Introduction and overview: Monocyte Activation Tests

Thomas Hartung & team

Center for Alternatives to Animal Testing

A CENTURY OF SAVING LIVES
MILLIONS AT A TIME

JOHNS HOPKINS
BLOOMBERG SCHOOL
OF PUBLIC HEALTH

Gold standard rabbit test

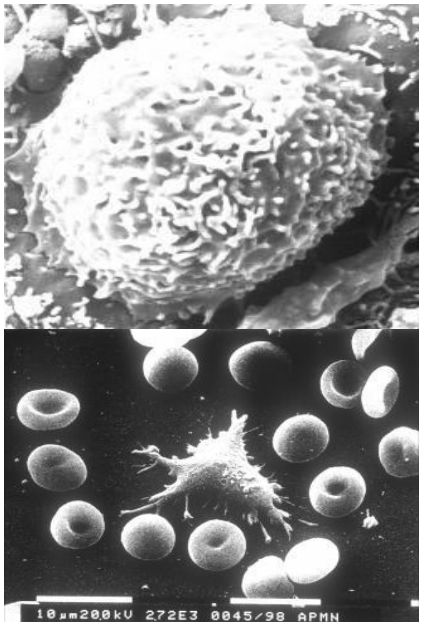
Pyrogen (bacterial and fungal contamination) testing still consumes about 400.000 rabbits per year

Limulus: 90% of pyrogen testing today

Limulus assay is restricted to Gram-negative endotoxin, disturbed by many substances and does not reflect human pyrogen potency

Problem solved?

My primary interest in 1992 - 1999



neutrophil

monocyte

G-CSF

Neupogen (Amgen)

Anti-infectious and anti-inflammatory

From bench to bedside:

***in vitro*: macrophages, neutrophils**

***in vivo*: Salmonella, S. aure[illegible]nes, sepsis**

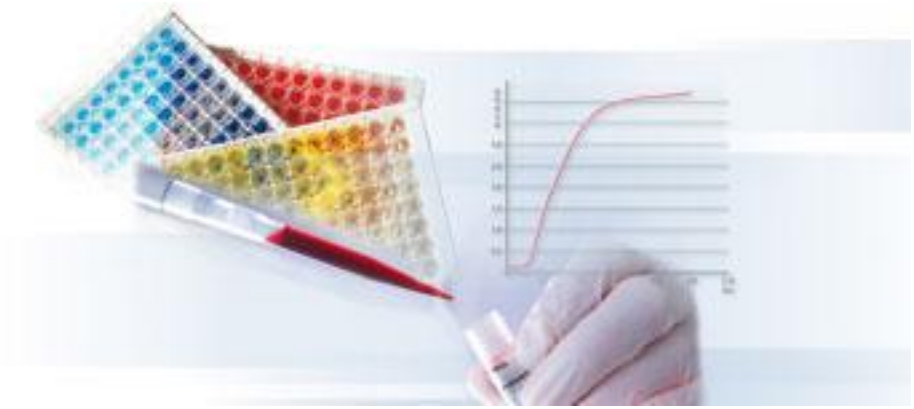
volunteer studies: ex vi[illegible] whole blood assay

clinical trials: sepsis proph[illegible]sis

Contribution to whole blood assays

- ***In vitro* and *ex vivo* immunopharmacology**
- **Pyrogen testing**
- **Cryopreserved blood**
- **Lymphokine release**

First lesson: Researchers need to know about the needs of applied sciences in order to bring their science to implementation. Academics often do not realize what goodies they have on their shelf.



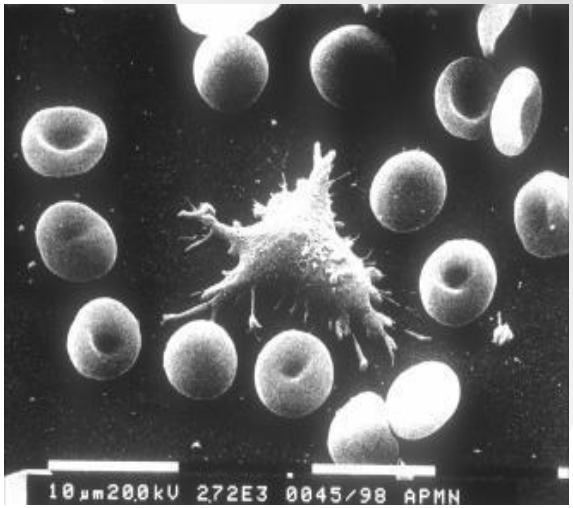
1995 Whole blood pyrogen test
2003 and 2004 Validated
2006 Validity statement
2009 Accepted Eur. Pharmacopoeia and with limitations by ICCVAM / FDA
PyroDetect (Biotest, now Merck-Millipore)

Second lesson: Patenting new methods is critical for their marketing, which gets them standardized, internationally available and visible. Premature publishing cancels this important driving force. The endurance required to attain optimization, validation and acceptance needs such drivers.

Novel Pyrogen tests based on the human fever reaction

Exogenous Pyrogen

1. Whole Blood (1995)
2. Leukocytes (1981, 1989)
3. Cell lines (1991, 1996, 1999)
4. Cryopreserved blood cells (1996)



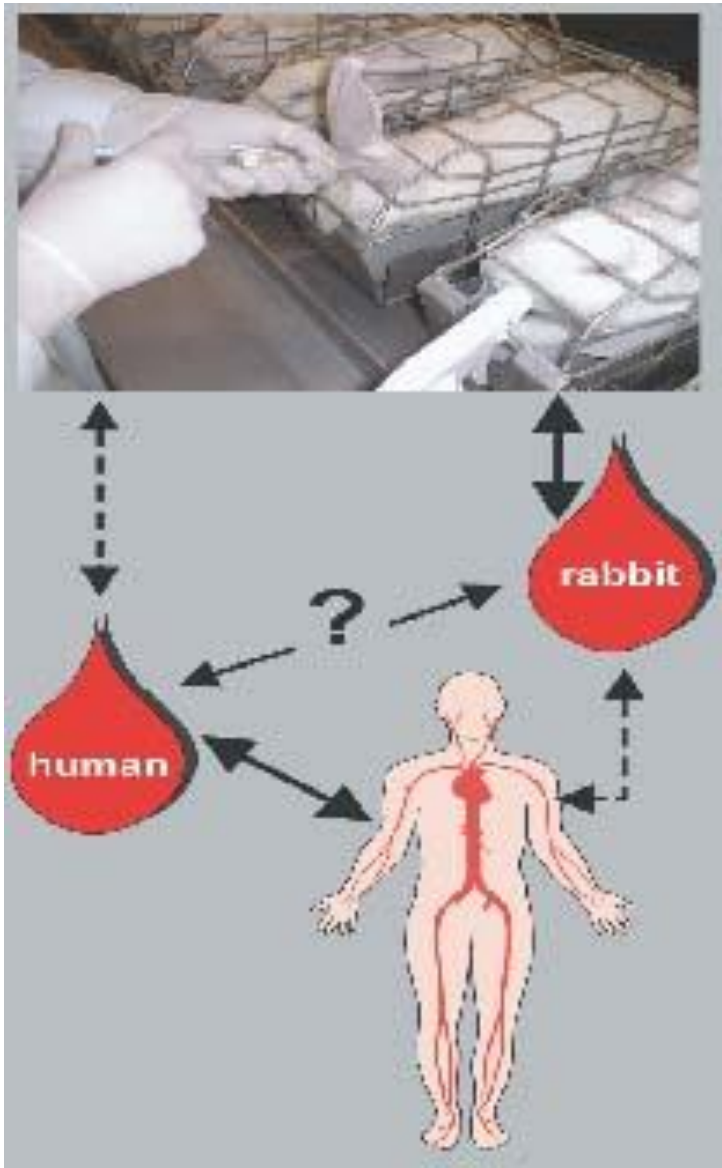
Endogenous Pyrogen

Fever

ELISA

WC2 Utrecht 1996

DZF award for blood pyrogen test



Food for Thought...

ALTEX 2015, 32:79-100

The Human Whole Blood Pyrogen Test – Lessons Learned in Twenty Years

Thomas Hartung

Validated 2006
Accepted FDA 2009, European Pharmacopoeia 2010, USP 2017, ISO 2018

Since then:

- **increase of rabbit pyrogen tests by 10,000 animals in Europe… to 170,000**
- **Less then 1% of pyrogen testing**

Validation and accpetance is not enough – we need implementation !!!

Tab. 1: Patents associated with the whole blood pyrogen test

…but my last patent expires ~~2017~~ 2020

	AT, BE, CH/LI,
	ed 06.04.1999, 04.2008
	05.11.2003 IL, SE), aluation, 5.04.2005

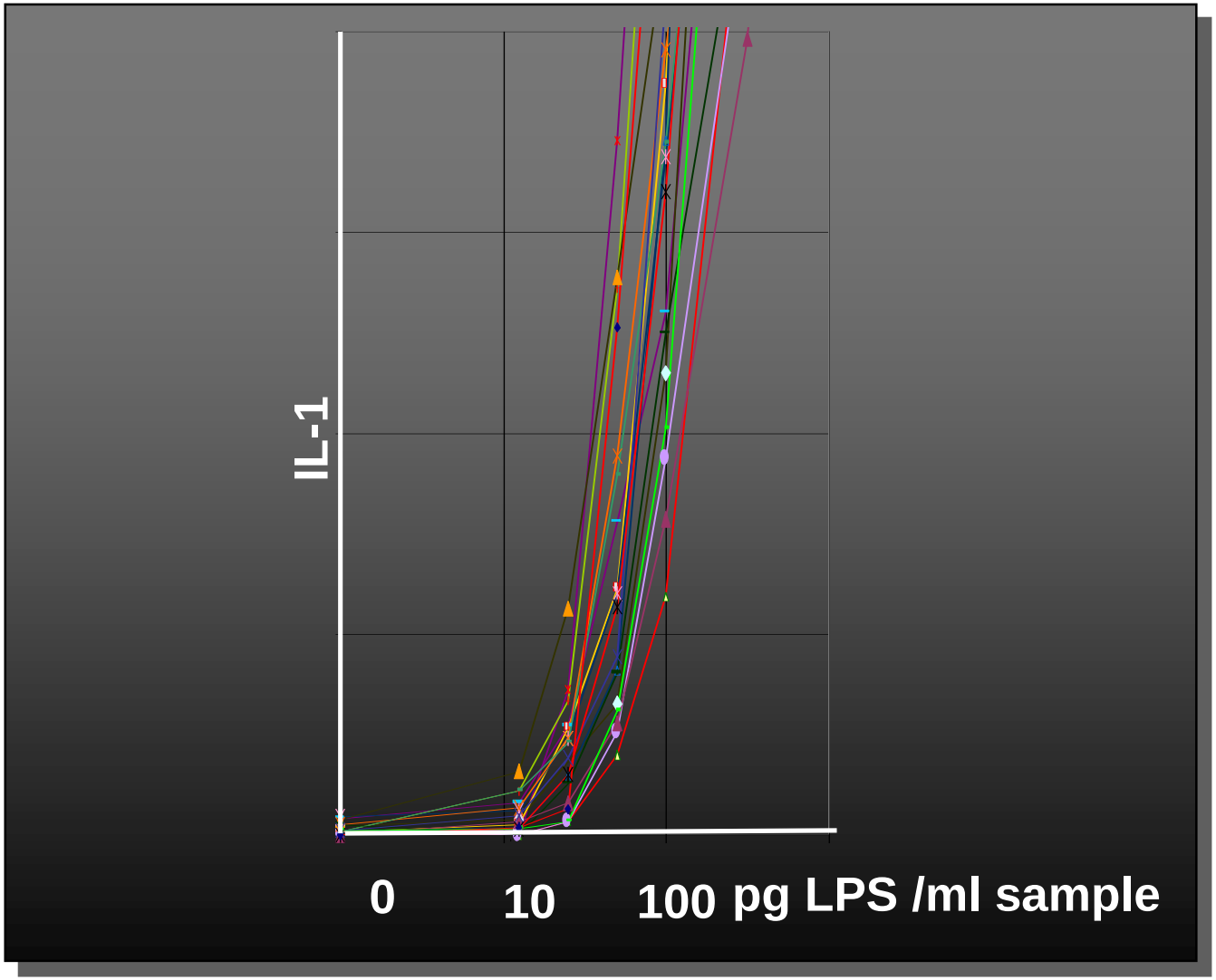
Tab. 2: The kit versions of the whole blood pyrogen test

Company	Kit Name	Time
DPC Biermann, Bad Nauheim, Germany	Pyrocheck	1996 - 2000
Charles-River Endosafe, Charleston, US	Endosafe IPT (In-vitro Pyrogen Test)	2001 - 2008
Biotest, Dreieich, Germany	PyroDetect	2009 - 2011
Merck-Millipore, Merck KG, Darmstadt, Germany	PyroDetect	2012 -

I have any conflict of interest you can imagine… including receiving royalties from Merck-Millipore for the commercial kit of whole blood pyrogen testing

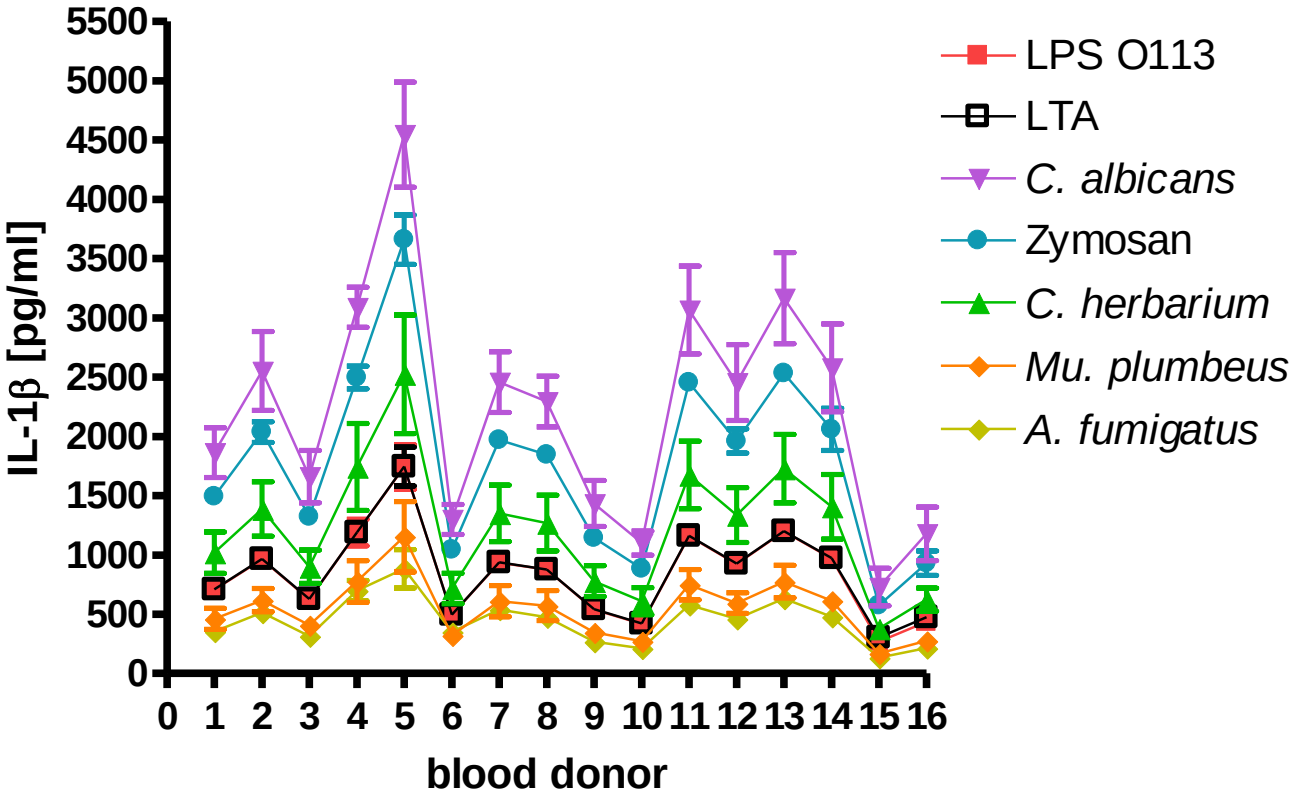
First independent evaluation (1997)

LPS response of 19 blood donors recruited by the blood bank of the University of Heidelberg



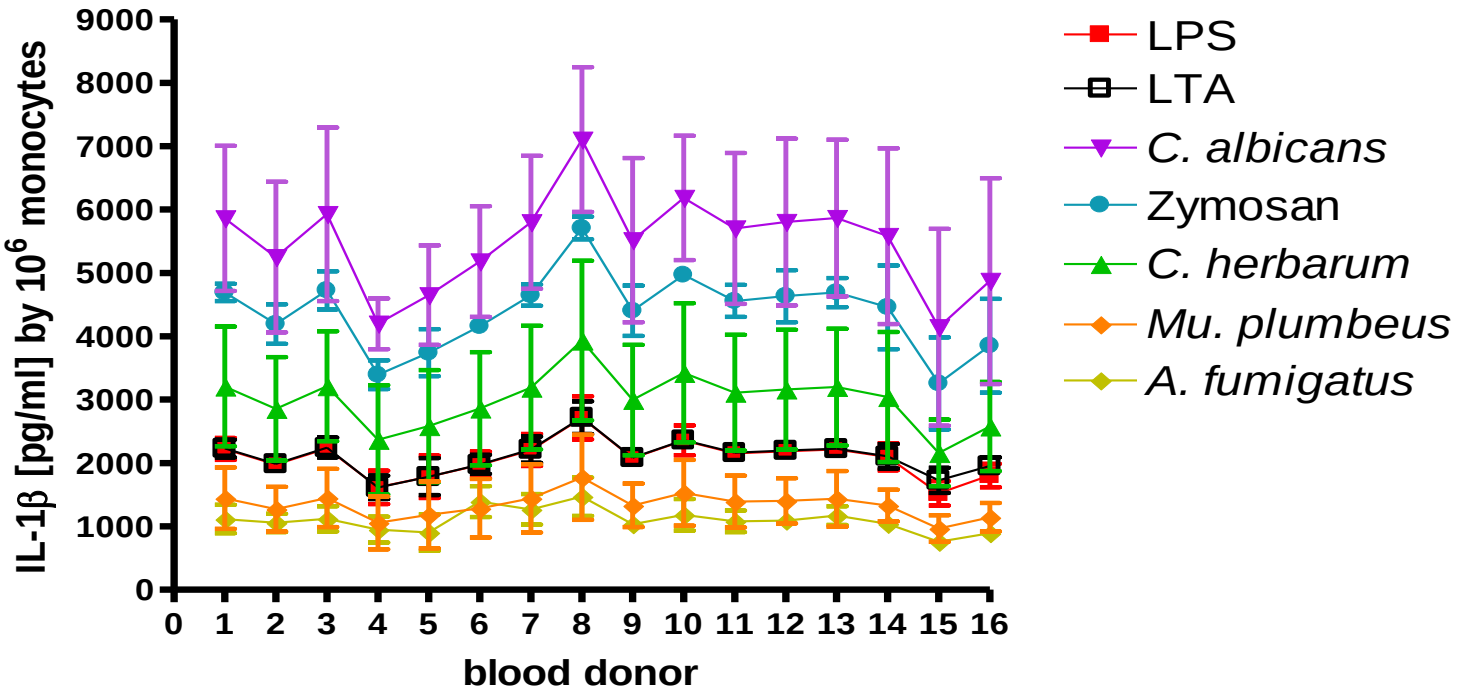
Weigand et al., 1998

Comparison of different donors



➡ Different absolute but similar relative response of donors

Relative response of donors



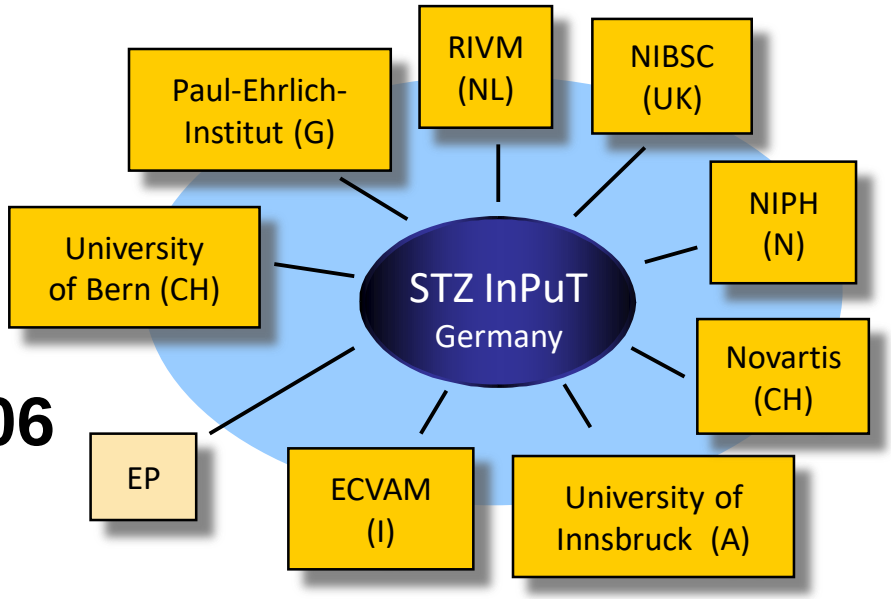
➡ Differences caused mainly by different monocyte counts

Hoffmann et al. (2005). International validation of novel pyrogen tests based on human monocytoid cells.

***J Immunol Methods*, 298: 161-73**

Collaborative study

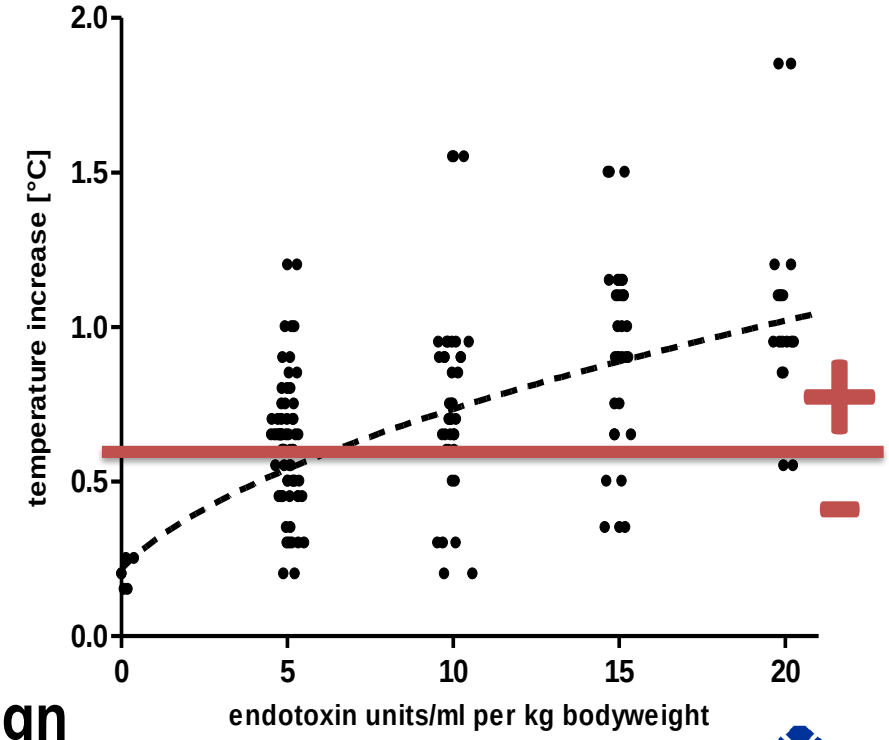
- **EU FP5 project**
- **2000-2003**
- **10 partners**
- **6 *in vitro* tests**
- **Validated by ESAC 2006**



The rabbit test as „reference“

The temperature reaction of a sensitive strain (n = 171)

- **limit temperature increase in Europe: 0.55°C**
  - ➡ **5 EU/10 ml = 0.5 EU/ml defined as threshold**
- **13 drugs with five contaminations each (0, 0.25, 0.5, 1 EU/ml)**



challenging original study design

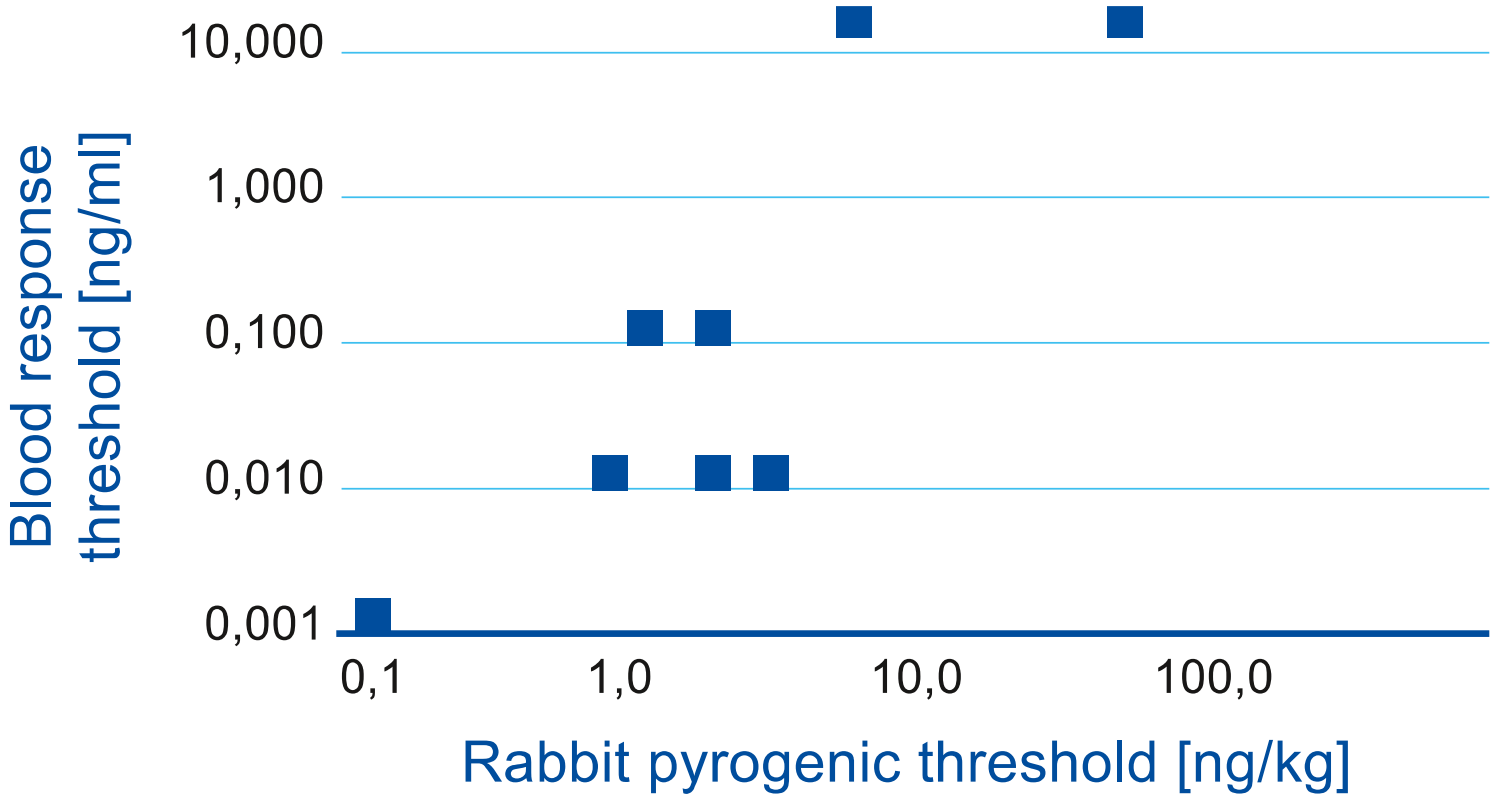
➡ **(58% specific, 88% sensitive for rabbit)**

Comparison of approaches

	advantage	disadvantage
Cell lines	No donor	High variability Laborious
PBMC	Most sensitive	Handling artefacts Infection risk
Whole blood	Cell suspension Physiological Strongly buffered	Infection risk
Cryo-blood	Standardisation Availability	(DMSO present)

MAT in contrast to BET

LAL reflects in vivo potency of different LPS



Fennrich et al., Dev. Biol. Stand. 1999, 101:131-139

Tab. 14: Result of LAL-testing of the 10 spiked drugs used in the MAT validation study

> ***Fourth lesson: A substantial number of products might have transitioned from rabbit testing to LAL because of flawed product-specific validations (not allowing time for endotoxin masking).***

Σ		12	12	24
NIBSC		"Truth" –	"Truth" +	Σ
PM	+	4	2	6
	–	**8**	**10**	18
Σ		12	12	34

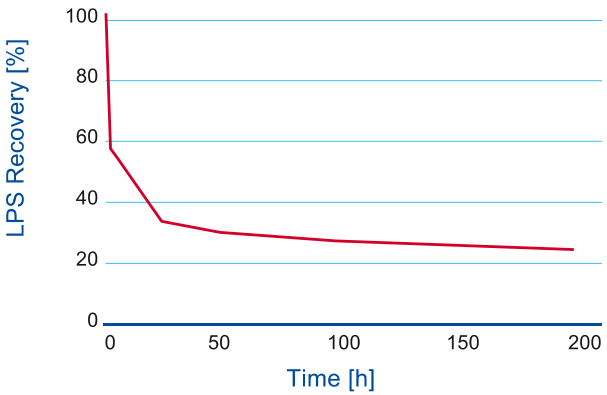
00%

sens: 83%

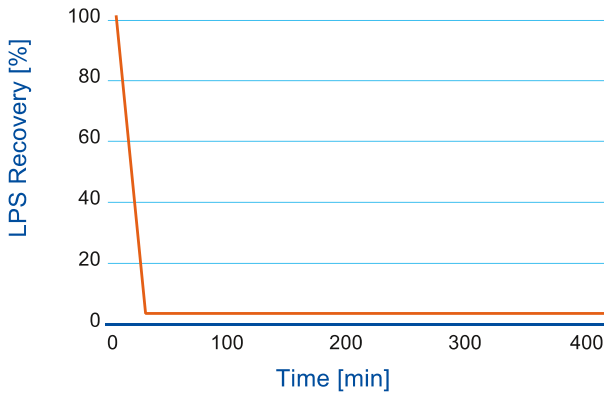
spec: 33%

Masking of endotoxin in LAL

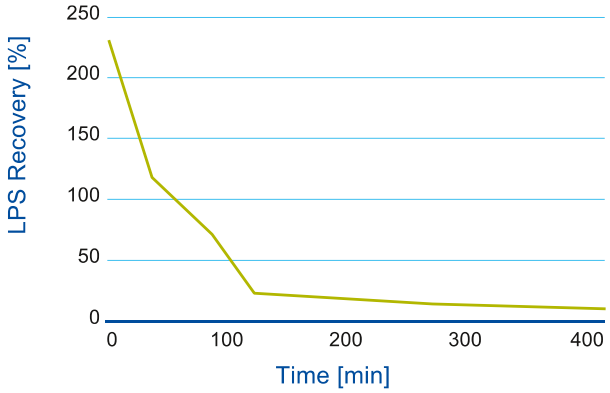
BSA/Citrate



Tween/Citrate



Tween/BSA /Citrate



Presented by Dr. Wolfgang Mutter, Hyglos, Bernsried, Germany, at VAAM expert group „Qualitätssicherung & Diagnostik“ at 20th of September 2013 in Villingen-Schwenningen, Germany

Tab. 6: Limitations of rabbit pyrogen tests and LAL versus the MAT

Rabbit pyrogen test	MAT
Animal use	None

Fifth lesson: MATs overcome often neglected problems of the LAL – they are not only the solution for those products / applications for which we cannot transition from rabbits to LAL.

Test is not applicable for certain drugs: Radiopharmaceuticals (short half-life), Chemotherapy (toxic), sedatives/analgesics (lower body temperature), Cytokines (increase body temperature), Antibiotics/plasma proteins/antigens (allergic reactions)	So far all assessed drugs were testable with appropriate dilution; the adsorption-wash-in-vitro-pyrogen-test was developed for cytotoxic substances (Daneshian et al., 2006)
LAL	**MAT**
Restricted to endotoxins	Coverage of Gram-positive (Hasiwa et al., 2013a) and fungal pyrogens (Schindler et al., 2009)
Disturbed by endotoxin-binding components (often not seen because of spike just before testing), e.g., blood components, lipids, solid materials (medical devices), cell therapies	Not observed
Potencies of pyrogens and synergies not reflected; difficult to correlate with rabbit test	Correlates with rabbit (Fennrich et al., 1998)
False-positive for glucans, cellulose and many herbal preparations	No
Not a full *in vitro* substitute; US landings down from 3 to 0.7 million (1998-2005), see also (Anderson et al., 2013)	No animal use or suffering

Comparison rabbit test and MAT for human serum albumin

Spreitzer et al., ALTEX 2002

	Rabbit pyrogen test (n = 261)		WB-IL-1		
Sixth lesson: Tests based on human-relevant mechanisms and measuring biomarkers of pathophysiology can be better than animal tests. They are also often enabling technologies, which allow uses not possible with the animal test.					
5 EU	**5**	**23**	**1**	**29**	**-**
10 EU	**10**	**21**	**8**	**29**	**-**

29 different batches of albumin were tested pure and spiked with 5 or 10 IU/ml

ICCVAM Recommendations: Future Studies

Mav 2008

Eighth lesson: Applying scrutiny to tests that are to be validated but not to the ones in use (the point of reference), which were never validated, and not acknowledging their shortcomings, creates hurdles for new tests where common sense would argue differently. Squeezing out of a group of experts only critical arguments about the new test and not asking them to compare it to the existing one does not do justice to new tests. Conflict of interest of experts needs to be considered more seriously.

Estimated costs $5-25 million

Assessment of pyrogenic contaminations with validated human whole-blood assay

Mardas Daneshian[1], Sonja von Aulock[1,2] & Thomas Hartung[1,3]

Nature Protocols 2009, 4:1709-1721

DB-ALM Protocol n° 133 : Human Whole Blood/Interleukin (IL)-1 Beta In Vitro Pyrogen Test (WBT)

Pyrogenicity Testing

https://ecvam-dbalm.jrc.ec.europa.eu/methods-and-protocols/biological-endpoint/mediator-release:-cytokines/key/em_938

23 June 2016, Strasbourg, France

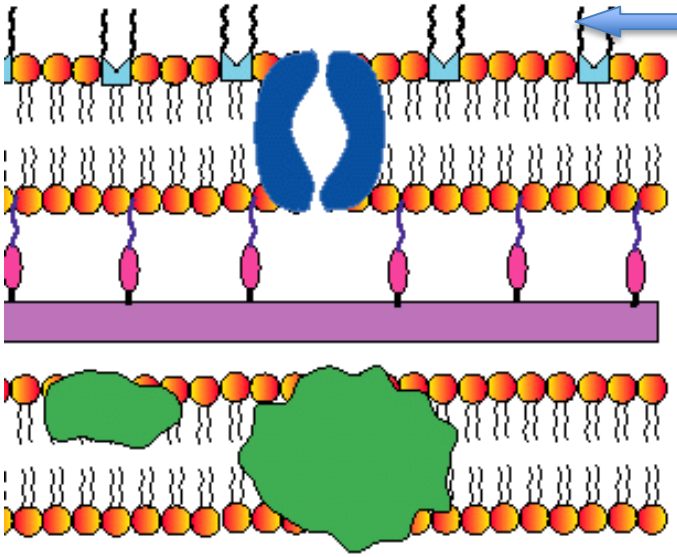
European Pharmacopoeia Commission adopts revised general chapter on Monocyte-activation test to facilitate reduction in testing on laboratory animals

2017 USP

2018 ISO

Bacterial pyrogens

Gram-negative bacterial cell wall

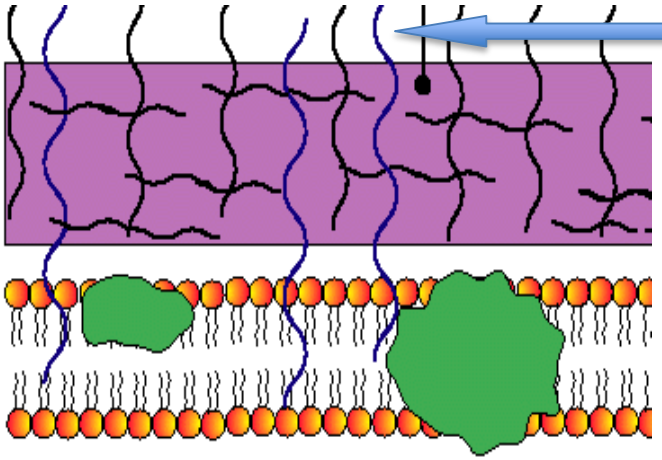


LPS

(Lipopoly-saccharide)

Medline publ. ~ 50 000

Gram-positive bacterial cell wall



LTA

(Lipoteichoic acid)

Medline publ. ~ 1 000

New challenges

Patient safety: non-endotoxin pyrogens

New expression systems in gene technology (Gram-positive bacteria, insect and fungal cells)

Medical devices

Cellular therapies

Environmental pyrogens

Seventh lesson: There are products on the market that are contaminated with non-endotoxin pyrogens. The extent of this problem will only be known when MAT-like technologies are introduced for screening.

transatlantic think tank for toxicology

ALTEX 2013, 30:169-208

t4 Report*

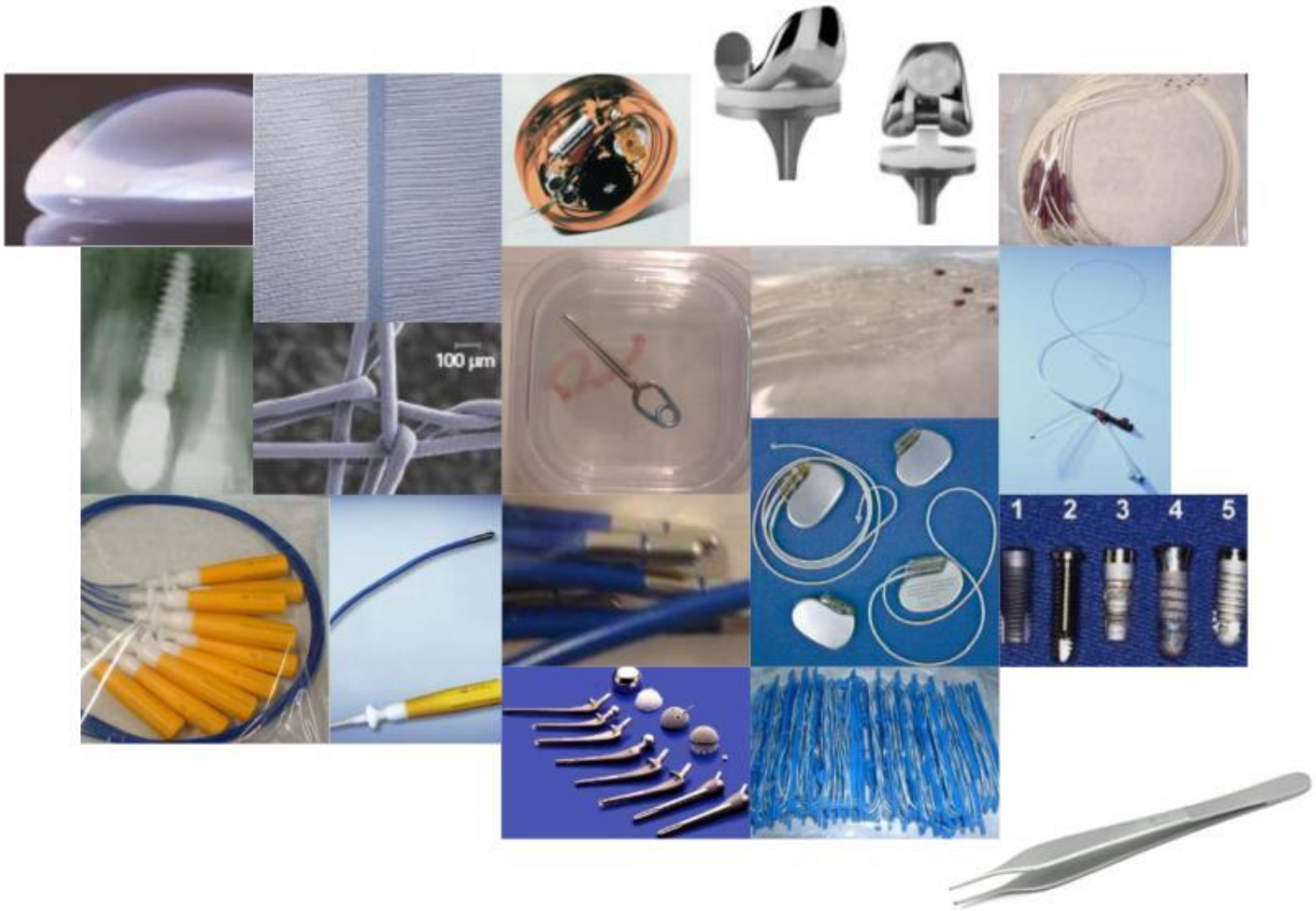
Evidence for the Detection of Non-Endotoxin Pyrogens by the Whole Blood Monocyte Activation Test[1]

Nina Hasiwa [1,2], Mardas Daneshian [1], Peter Bruegger [4], Stefan Fennrich [5], Astrid Hochadel [2], Sebastian Hoffmann [6], Felix E. Rivera-Mariani [3], Christoph Rockel [7], Stefanie Schindler [8], Ingo Spreitzer [9], Sandra Stoppelkamp [5], Kranthi Vysyaraju [3], and Thomas Hartung [1,3]

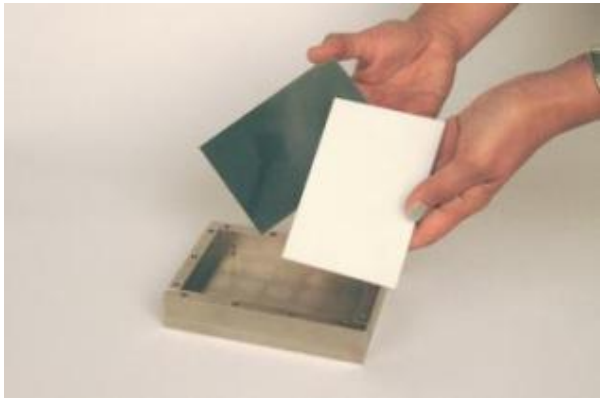
Submitted to FDA / ICCVAM Dec 2012

Never discussed

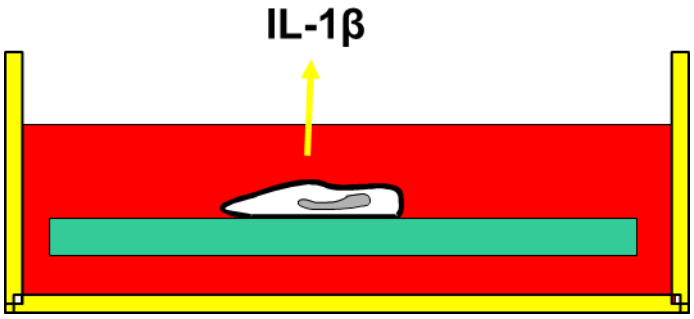
Examples for medical devices



15-well steel block

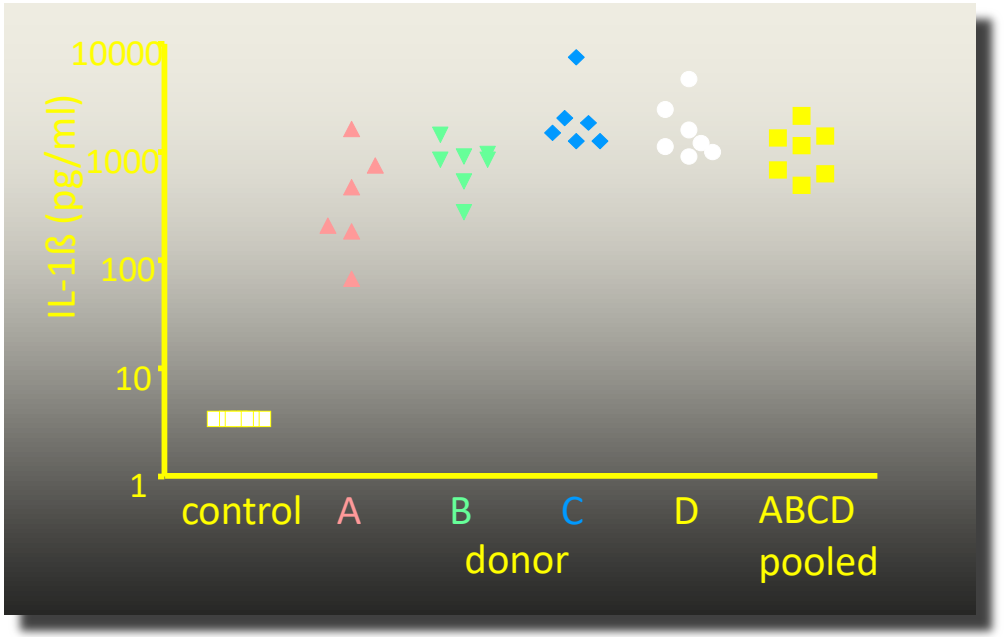


STATIC CONTACT



15-well steel block

Contamination of steel by manual handling

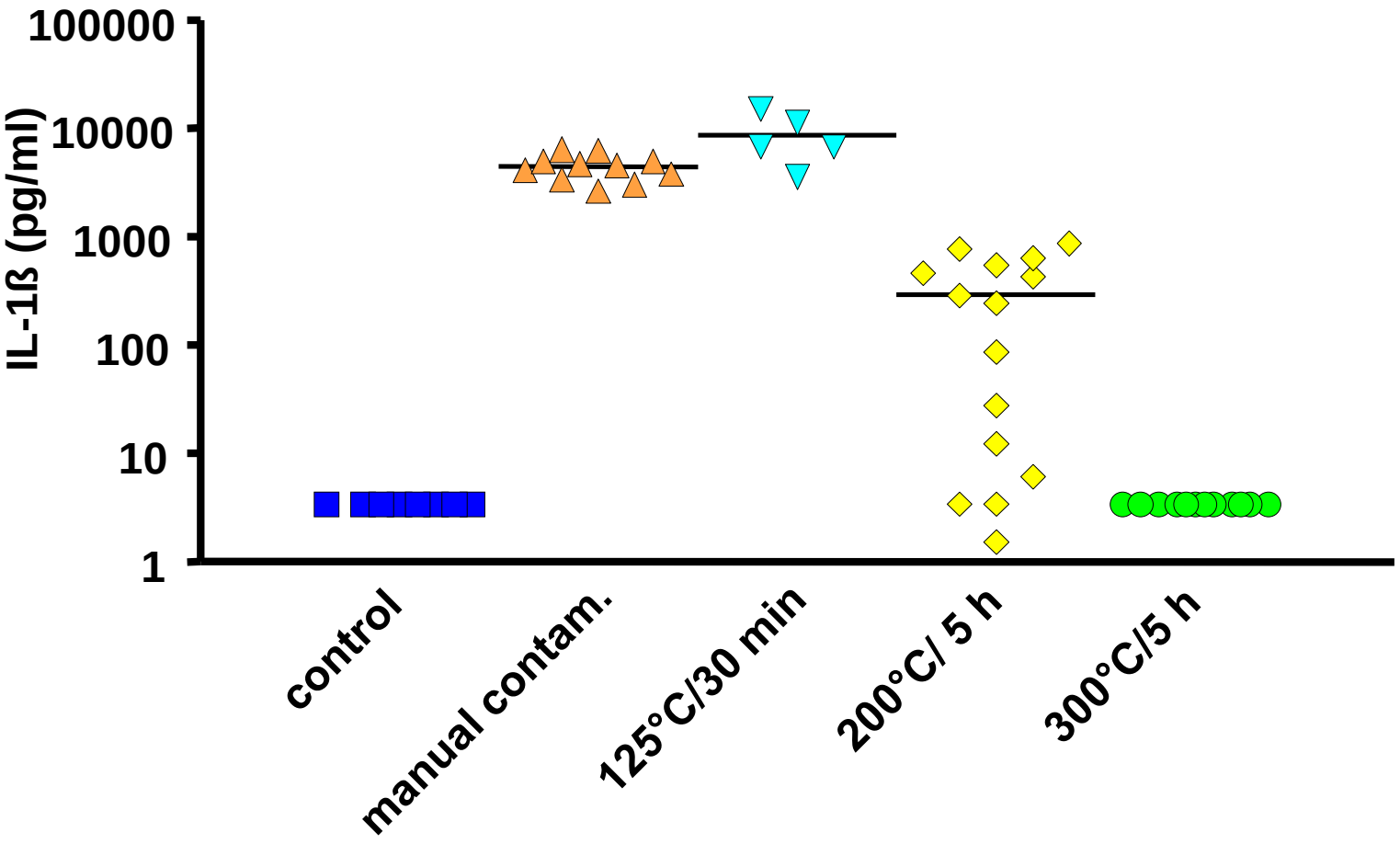


An in vitro pyrogen safety test for immune-stimulating components on surfaces

Marina Hasiwa[a,b], Karin Kullmann[a,b], Sonja von Aulock[b], Christoph Klein[a], Thomas Hartung[a,b,*]

Biomaterials 2007, 28:1367–1375

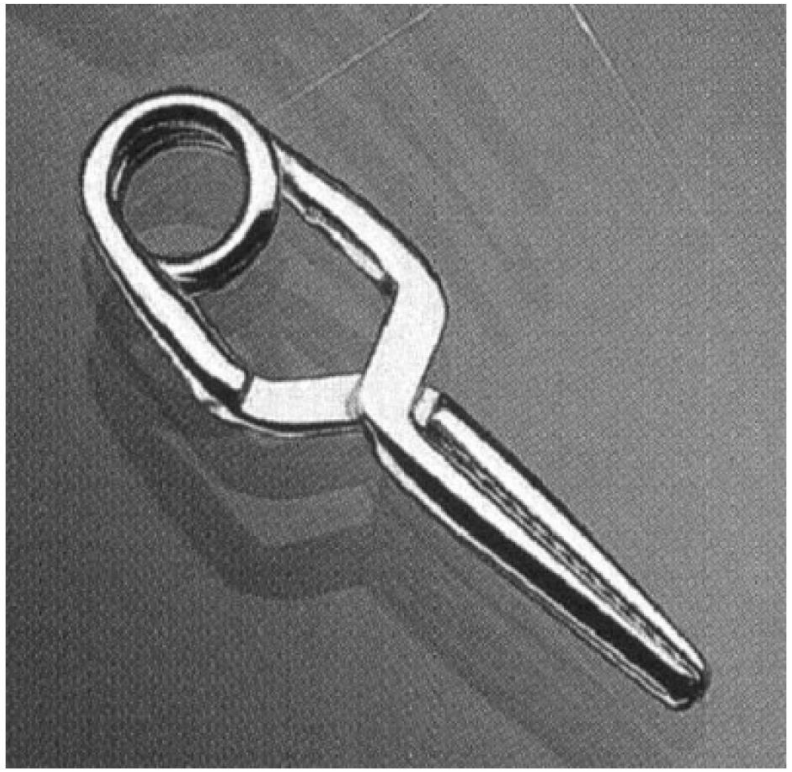
Elimination of pyrogenic residuals on titanium plates contaminated by manual handling



In vitro pyrogen test—A new test method for solid medical devices

Francesca Mazzotti,[1] Julia Beuttler,[1] Richard Zeller,[2] Ulrich Fink,[2] Stefanie Schindler,[1] Albrecht Wendel,[1] Thomas Hartung,[1,3] Sonja von Aulock[1]

J Biomed Mater Res 2007, 80A:276–282



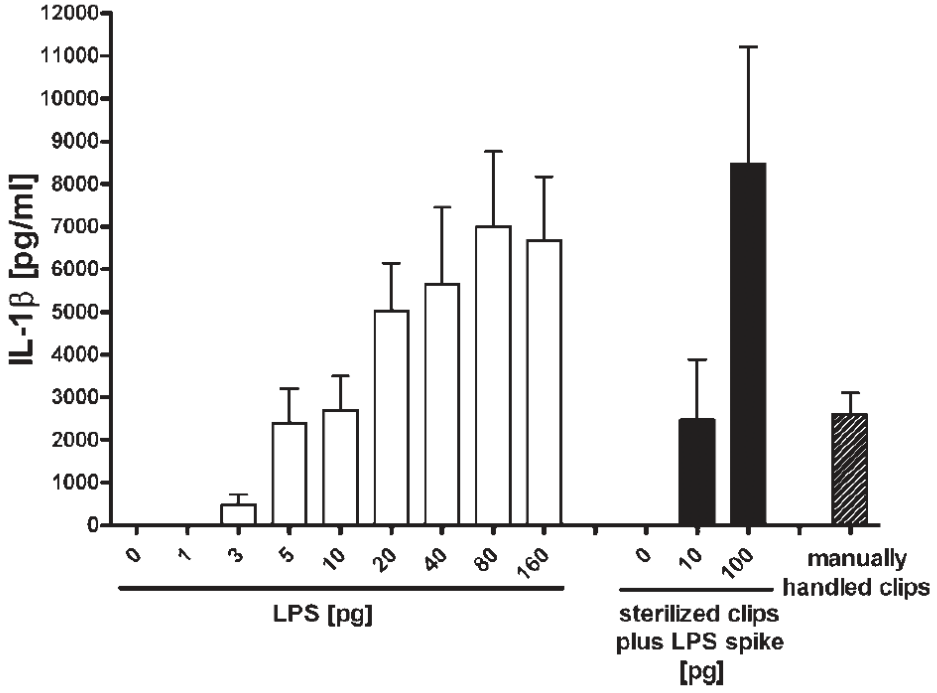
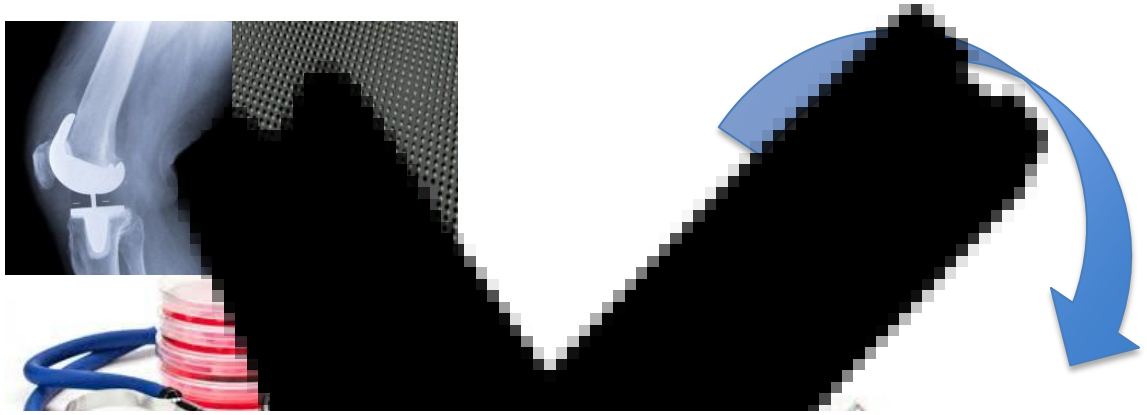
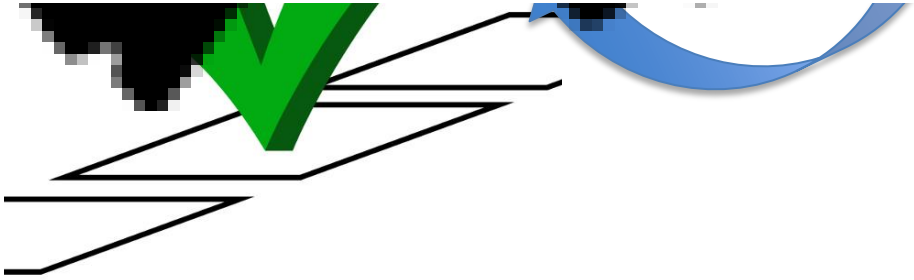


Figure 1. Yasargil titanium aneurysm clip.

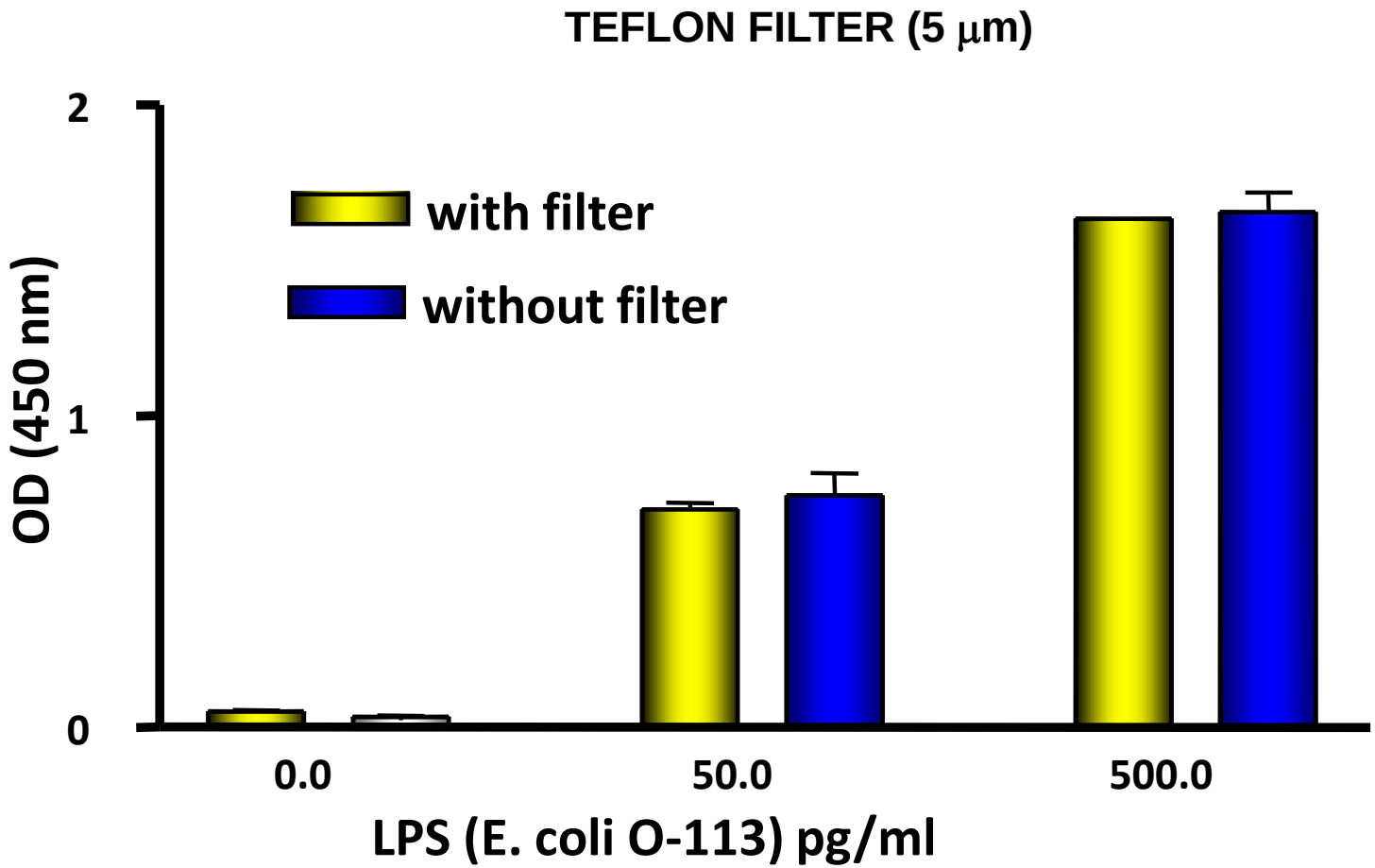


Bacteria & pyrogens

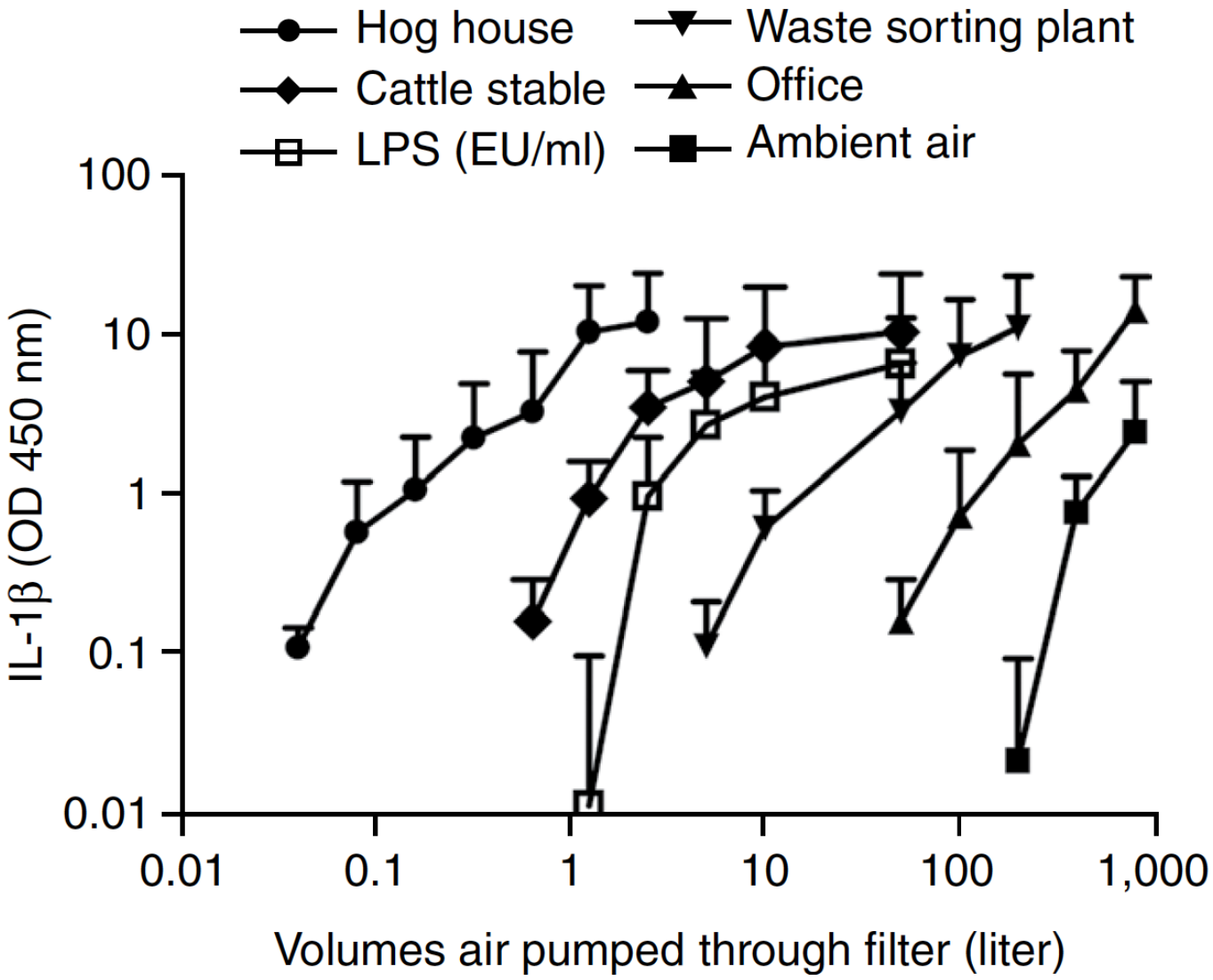
Eleventh lesson: Pyrogen testing on the surface of medical devices is a paradigm shift advancing the safety of these products, but implementation is slow. Nanomedicines might change this. A key point is that the effect of pyrogen contamination for patients has not been studied and thus hazard is somewhat hypothetical.



Endotoxin determination on PTFE filters



Monitoring Air Quality



Nature Protocols 2009, 4:1709-1721

Air-born pyrogens as risk factor for asthma and COPD

- **Human relevant test system**

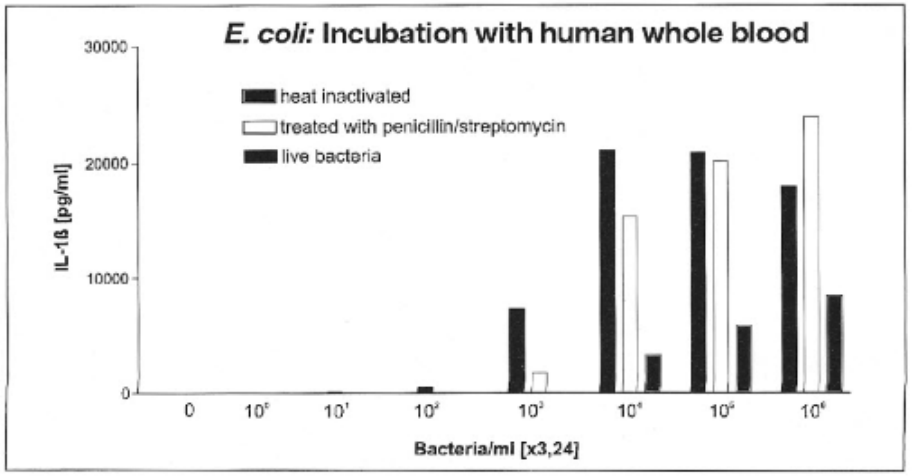
frontiers in Pharmacology

ORIGINAL RESEARCH
published: 30 March 2017
doi: 10.3389/fphar.2017.00157

Check for updates

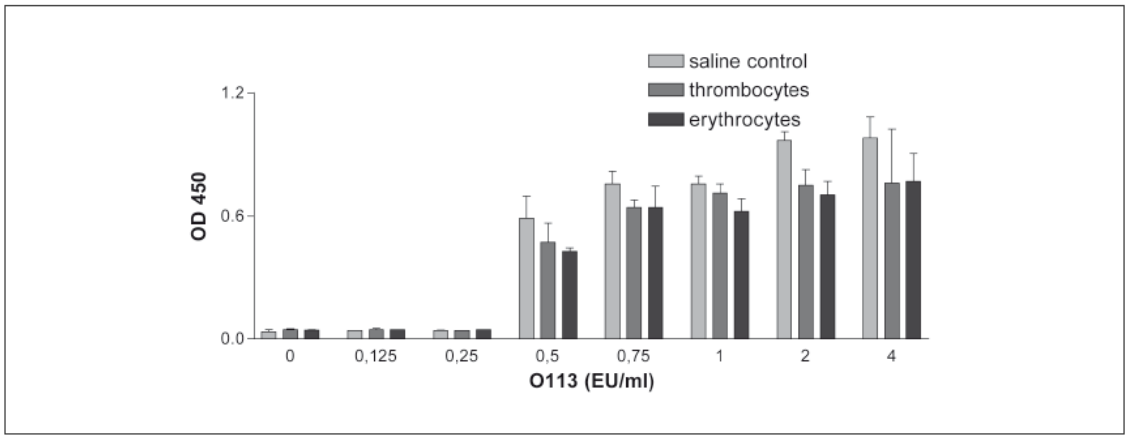
Whole Blood Cytokine Response to Local Traffic-Related Particulate Matter in Peruvian Children With and Without Asthma

*Jesse P. Negherbon[1], Karina Romero[2], D'Ann L. Williams[1], Rafael E. Guerrero-Preston[3], Thomas Hartung[1,4], Alan L. Scott[5], Patrick N. Breysse[1], William Checkley[2,6] and Nadia N. Hansel[1,6]**



US: 30 million blood transfusions /a
None tested for pyrogens
About 1.000 fatalities /a

Retrieval of live bacteria



Endotoxin retrieval in erythrocyte and platelet concentrates

Fig. 3: Retrieval of endotoxin spikes in red blood cell and platelet concentrates
Clinical erythrocyte and platelet concentrates were spiked with reference LPS at the concentrations indicated. WBT was carried out directly on these samples.

FDA study pr elected

Tenth lesson: Bacterial and pyrogen contaminations of blood products and cell therapies kill thousands of people per year (in the US alone about 1,000 people die from platelet-rich plasma). The whole blood pyrogen test seems to be a solution, but nobody exploits it.

Lessons learned

- alternative methods can outperform animal tests
- Mechanism-based & human cells
- importance of commercialization
- 15 years from development to acceptance
- Importance International harmonization
- lack of enforcement of implementation
- enabling technology
- importance of public-private partnership

Tab. 7: A little who-is-who in the development of the whole blood pyrogen test

Name	Role
Ingeborg S. Aaberge (with L. M. Naess and B. Mogster)	Partner in validation study at NIPH, Oslo, Norway
Wilhelm Berg	Patent attorney, Germany
Susanne Berthold (with P. Zwerenz, W. Klingler and R. Dostatni)	Pyrogen project manager at DPC Biermann, Germany
Audrey Borel (with G. Schmitz)	Pyrogen project manager at Merck-Millipore, France
Rogier Bos (with M. vam Aalderen, M. Gommer and R. Nibbeling)	Partner in validation study at RIVM, Utrecht, The Netherlands
Peter Brügger (with E. Frey)	Partner in validation study at Novartis, Switzerland
Sandra Coecke (with G. Bowe, J. Casado and J. de Lange)	ECVAM, partner in validation study
Mardas Daneshian	PhD on fungal and airborne pyrogens as well as problematic test products (dialysis, cytotoxic and lipophilic drugs); joint first author background review on non-endotoxin pyrogens (Hasiwa et al., 2013a)
Stefan Fennrich	Project manager for the pyrogen test in our group for many years, continues to promote the technology from University of Tübingen, Germany
Matthias Fischer	Paul-Ehrlich-Institute; early evaluation of the test (Fischer et al., 1998)
Marlies Halder	Led the ECVAM peer-review and hand-over to ICCVAM
Thomas Hartung	Inventor and principal investigator
Nina Hasiwa	PhD on pyrogen testing of medical devices and removal from surfaces; joint first author background review on non-endotoxin pyrogens (Hasiwa et al., 2013a)
Kilian Hennes	Cryoblood validation study partner at Qualis, now Zwisler Laboratories, Konstanz, Germany
Sebastian Hoffmann	Biometry of the validation studies and modeling of rabbit responses (Hoffmann et al., 2005, 2006)
Foster Jordan (with B. Knörzer)	Kit marketing by Charles-River Endosafe (CEO)
Thomas Jungi (with M. Brcic)	Partner in validation study at University of Berne, Switzerland
Felix Rivera-Mariani (with P. Breysse and J. Negherbon)	Post-doc JHSPH on airborne pyrogens
Thomas Montag-Lessing	Early partner at the Paul-Ehrlich-Institute in the development and validation of the test
Siegfried Morath (with C. Draing, S. Deininger, R. R. Schmidt, I. Figueroa-Perez, A. Stadelmaier, A. Geyer, A. Kinsner, K. Hoebe, S. Traub, C. Hermann, S. von Aulock and others)	Developed in his PhD the isolation procedure for lipoteichoic acid, the key non-endotoxin pyrogen (Morath et al., 2001) used as reference material in the kits; showed the activity of the respective synthesized structures (Morath et al., 2002; Deininger et al., 2007) and enabled their biological characterization.
Rolando Perdomo Morales (with Z. Pardo-Ruiz)	Adaptation and evaluation in Cuba
Stephen Poole (with Y. Mistry)	Partner in validation study at NIBSC, UK and developer of one-plate version (Poole et al., 2003)
Octavio Presgrave (with I. Fernandes Delgado and others)	Adaptation and evaluation in Brazil (BraCVAM)
Wolfram Riedel	Recognized the potential of the technology and brokered the first license with DPC Bierman, Bad Nauheim, Germany
Stefanie Schindler	PhD and DVM/PhD thesis on the test including optimization of cryopreserved blood, validation studies, testing of lipophilic samples and airborne pyrogens.
Anke Schulz (with B. Holtkamp and G. Schmitz)	Pyrogen project manager at Biotest, Germany
Ingo Spreitzer (with B. Loeschner, S. Müller and others)	PhD on non-endotoxin pyrogens; then work on pyrogenicity testing at the Paul-Ehrlich-Institute
Sonja von Aulock	Studied the variance of whole blood responses in large donor collectives (von Aulock et al., 2003, 2004).
Markus Weigandt, (together with M. Jahnke, P. Lexa and H.-G. Sonntag)	First independent evaluation of the test as thesis prompted by European Pharmacopoeia and financed by Roche-Boehringer Mannheim (Weigandt et al., 1998; Jahnke et al., 2000)
Albrecht Wendel	Co-inventor providing the infrastructure for R&D and partner for the commercialization
Gabriele Werner-Felmayer (with A. Peterbauer and P. Loitzl)	Partner in validation study at Innsbruck University, Austria

It takes a village…

Success has many fathers, failure is an orphan…

More than 60 people critically involved