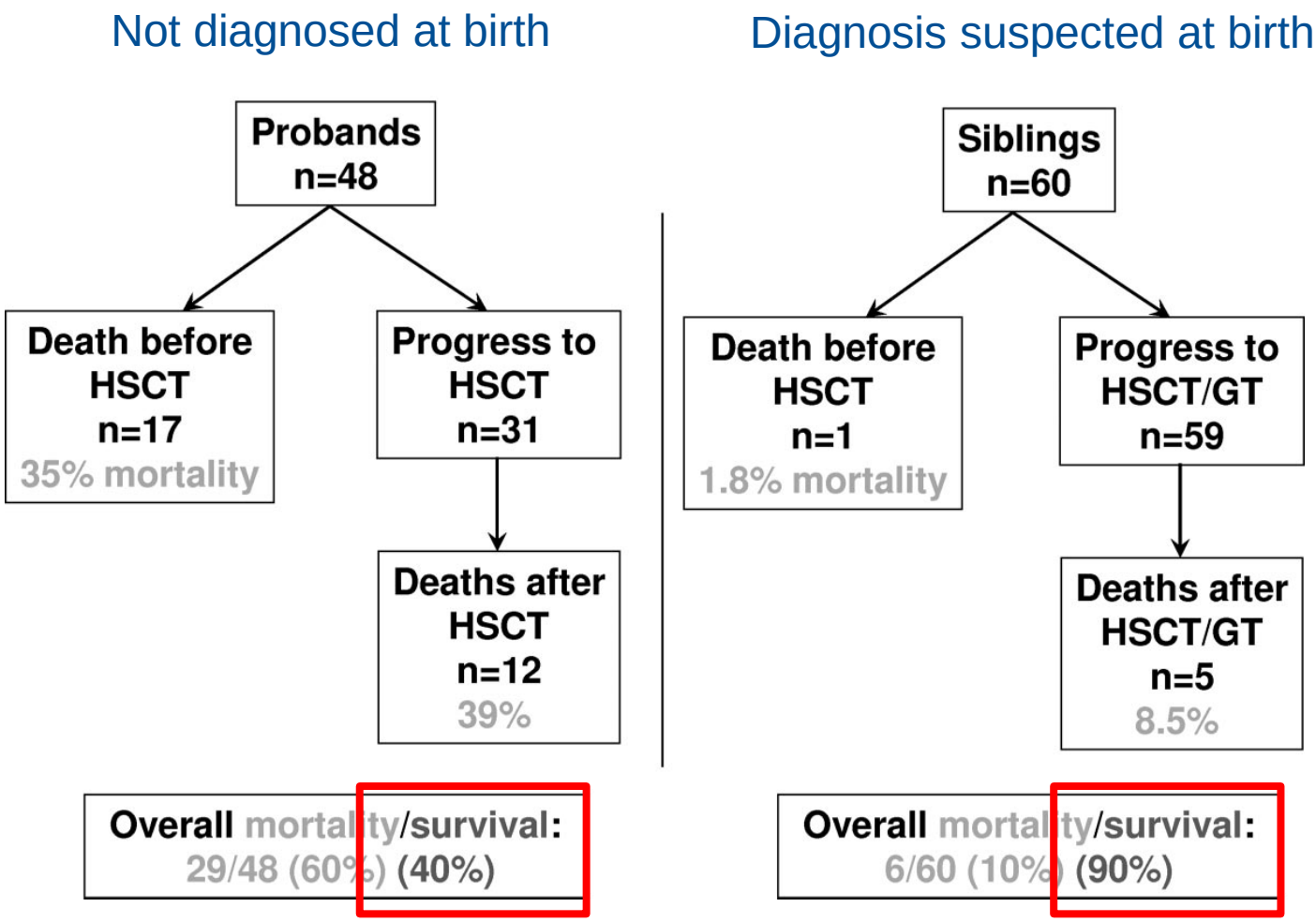
• Rheumatoid arthritis	1 : 100-200
• Gluten enteropathy	1 : 200-800
• **Primary immunodeficiencies (PID)**	**1 : 250-500**
• Type 1 diabetes	1 : 600-800
• Multiple sclerosis	1 : 800-1000

Diagnostic delay of all PID entities by age

Age group	Diagnostic delay (years) Median (IQR)	*p* value
< 16	1.2 (0.3, 4.6)	<0.001
16 +	3.2 (0.8, 9.7)	

- Differing PID awareness of medical specialists AND patients
- Adult-onset of distinct immunodeficiency syndromes
- Single-gene defects vs polygenic / complex predisposition
- Epigenetic, environmental and iatrogenic factors

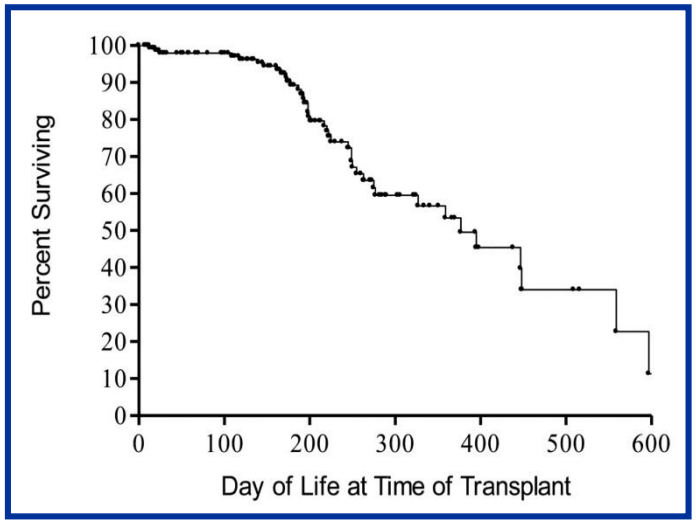
Rationale of neonatal screening for SCID



Severe Combined Immunodeficiency (SCID)
is a Pediatric Emergency

Untransplanted patients die within
the first year of life

Early (<3.5 months of age) identic/non-identic transplants
are life saving



Buckley RH et al. NEJM, 1999

1982-2011, 169 SCID

Age at transplant <3.5 mo (n=49): 94% live

3 pts. >30 yrs.

Newborn screening for severe PID

Newborn screening for severe PID

Clinical presentation

- Heterogeneous group of inborn disorders
- Lack of functional T cells +/- B and NK cells
- Overwhelming fungal, viral and bacterial infections
- Fatal <12 mo if undiagnosed and untreated
- Pediatric emergency

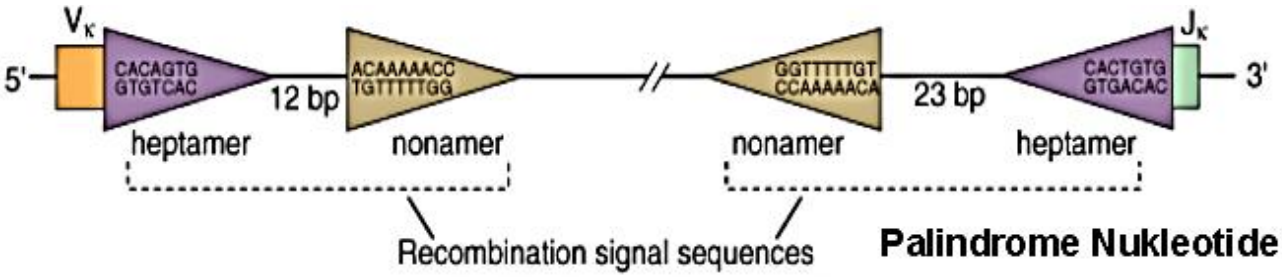
Hallmarks applicable to neonatal screening

- Severe T cell lymphopenia
- Severe B cell lymphopenia

„Screening“ for severe T-lymphopenia

Biomarker analysis	**Flow cytometry**	**Molecular cytometry**
T lymphocyte correlated proteins (IL-7 ELISA, CD3/CD45 assay)	Surface markers	TRECs (T cells)
• *false negatives*	• *sample source* • *throughput ?* • *analysis ?*	KRECs (B cells)
		Signature transcripts

Somatic recombination



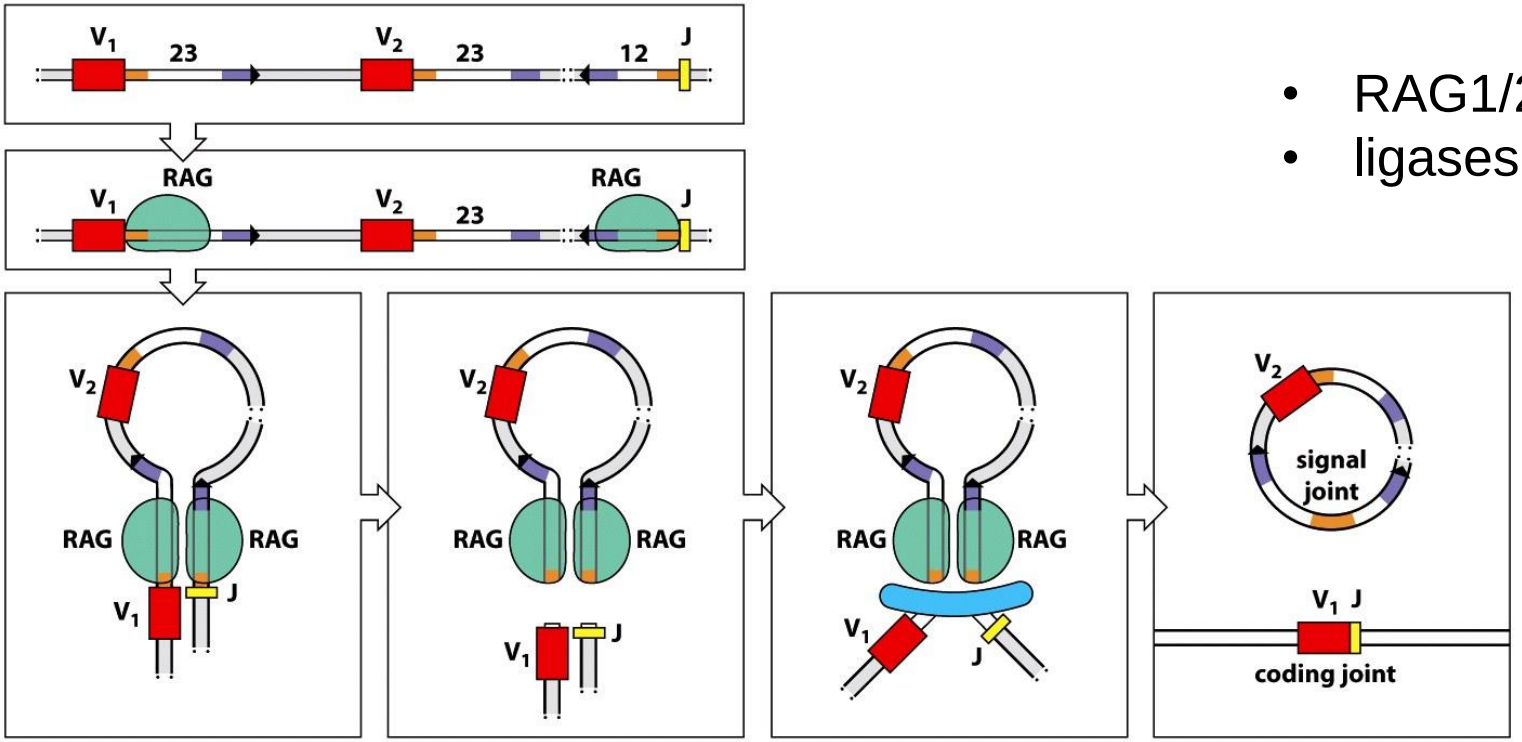
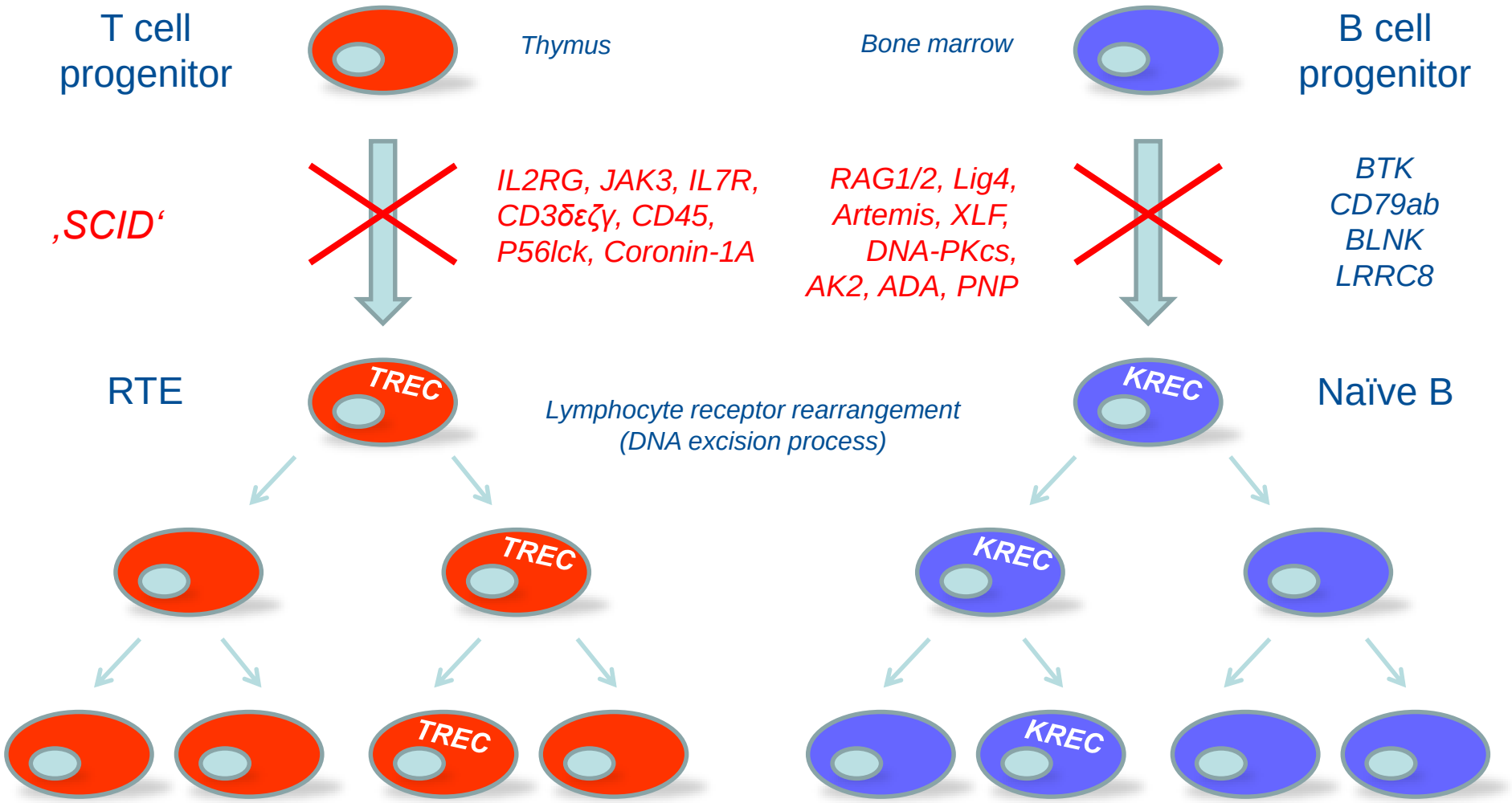


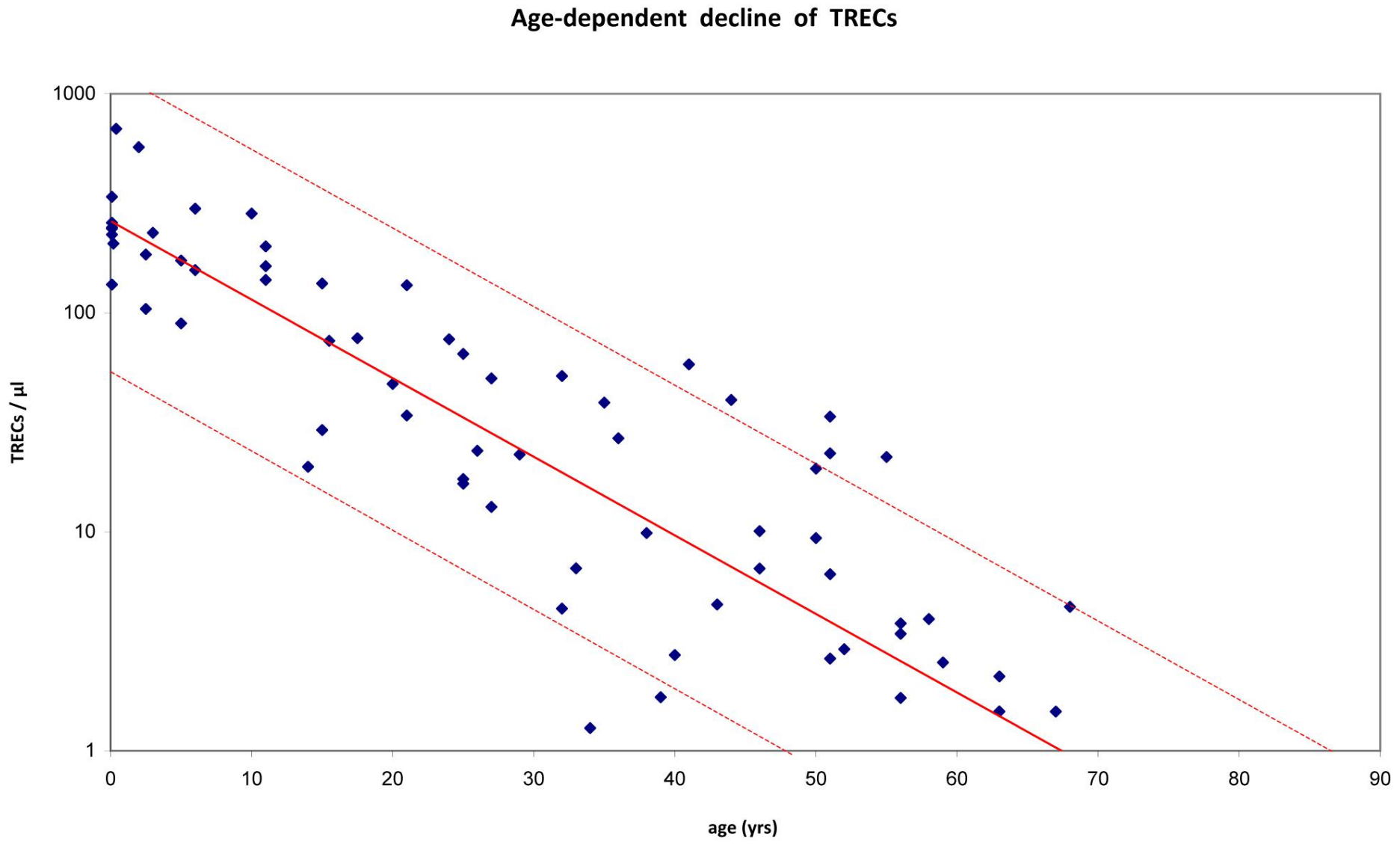
Figure 4.20 The Immune System, 3ed. (© Garland Science 2009)

- RAG1/2 proteins
- ligases

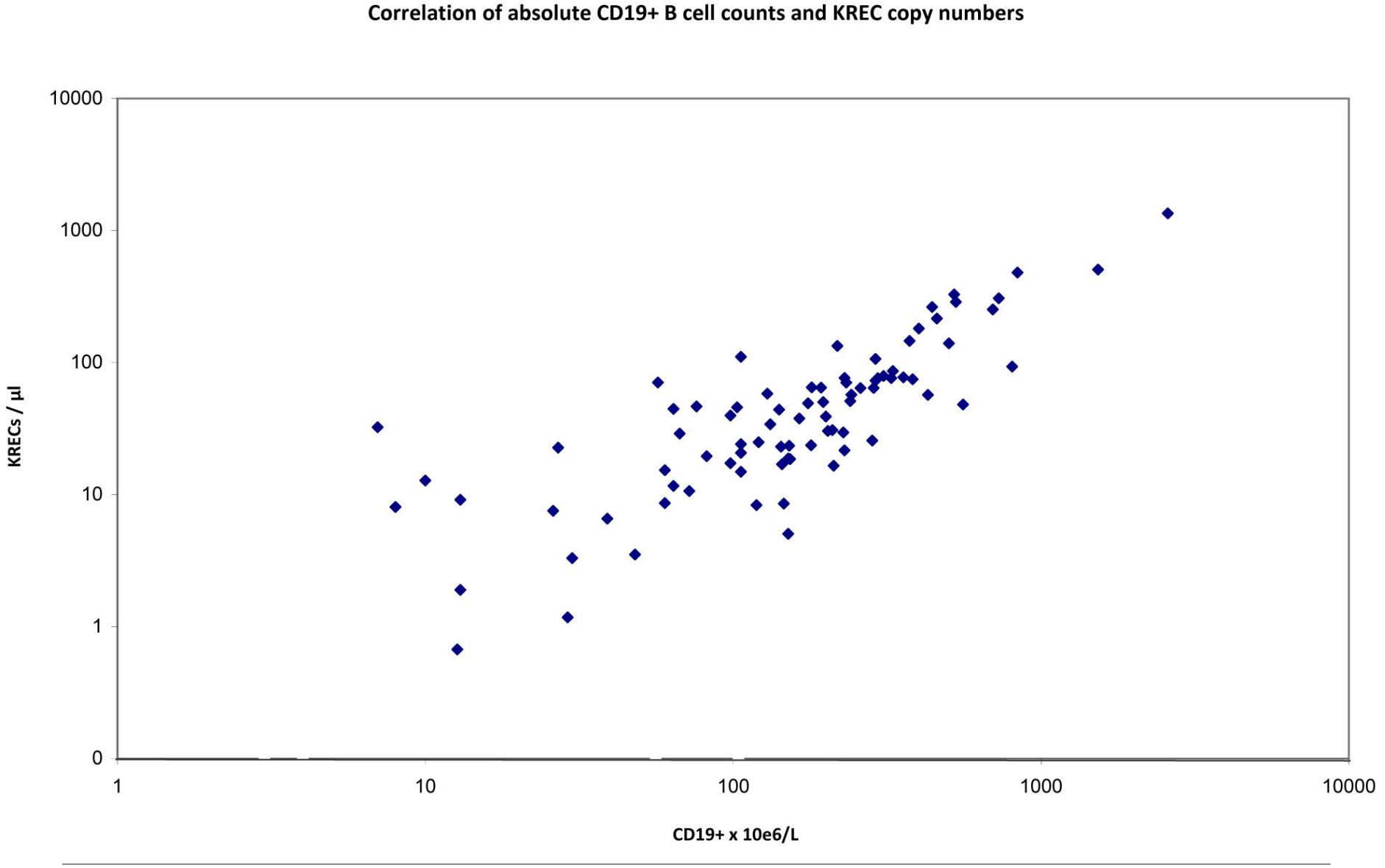
Genomic alterations in severe PID



Thymic output correlates with TREC copy numbers

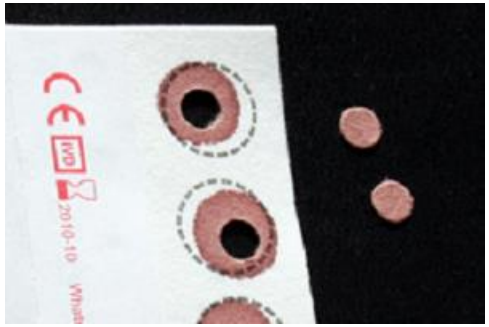


KRECs represent the homeostatic replication of B cells



TREC-KREC-ACTB screening assay

Dried blood spot sample



- regular Guthrie cards (*e.g.* 903 paper)
- single 3.2 mm dried blood spot punch

Robotic sample processing

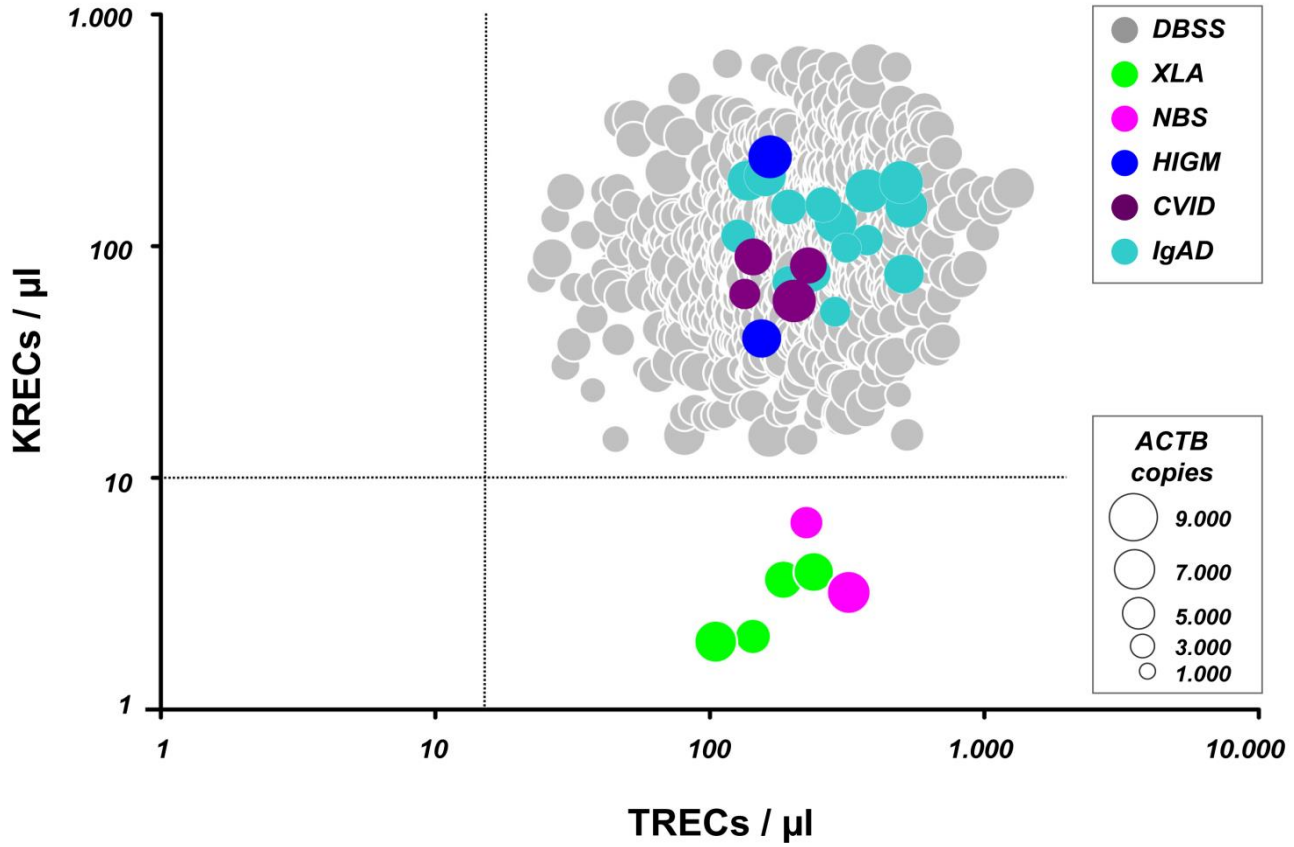
- removal of PCR inhibitors
- heat-elution of genomic DNA

DNA

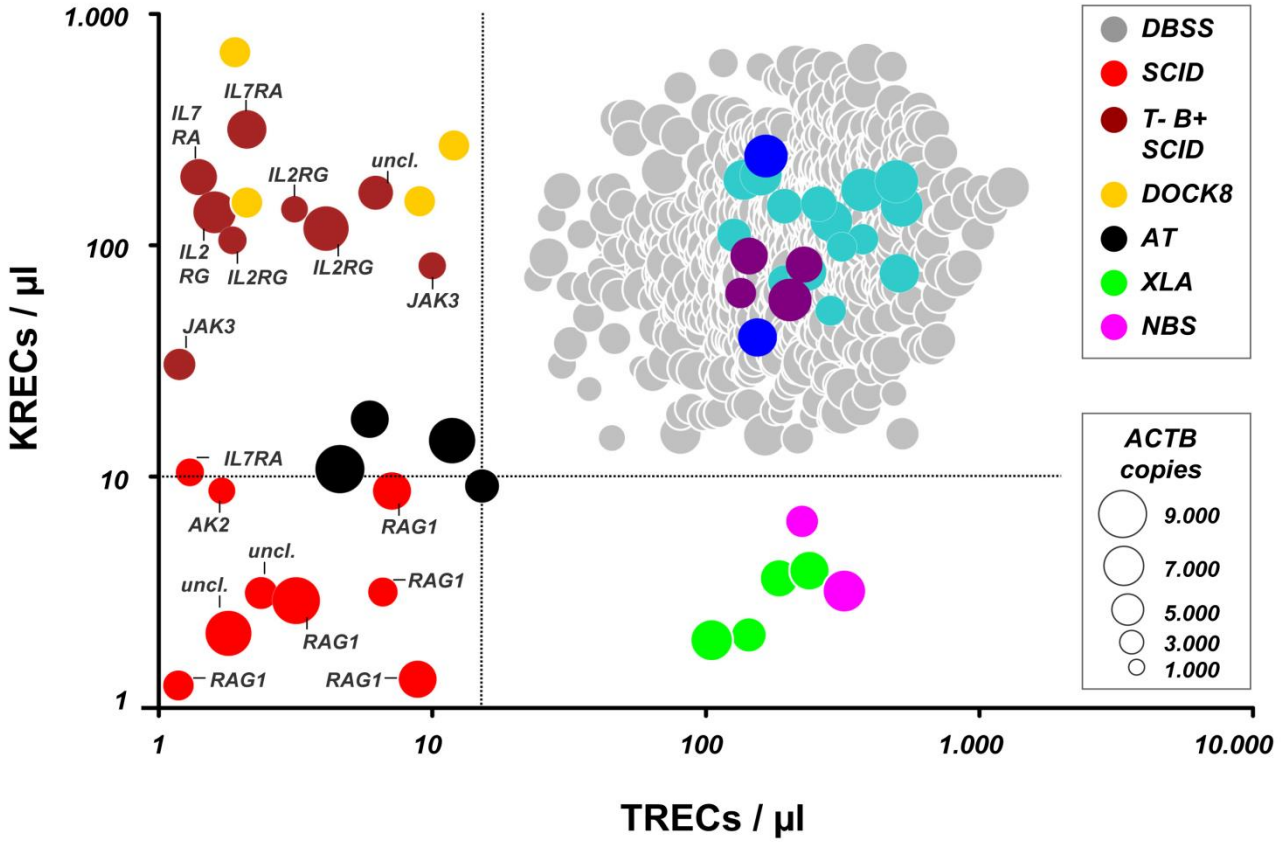
Real-time quantitative PCR

- real-time triplex PCR
- plasmid standard for absolute quantitation
- possibility for multiplexing

Proof-of-principle (*retrospective study 2011*)



Proof-of-principle (*retrospective study 2011*)

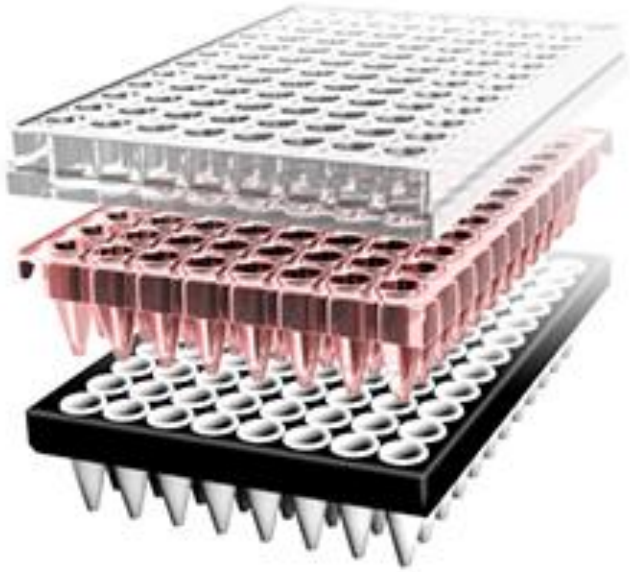


Improved TREC-KREC assay *SCREEN-ID*

- Improved low-copy resolution
- Lowered cut-offs
- Reduced recall rate
- Reduced cost (€ 1.45 in our hands)

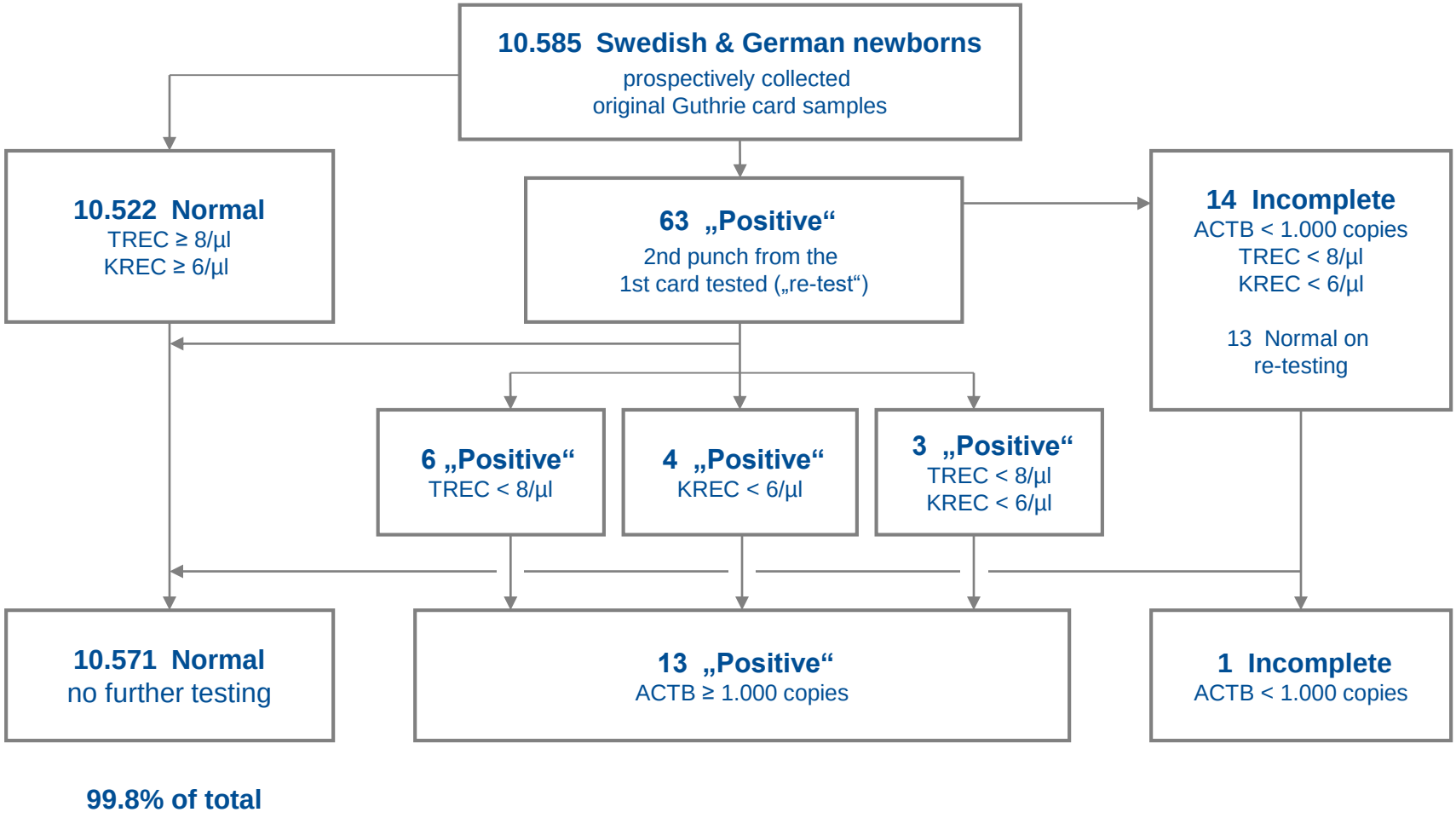
MABTECH Diagnostics

SCREEN-ID workflow (~2 hrs)



Filter plate	Rinsing of dried blood spots (15 min)
Elution plate	Elution of nucleic acids from the dried blood spots (30 min)
	Filter plate is re-used to withdraw eluted dried blood spots
qPCR plate	Absolute quantitation of TREC, KREC and beta-actin copy numbers using triplex-qPCR (60 min)

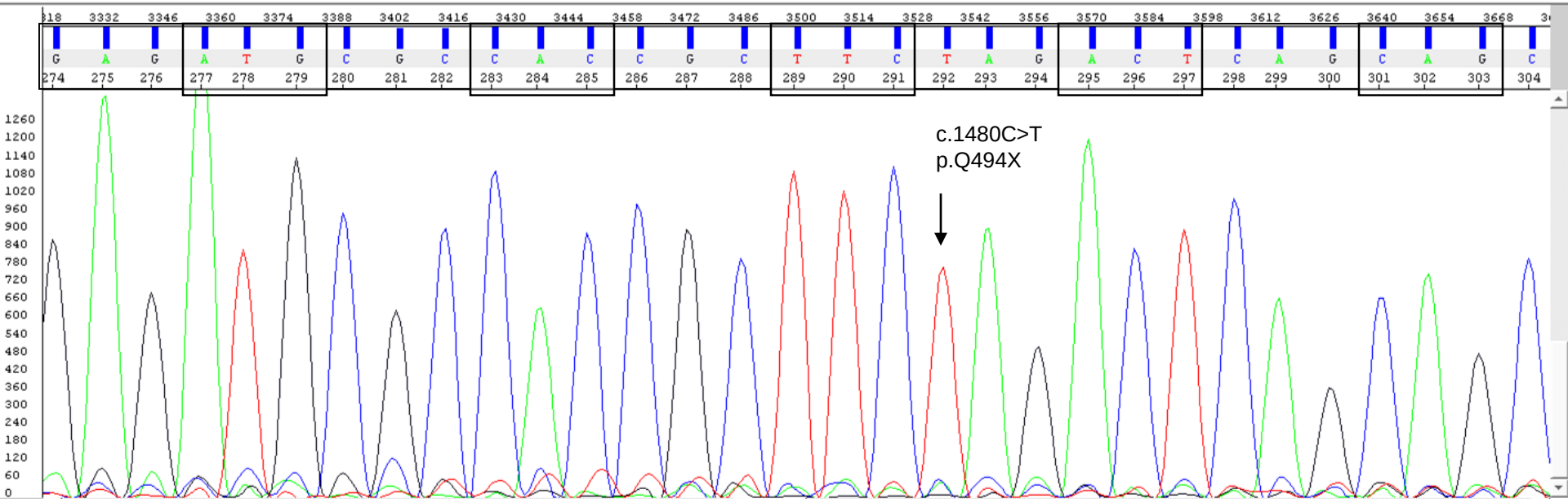
TREC-KREC assay (*prospective study 2012*)



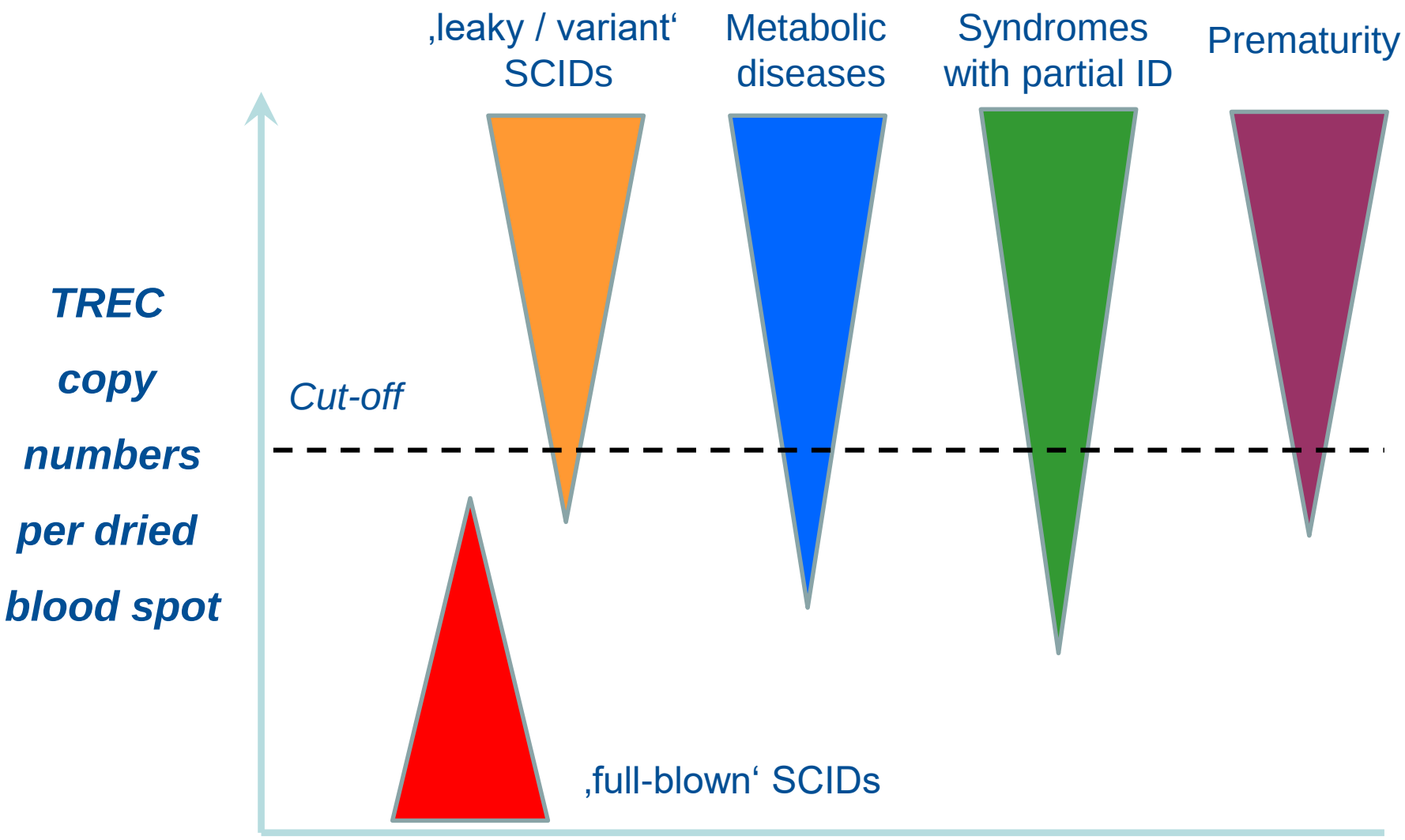
The first XLA patient identified by newborn screening

Stockholm
2012

***BTK* gene** (exon 15)



„False-positives“ in the TREC assay

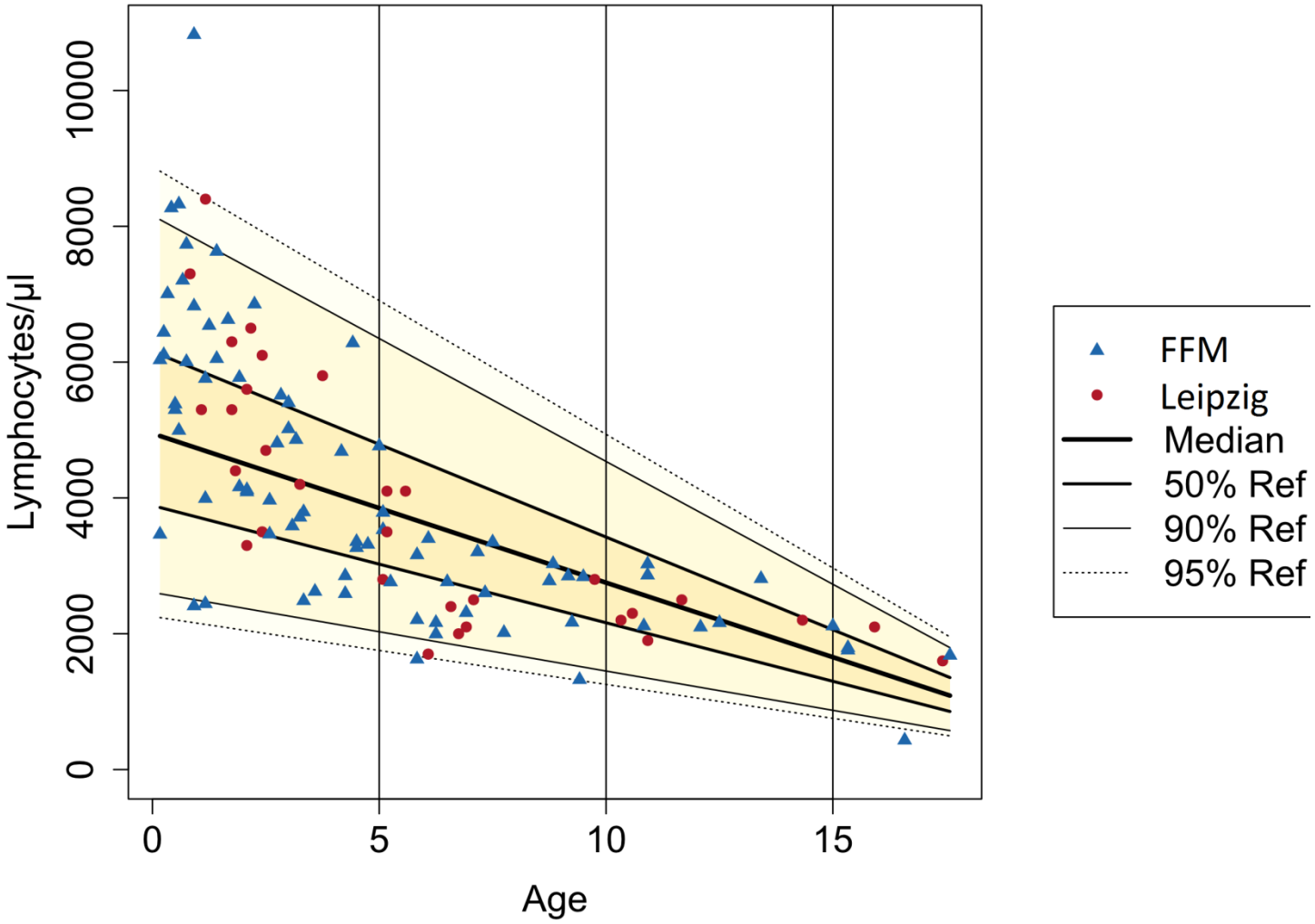


- Genetic screening for PID can be misleading and must be confirmed and/or supplemented by flow cytometry.
- Occasionally, PID present inedependent on genetic screening

Needed:

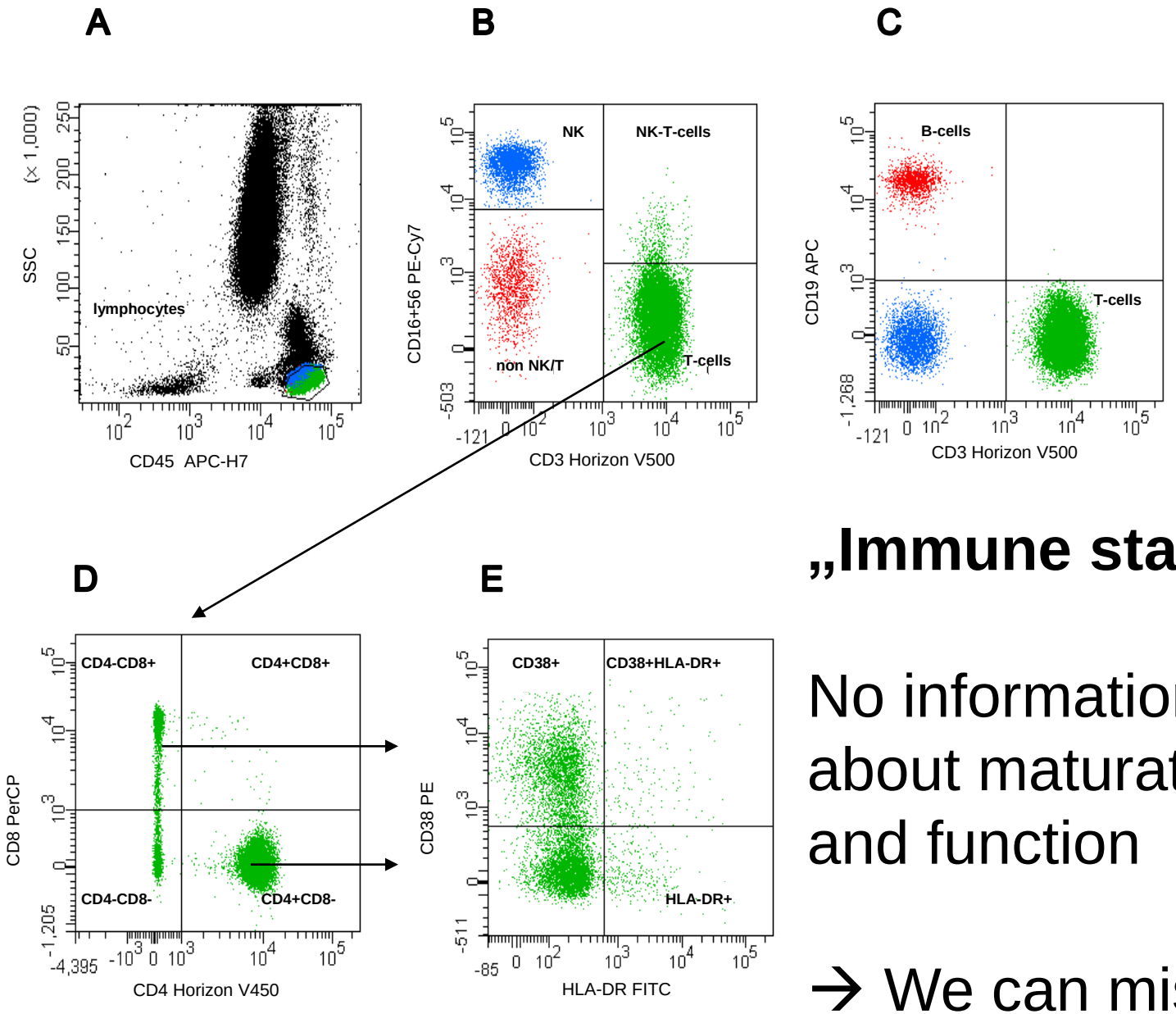
- PID screening with flow cytometry

Challenge # 1: missing reference ranges in children and highly individual findings



Challenge # 2: step-by-step approaches.

A) Risky: common immune status:



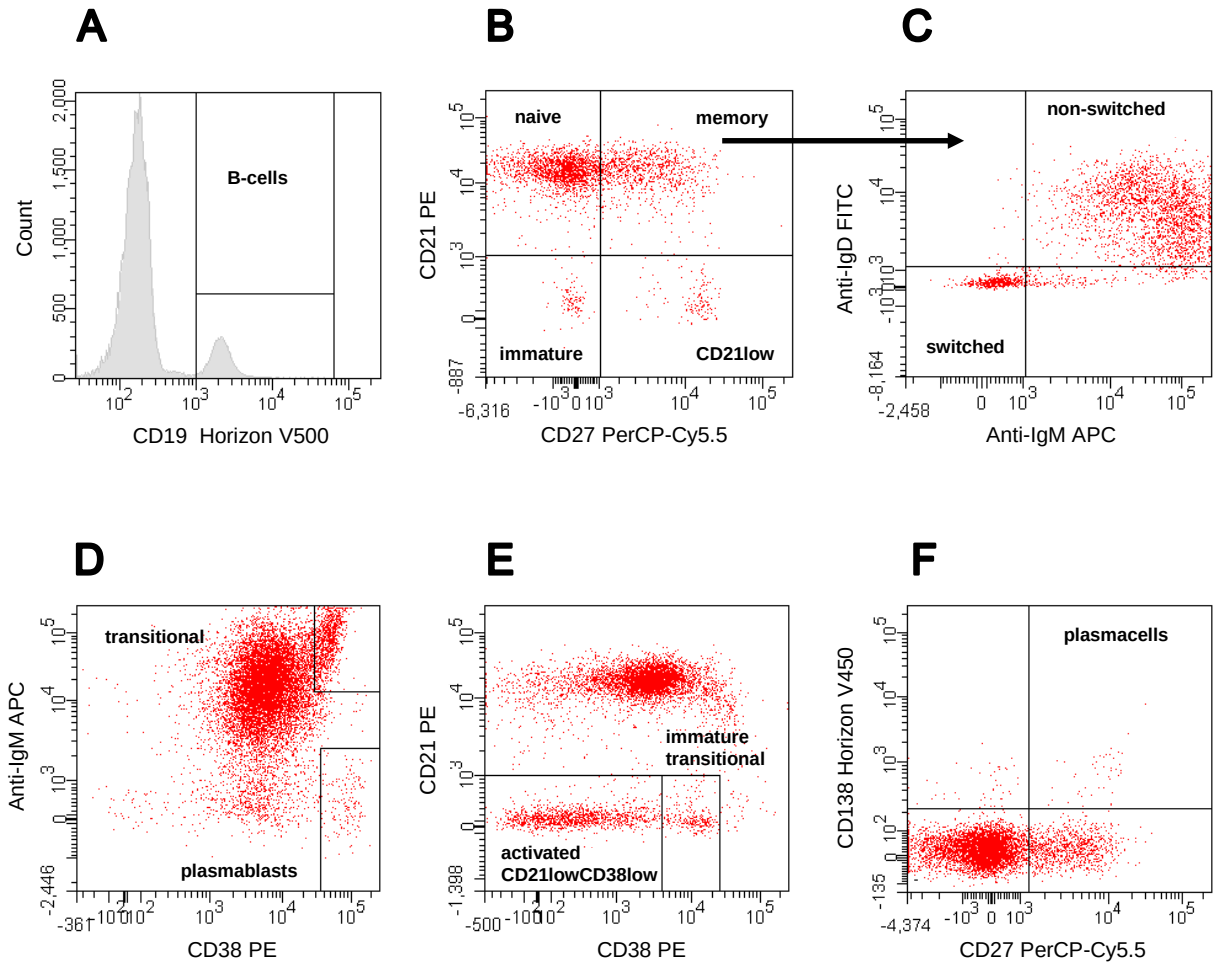
„Immune status“:

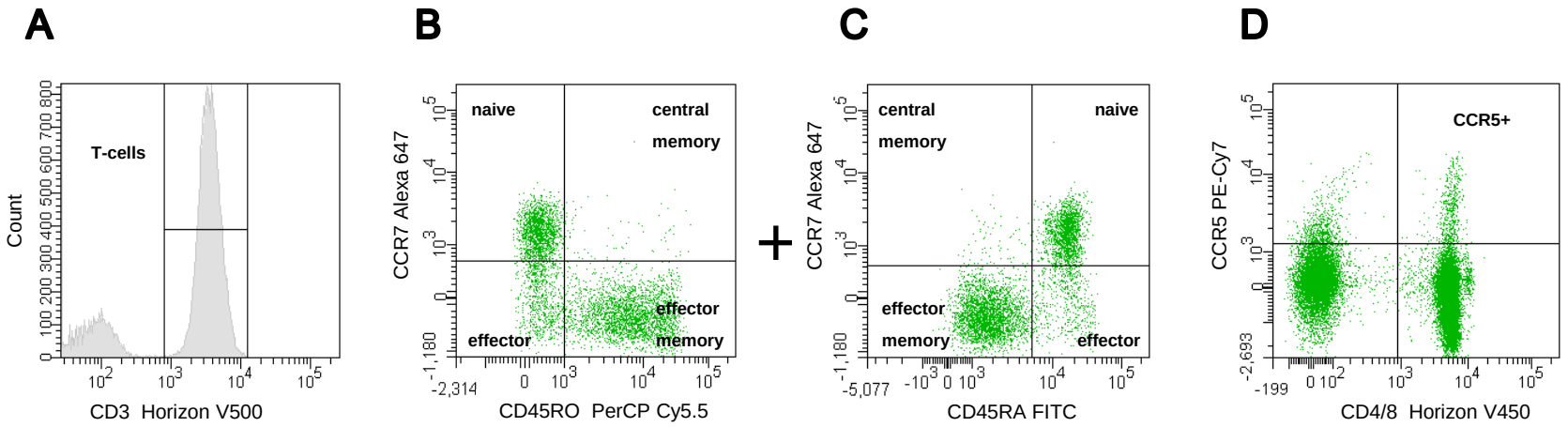
No information about maturation and function

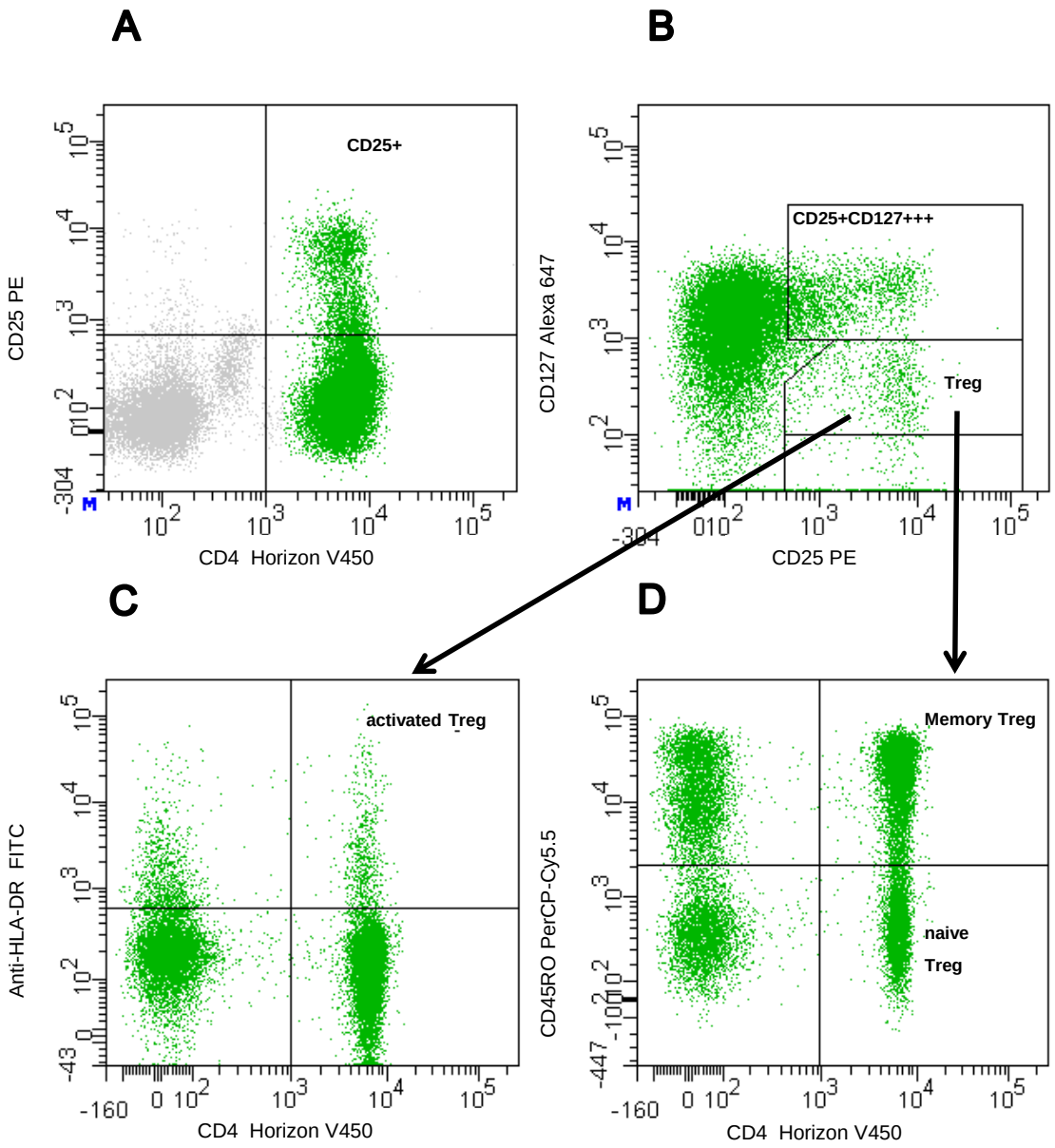
→ We can miss PID!

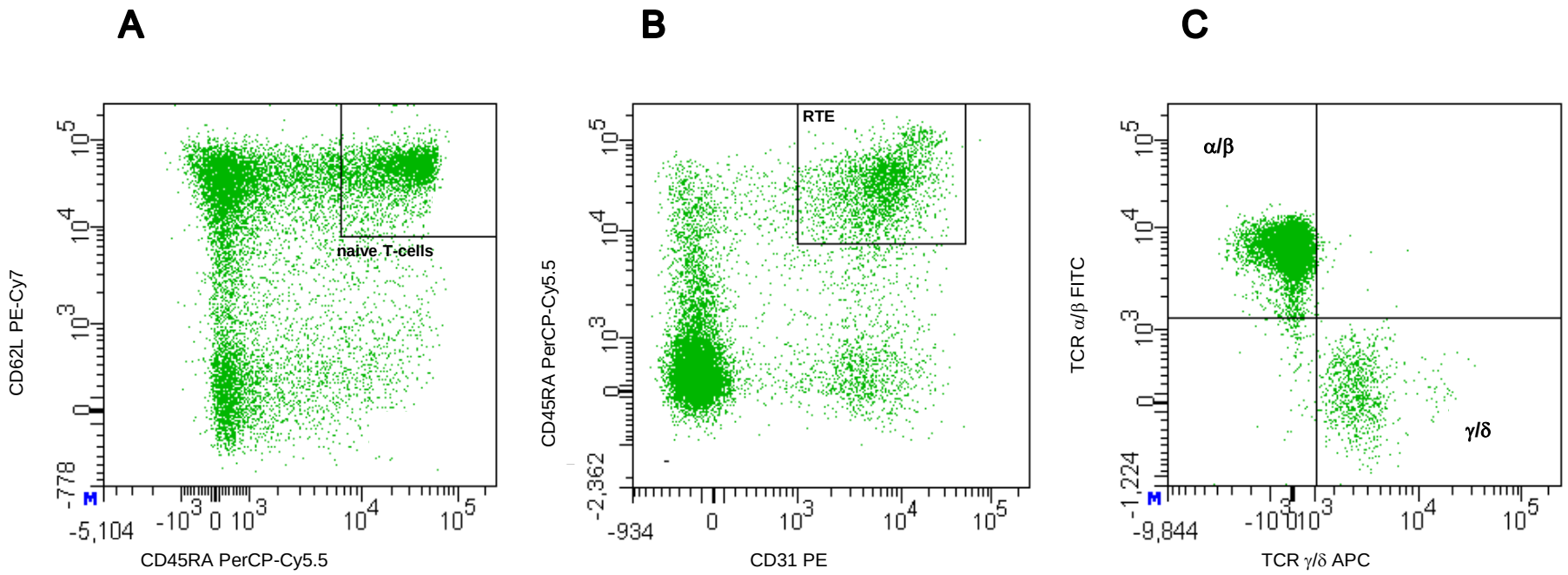
Challenge # 2: step-by-step approaches.

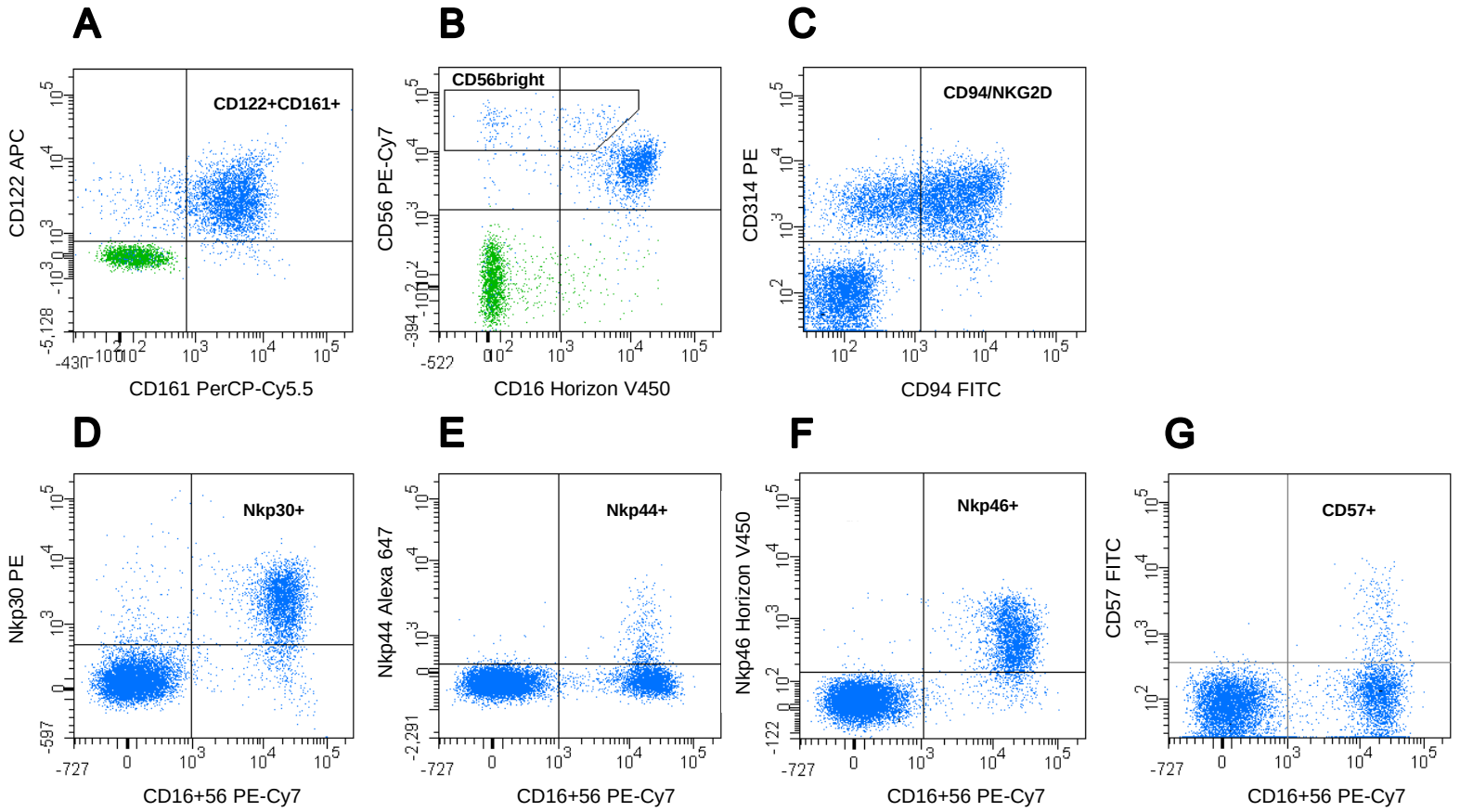
B) expensive: various tubes needed:


A
B
C
Count
B-cells
CD19 Horizon V500
CD21 PE
naive
memory
immature
CD21low
CD27 PerCP-Cy5.5
Anti-IgD FITC
non-switched
switched
Anti-IgM APC
D
E
F
Anti-IgM APC
transitional
plasmablasts
CD38 PE
CD21 PE
immature
transitional
activated
CD21lowCD38low
CD38 PE
CD138 Horizon V450
plasmacells
CD27 PerCP-Cy5.5


A
B
C
D
T-cells
Count
CD3 Horizon V500
naive
central
memory
effector
memory
effector
CCR7 Alexa 647
CD45RO PerCP Cy5.5
central
memory
naive
effector
memory
effector
CCR7 Alexa 647
CD45RA FITC
CCR5+
CCR5 PE-Cy7
CD4/8 Horizon V450


A
B
CD25+
CD25 PE
CD4 Horizon V450
CD25+CD127+++
Treg
CD127 Alexa 647
CD25 PE
C
D
activated Treg
Anti-HLA-DR FITC
CD4 Horizon V450
Memory Treg
naive
Treg
CD45RO PerCP-Cy5.5
CD4 Horizon V450


A
B
C
CD62L PE-Cy7
CD45RA PerCP-Cy5.5
naive T-cells
-778
-5,104
CD45RA PerCP-Cy5.5
RTE
-2,362
-934
CD31 PE
TCR α/β FITC
α/β
γ/δ
-1,224
-9,844
TCR γ/δ APC

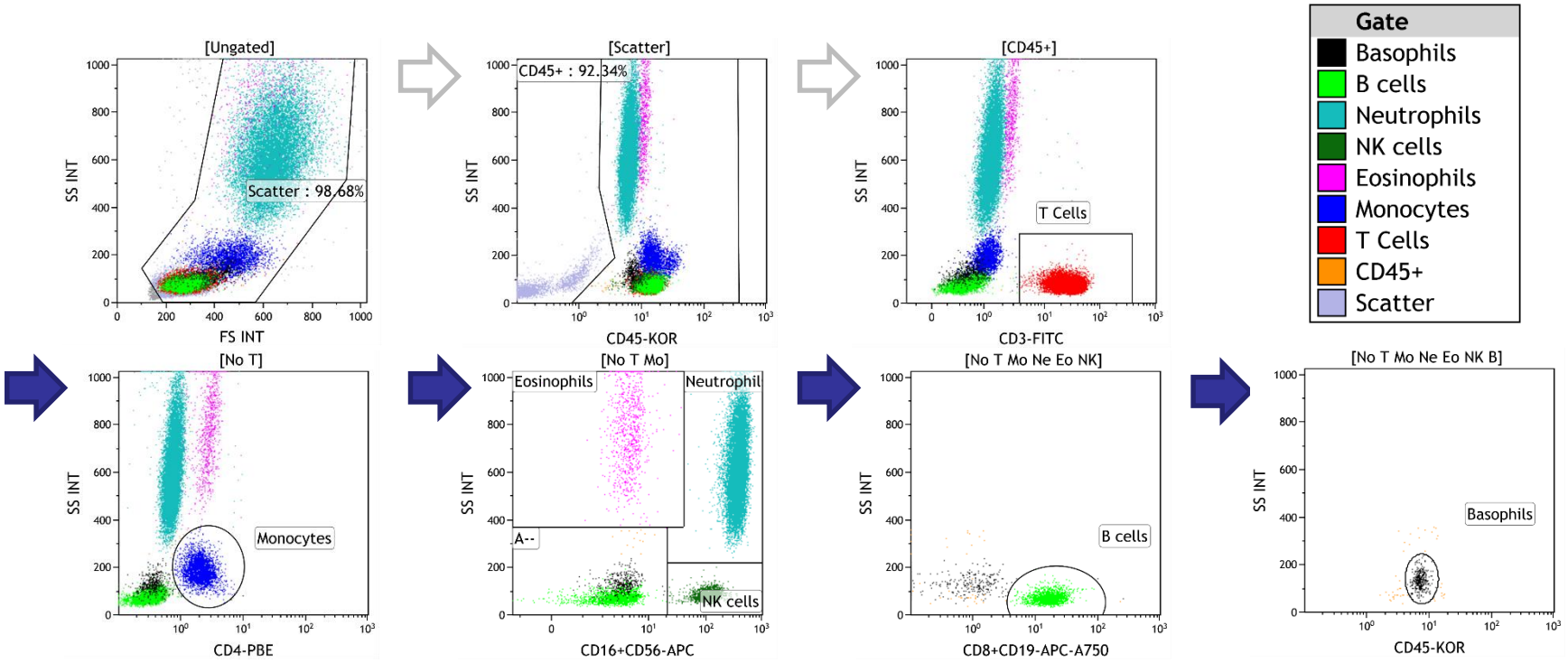

A
CD122+CD161+
CD122 APC
CD161 PerCP-Cy5.5
B
CD56bright
CD56 PE-Cy7
CD16 Horizon V450
C
CD94/NKG2D
CD314 PE
CD94 FITC
D
Nkp30+
Nkp30 PE
CD16+56 PE-Cy7
E
Nkp44+
Nkp44 Alexa 647
CD16+56 PE-Cy7
F
Nkp46+
Nkp46 Horizon V450
CD16+56 PE-Cy7
G
CD57+
CD57 FITC
CD16+56 PE-Cy7

Our proposal: Validation of a one-tube orientation approach

New: novel Duraclone tube: 1 tube only

	Violet Excitation (405nm)		Blue Excitation (488nm)				Red Excitation (633-640nm)	
Specificity 1	**CD4**	**CD45**	**CD3**	**IgD**	**CD27**	**CD38**	**CD16**	**CD19**
Clone 1	*13B8.2*	*J33*	*UCHT1*	*IA6-2*	*1A4CD27*	*LS198-4-3*	*3G8*	*J3-119*
Specificity 2	x	x	x	**CD45RO**	x	x	**CD56**	**CD8**
Clone 2	x	x	x	*UCHL1*	x	x	*N901*	*B9.11*
Fluorochrome	Pacific Blue	Krome Orange	FITC	PE	PC5.5	PC7	APC	APC-AF750

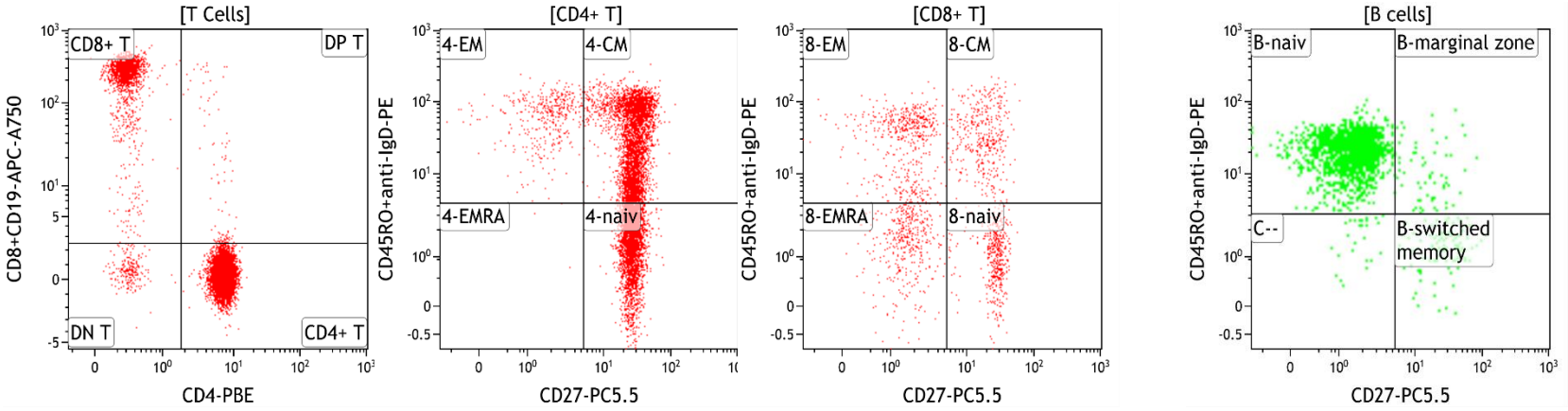
Principal populations



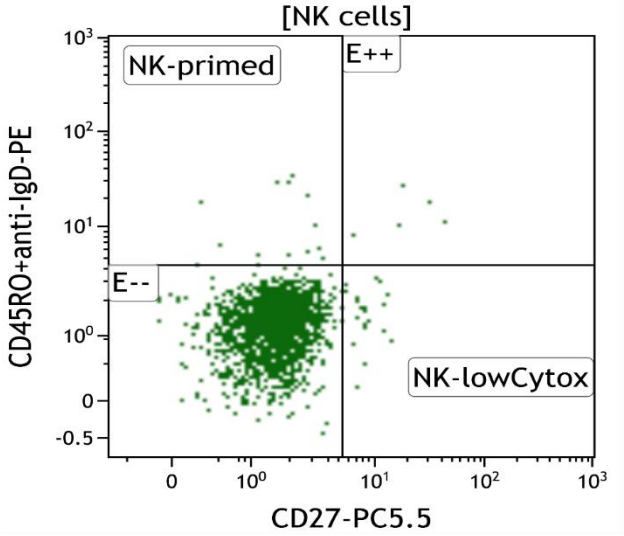
Continue with gated population Continue without gated population

T Cell subpopulations

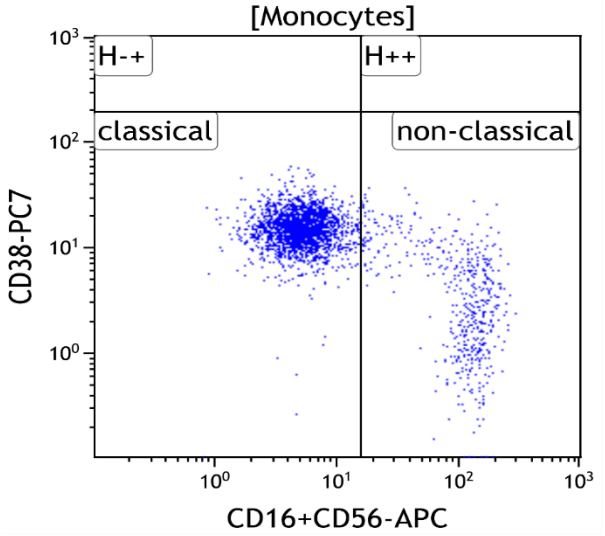
B Cell subpopulations



NK Cell subpopulations



Monocyte subpopulations



Conclusion

The presented antibody panel is a potent candidate for a rapid primary immune deficiency screening tool in the immunology lab:

- easy implementation due to pipetting-free dry panel format, no-wash protocol
- comprehensive information on cellular proportions / concentrations
- coverage of important late cellular differentiation stages
- compatibility with common instrumentation

Also, secondary immune deficiencies may be subject to this rapid screening method. Further work will now focus on cross-instrument standardization.

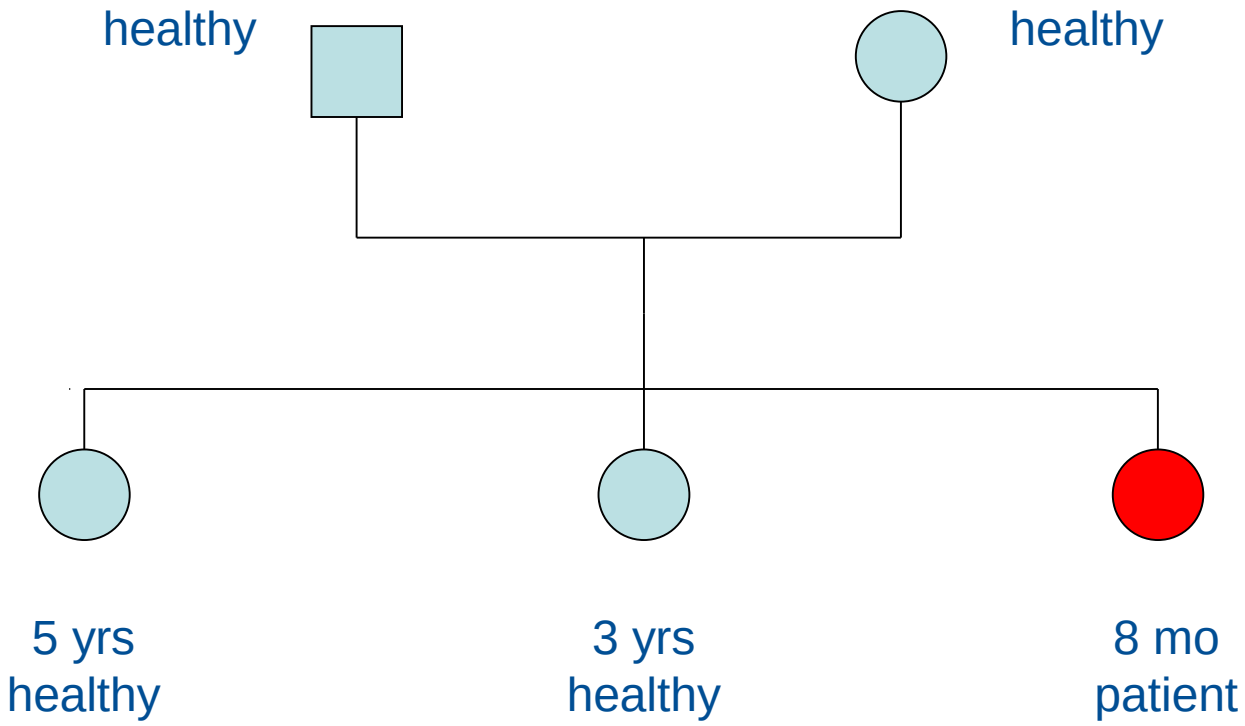
Functional T cell phenotyping in PID

Challenge # 3: Functional abberations in immune cells

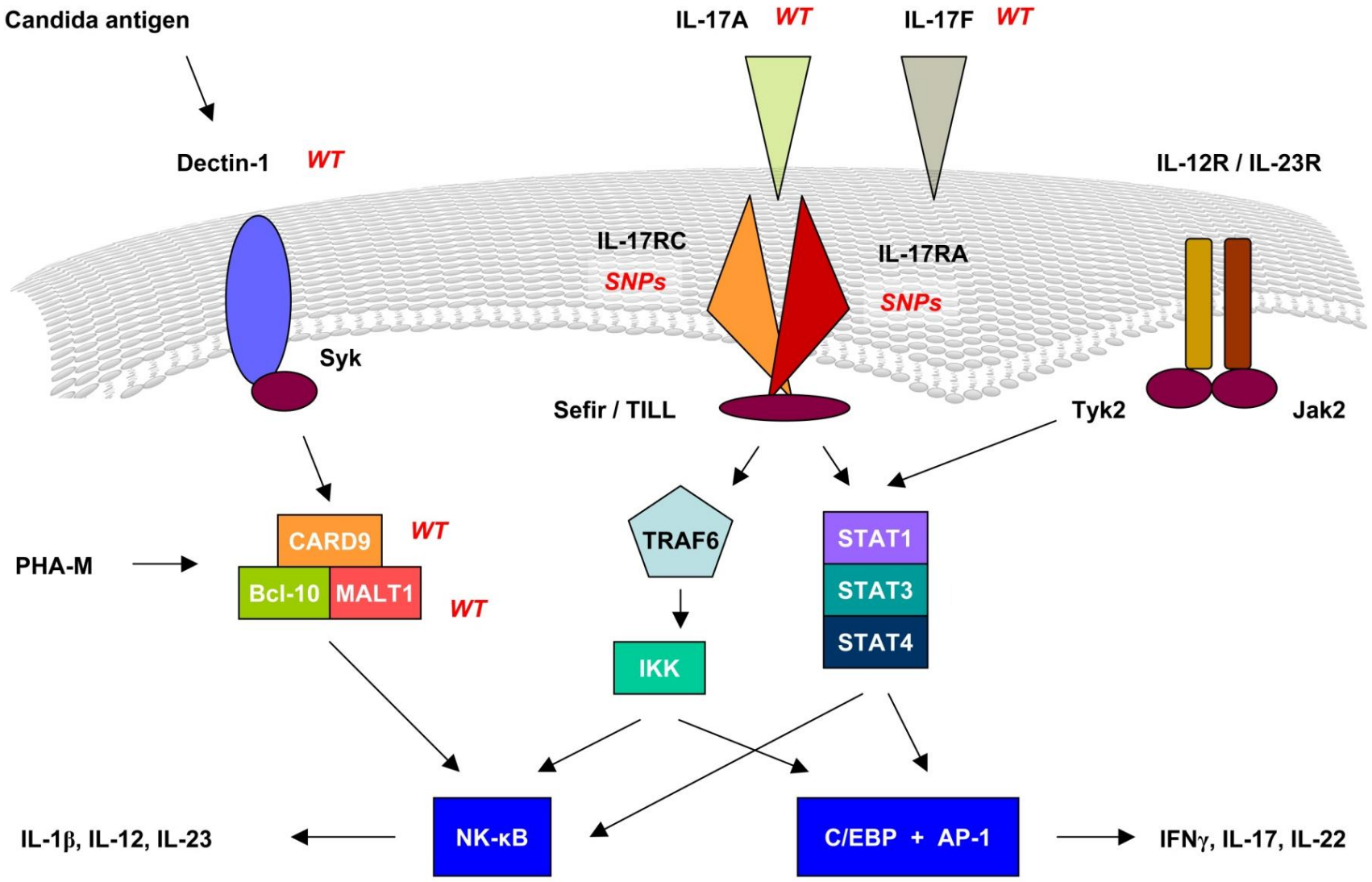
Clinical case: a baby-girl with severe fungal infections

- Treatment-resistent diaper candidiasis
- 2 episodes of pneumonia in 1st y of life

Clinical case: pedigree



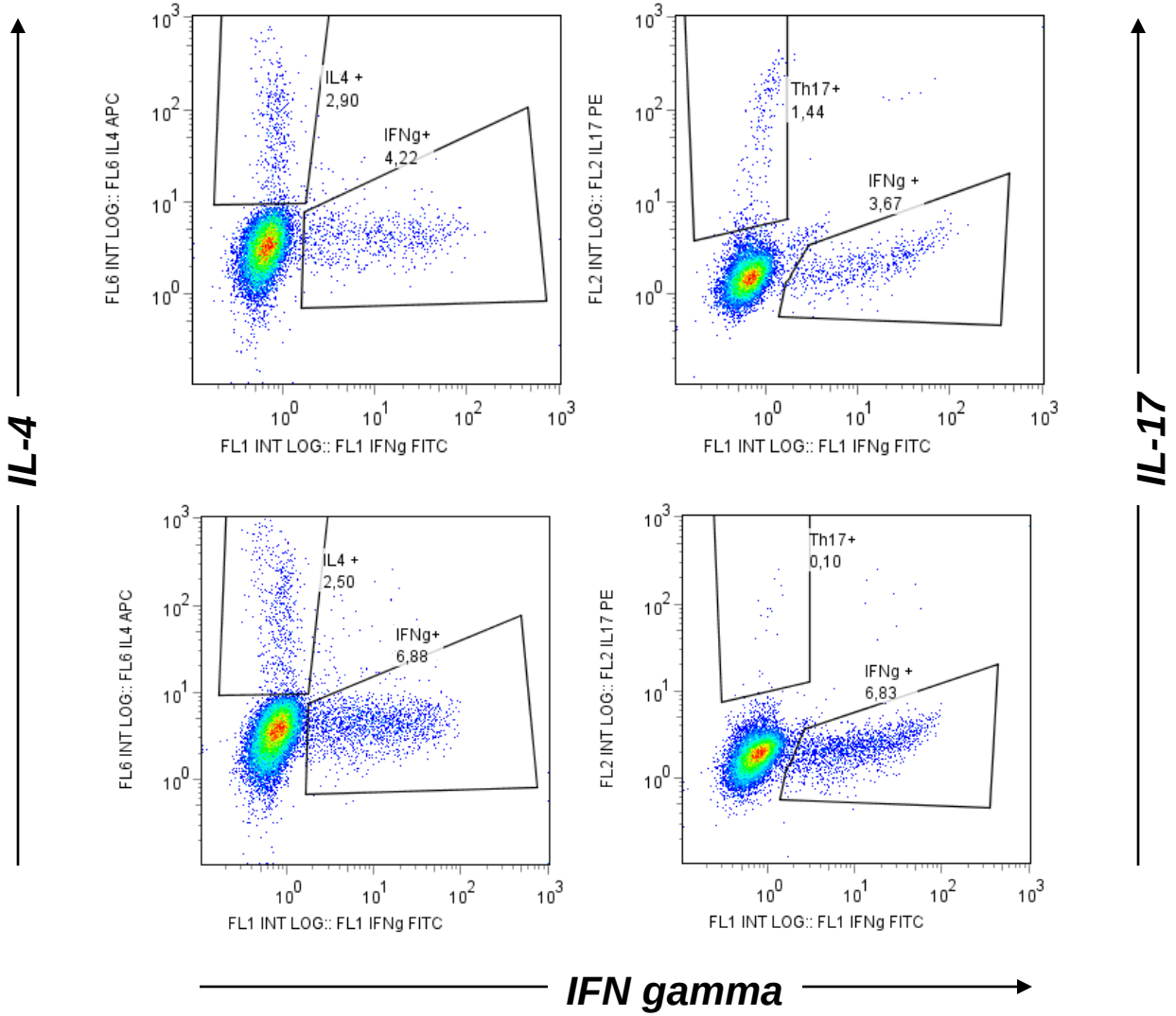
Chronic mucocutaneous candidiasis: disease mechanisms



Analysis of Th17 cells (ICS, PMA+Iono)

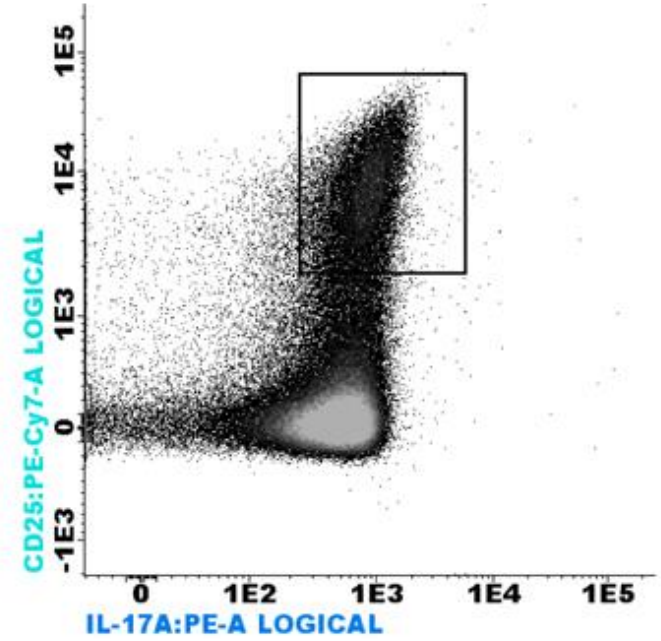
healthy sibling

patient

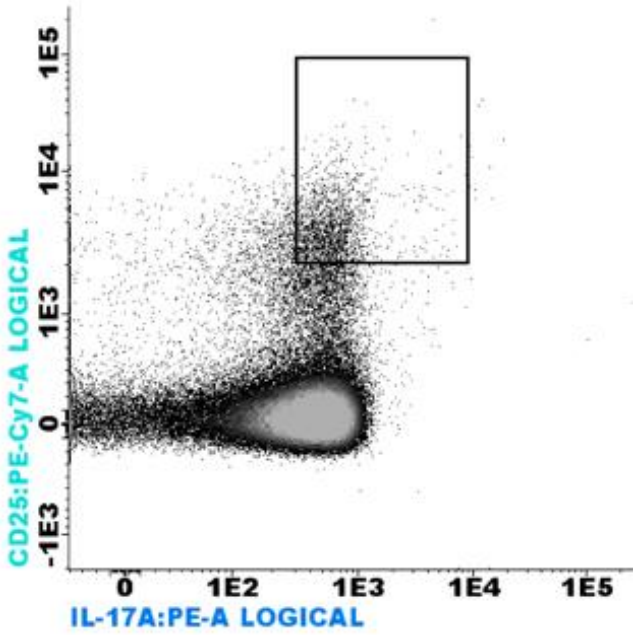


Analysis of specific Th17 responses

Leucocytes cultured in presence of HKCA for 3 days



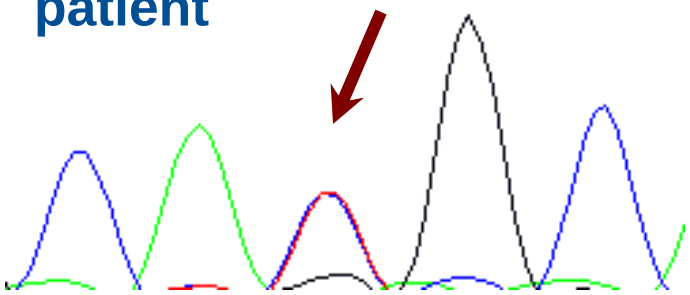
healthy sibling



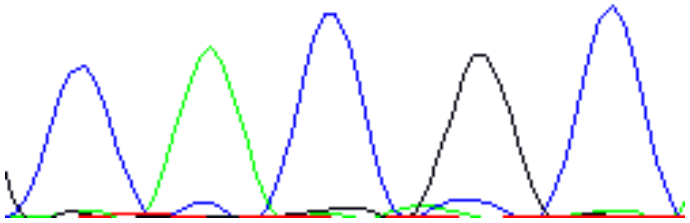
patient

Lack of Th17 cells and CMC ?

patient

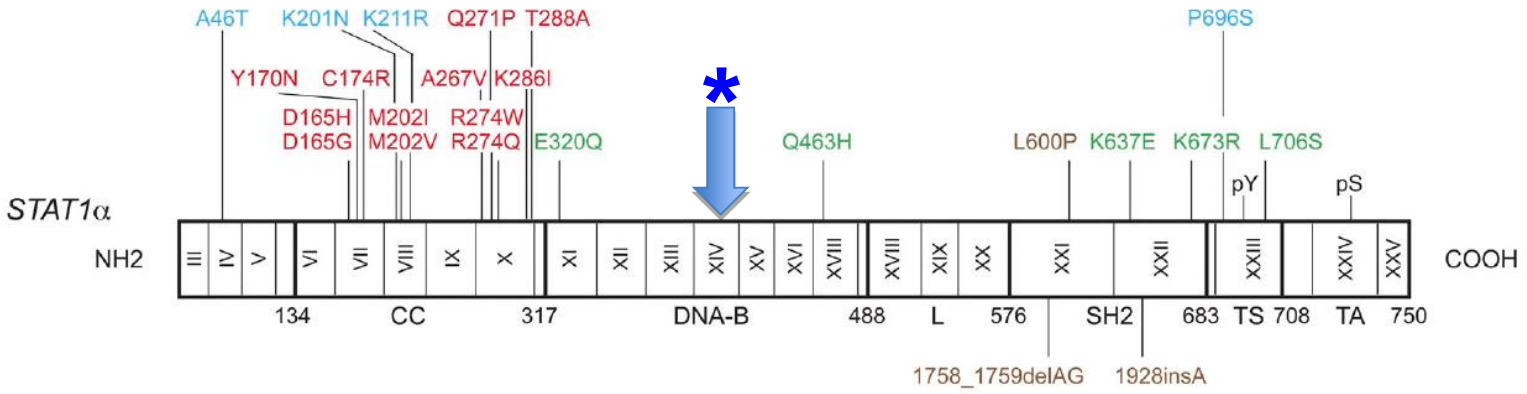


healthy sibling



***STAT1* gene**
c.1154 C>T (he)

STAT1 deficiency and clinical phenotypes



Red: AD: CMC phenotype

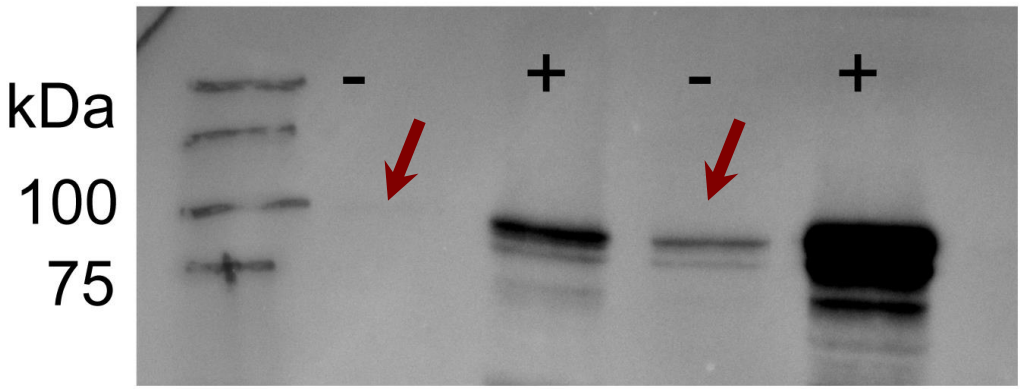
Blue: AR: MSMD/viral phenotype (partial STAT1 deficiency)

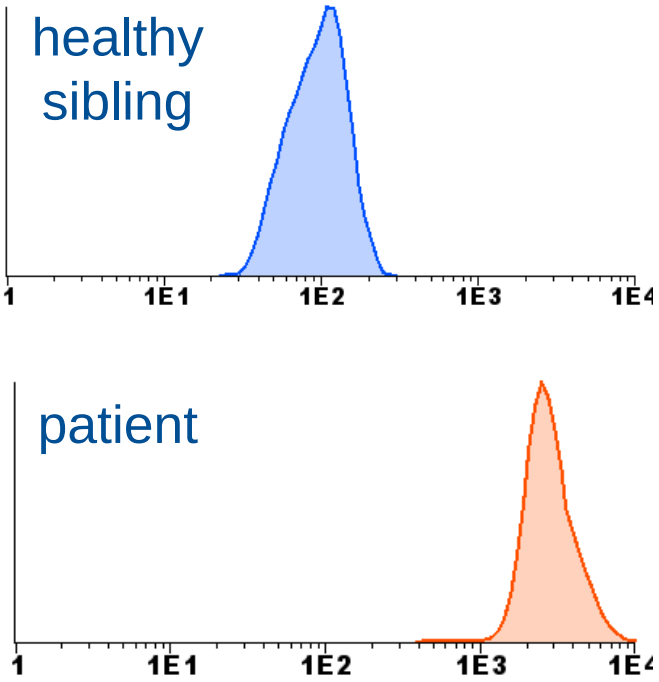
Green: AD: MSMD phenotype (partial STAT1 deficiency)

Brown: AR: MSMD/viral phenotype (complete STAT1 deficiency)

STAT1 p.T385M is a gain-of-function mutation

constitutive STAT1 phosphorylation
(*anti*-pTyr701 Ab)





Lymphocytes +/- IFNγ für 3
20 µg Lysate per lane

BD Phosflow pSTAT1
staining of CD4⁺ T cells

Challenges for laboratories

- Clinical question? → clinical answer!
- Clinical advisory services
- Consider partial independency between lab findings and pathological consequences
- In children very broad reference ranges
- Severe therapeutic consequences!
- Cave surrogate markers!
- Precious blood samples
- Proficiency testing

Conclusions

- Increasing need for immune diagnostics
- Clinical awareness crucial
- Frequent diseases but heterogeneous ones
- Relevant findings for therapeutic decisions
- Disadvantages of initial screening tests

Thank you very much!

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