

3rd – 6th March 2004 in Freising-Weihenstephan, Germany

*1st International qPCR Symposium &
Application Workshop*

qPCR and transcriptomics
A new tool to understand live

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A short history of qPCR

1970-80	DNA polymerases need RNA primers
1975-85	the way to recombinant Taq polymerase
1983-86	development of PCR
1993	PCR: Nobel Price in Chemistry
1988-95	first developments of quantitative PCR external and internal standardization (cDNA or cRNA) competitive PCR
1993	first qPCR training course in Weihenstephan
1996	real time PCR equipment becomes available
1996-2004	qPCR goes life sciences
2004	1 st International qPCR Symposium & Application Workshop



Weihenstephaner
Fortbildungs-Seminare

Forschungszentrum für Milch und Lebensmittel Weihenstephan, 85354 Freising
Vöttinger Str. 45, Technische Universität

**„Low abundance“ Rezeptor-mRNA Quantifizierung mittels
RT-PCR/HPLC-UV**

**FORTBILDUNGSKURS MIT UNTERSTÜTZUNG DER SEKTION
MOLEKULARE UND ZELLULÄRE ENDOKRINOLOGIE DER
DEUTSCHEN GESELLSCHAFT FÜR ENDOKRINOLOGIE**

Zeit und Ort: 08.-09. Juli 1993 im Institut für Physiologie

Wissenschaftl. Leitung: Kerstin Hagen-Mann, Alberto Malucelli
Heinrich H.D. Meyer

Organisation: Sabine Peckel (Sekretariat)
Tel : 08161/713508

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Endocrinology and qPCR

	DNA	RNA	Proteins	Steroids etc
1960			RIA <i>fmoles</i>	
1970			RRA ELISA <i>amoles</i>	RIA EIA
1980	PCR	RT-PCR		
1990	<i>1-100 molecules</i>	comp PCR real time PCR		
2000			PCR-IA	

New research options: transcriptome,
paracrine regulation, gene silencing

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qPCR Technology

validation

qualitative validation

analyte identity

quantitative validation

accuracy (true value)

precision (reproducibility)

quantification range

pre-analytical steps

RT

clean up

matrix effects

reagents and kits

validation of other methods with qPCR

new developments

new detection methods

new dyes

duplex and multiplex qPCR

high throughput

miniaturisation (1536 well plates)

chip + microfluid technology

cost effectiveness

new combinations

PCR / MALDI-TOF MS

trehalose – GCrich DNA

time requirement

potential for the clinic

and for food surveillance

Validation of quantitative steroid receptor real-time RT-PCR

	AR	ER α	ER β	PR
product length	172 bp	234 bp	262 bp	227 bp
detection limit	12 molecules	2 molecules	10 molecules	14 molecules
quantification limit	120 molecules	165 molecules	106 molecules	760 molecules
quantification range (test linearity)	120 - 1.20*10 ¹⁰ molecules (r = 0.998)	165 - 1.65*10 ⁹ molecules (r = 0.995)	106 - 1.06*10 ¹⁰ molecules (r = 0.996)	760 - 7.60*10 ⁹ molecules (r = 0.998)
PCR efficiency	90.7%	81.2%	81.3%	93.9%
intra-assay variation	31.2% (n = 3)	18.7% (n = 4)	17.6% (n = 4)	5.7% (n = 4)
inter-assay variation	24.3% (n = 7)	28.6% (n = 4)	29.7% (n = 4)	25.7% (n = 4)
Species specific T_{melt} (°C)				
<i>Homo sapiens</i>	85.4	86.0	[87.9]	83.5
<i>Rattus norvegicus</i>	84.4	85.0	89.0	[82.9]
<i>Callithrix jacchus (primate)</i>	85.0	--	[89.9]	83.9
<i>Bos taurus</i>	85.5	85.3	90.1	83.8
<i>Ovis aries</i>	--	85.4	90.5	83.1
<i>Sus scrofa</i>	84.5	86.0	90.2	83.5

use of qPCR

disease diagnostics – pathogen quantification

human and veterinary medicine
phytopathology
food hygiene

transcriptomics

development biology
endocrinology
infection biology
drug response
gene silencing

basic research

gene polymorphism
microorganisms, plants,
animals and humans
pharmacogenomics
nutrigenomics
resistance mechanisms

other applications

biotechnology
conservation biology
forensic analyses

Pathogen analysis

- ⇒ hepatitis A virus and C virus, parvovirus in human transfusion plasma
- ⇒ adenovirus, clamymdia trachomatis, AIDS
- ⇒ apple proliferation disease, plum pox, Dutch-elm disease
- ⇒ mycobacterium av. Paratuberculosis
- ⇒ salmonella enteritidis

Transcriptomics

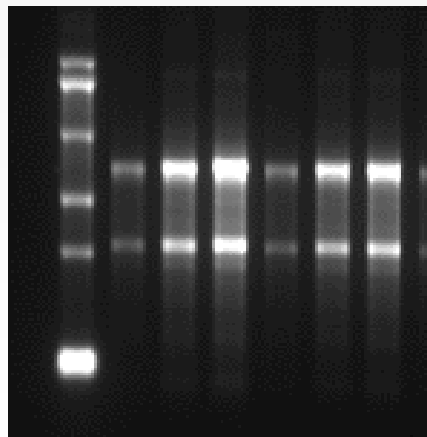
- ⇒ gene expression profiling in yeast depending on metabolic state
- ⇒ different susceptibility and response to malaria in African children
- ⇒ profiling of tumor biopsies and in leukemia patients
- ⇒ growth regulation in small intestine

Transcriptome

RNA sub-classes in a mammalian cell total RNA

ribosomal RNA	rRNA	80-85%	(5S, 18S und 28S)
transfer RNA	tRNA	10-15%	
messenger RNA bases)	mRNA	1-5%	(Ø length 1930

<i>high abundant</i>	< 100 genes	10-20 * 10 ³ copies/cell
<i>intermediate abundant</i>	~ 500 genes	200-400 copies/cell
<i>low abundant</i>	> 30.000 genes	<20 - 50 copies/cell



←	28S rRNA	3898-6333 bases
←	18S rRNA	1898-1976 bases
←	5S rRNA	120 bases

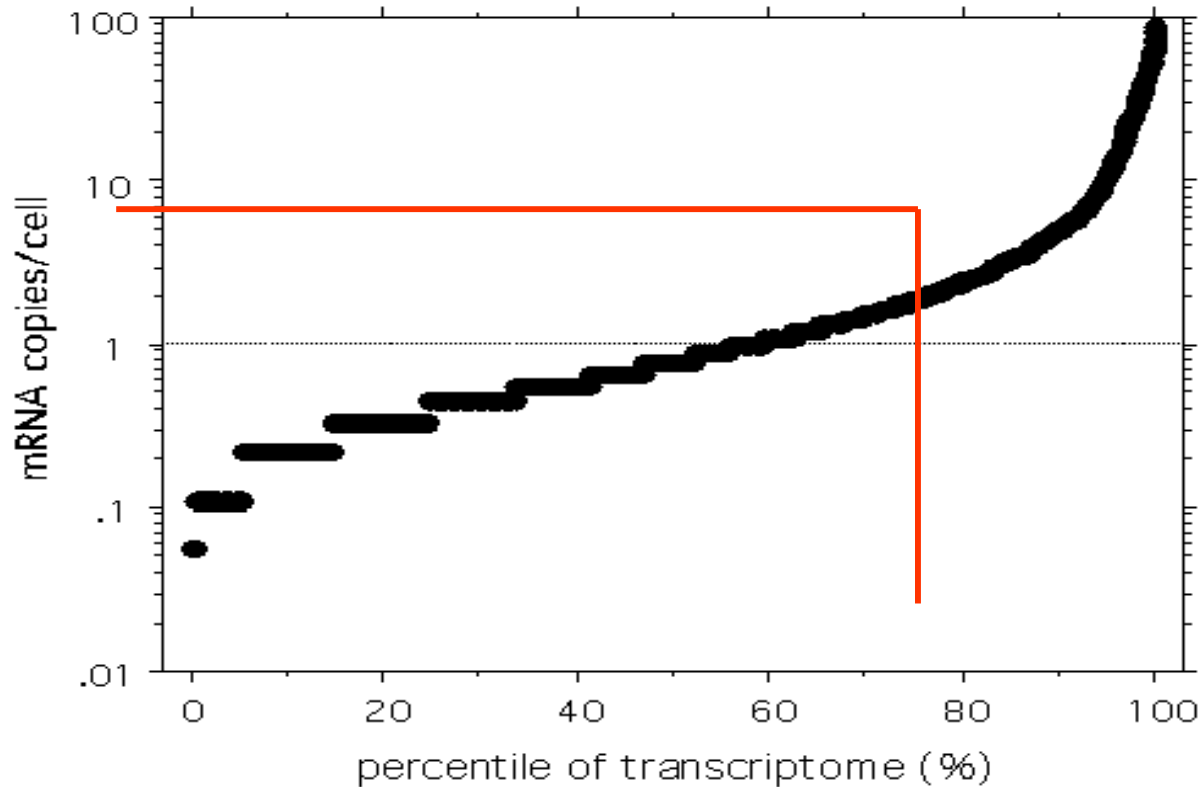
Transcriptomics in Yeast

5460 transcripts were investigated
estimated 15000 poly-A RNAs per cell

average level: 2.8 copies/cell

median level: 0.9 copies/cell

80% of the yeast transcriptome is expressed at 0.1-2
copies/cell



Molecular physiology
mRNA quantification via qRT-PCR

Physiology Weihenstephan

⇒ **Secondary features of estrogens**
expression of estrogen receptors

⇒ Androgenic activity of crop fungicides
gene expression assay in prostate cell lines

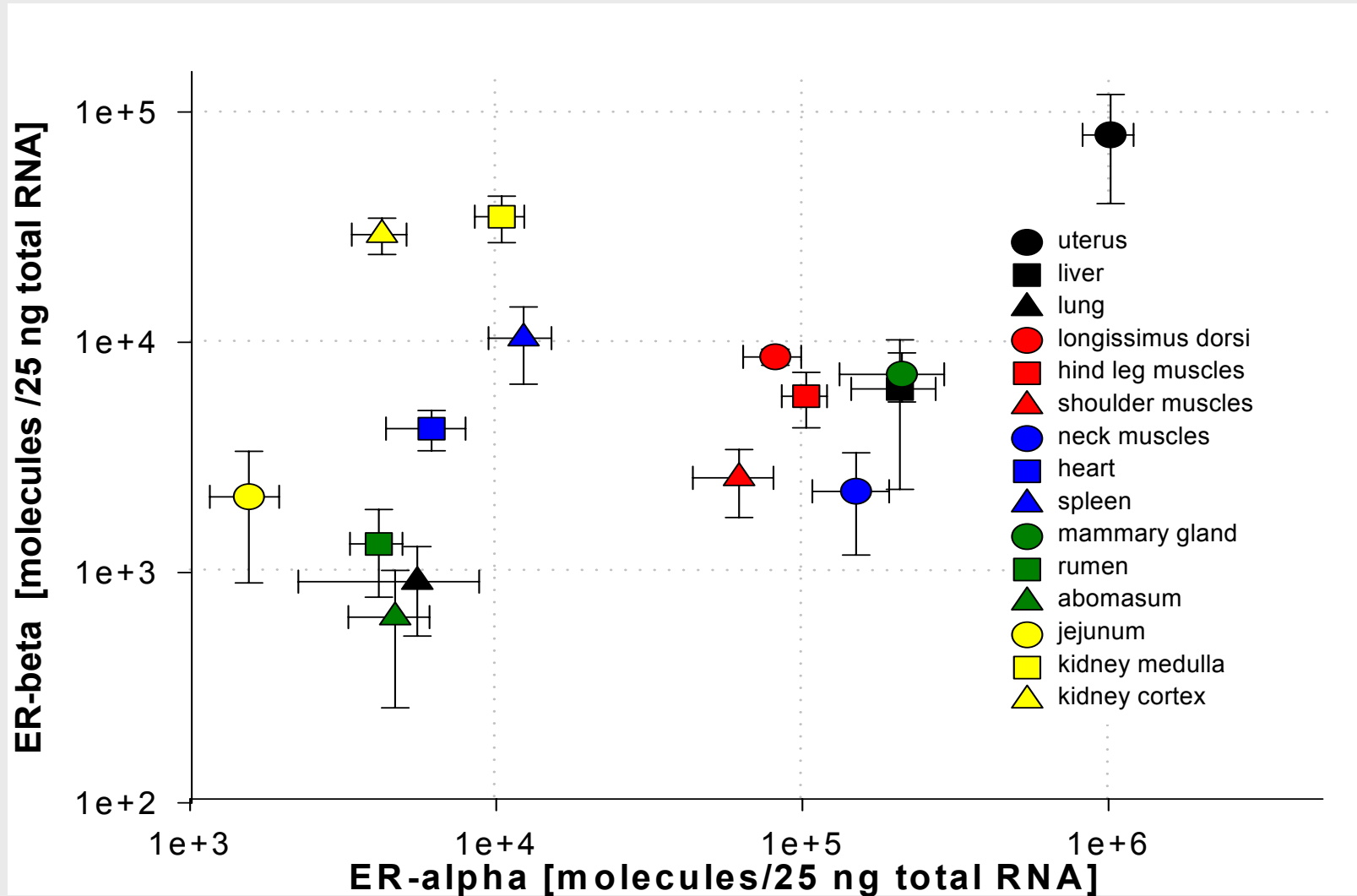
⇒ Mammary gland immunology
pro-inflammatory signals of somatic cells and epithelia

⇒ Embryo-maternal communication

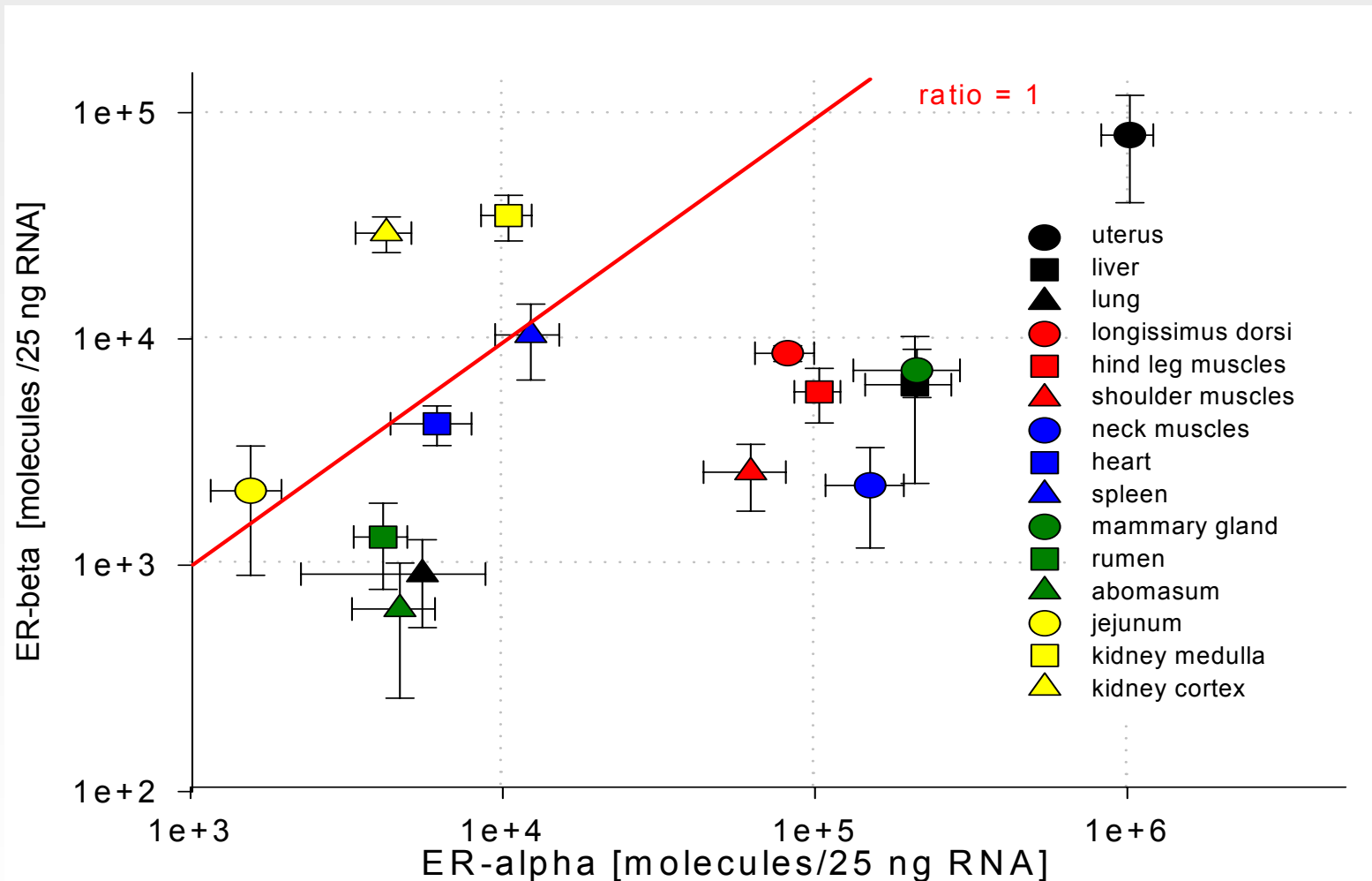


ER α and ER β expression pattern in bovine tissues

(n=8; mean with bi-directional SEM bars; in cDNA molecules / 25 ng total RNA)

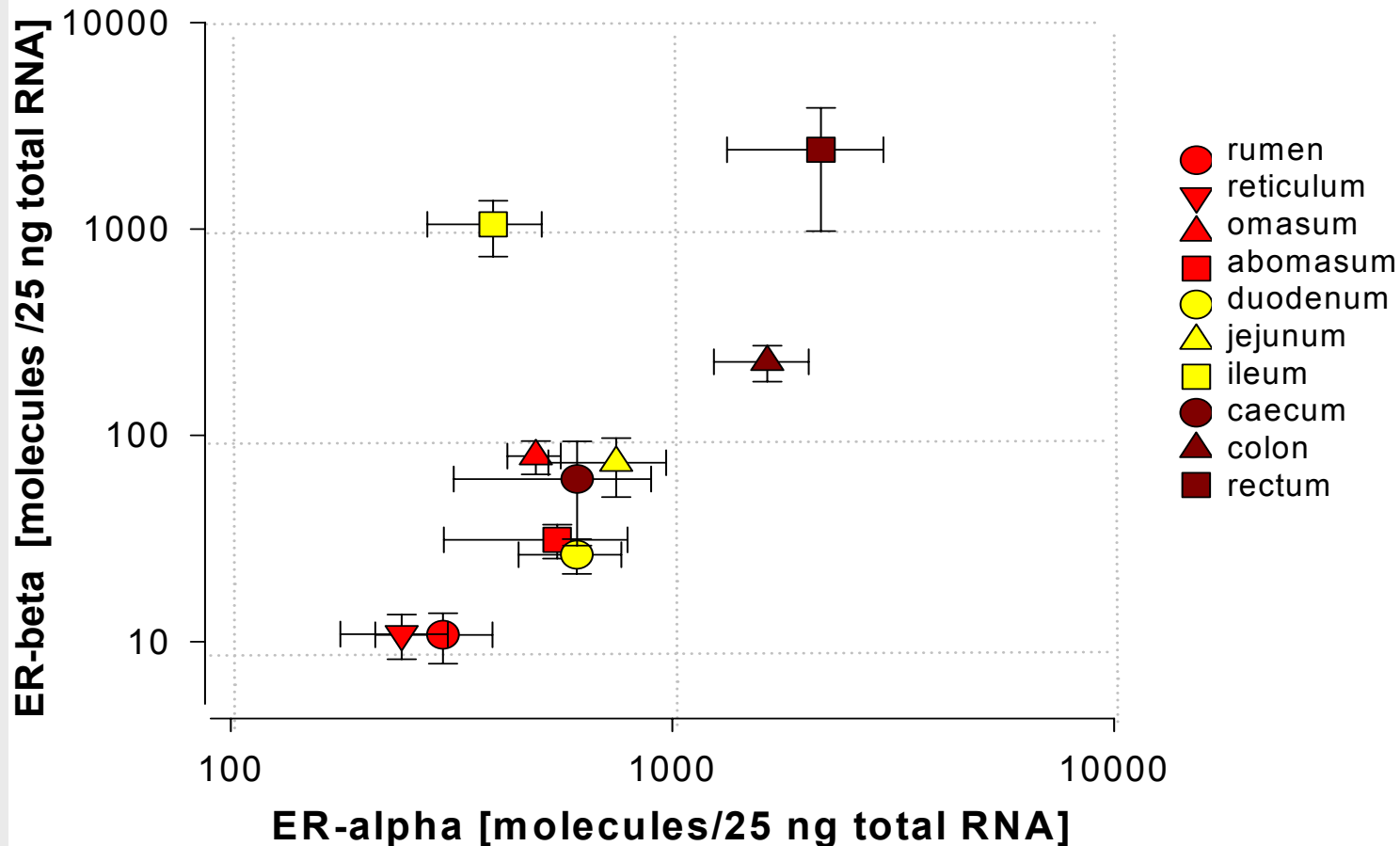


Estrogen receptors (ER α & ER β) expression pattern in numerous cattle tissues

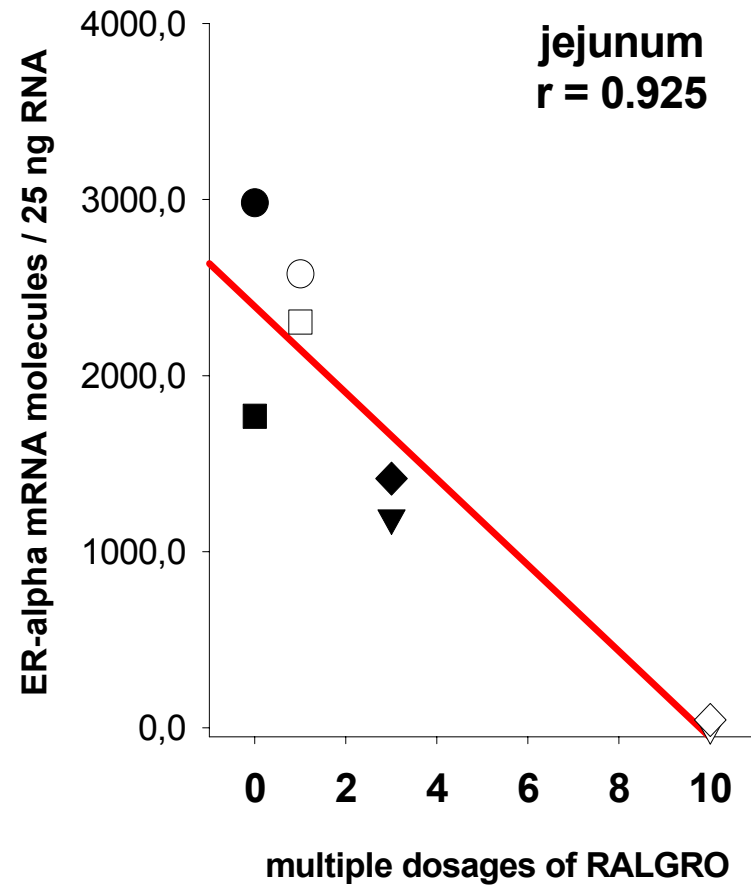
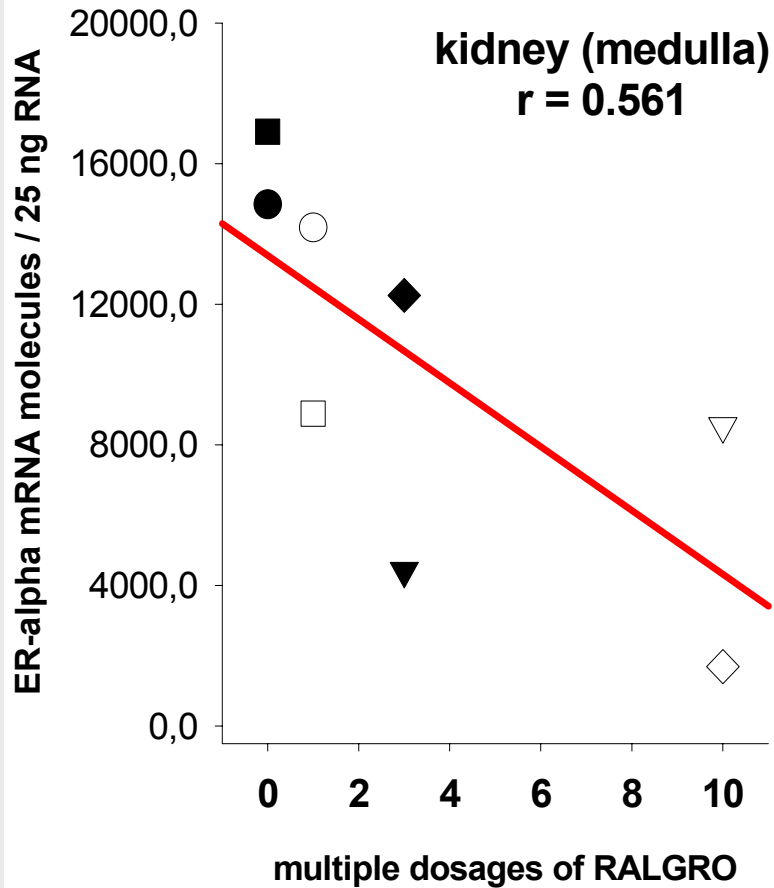


ERa and ERb expression pattern in bovine gastro-intestinal compartments

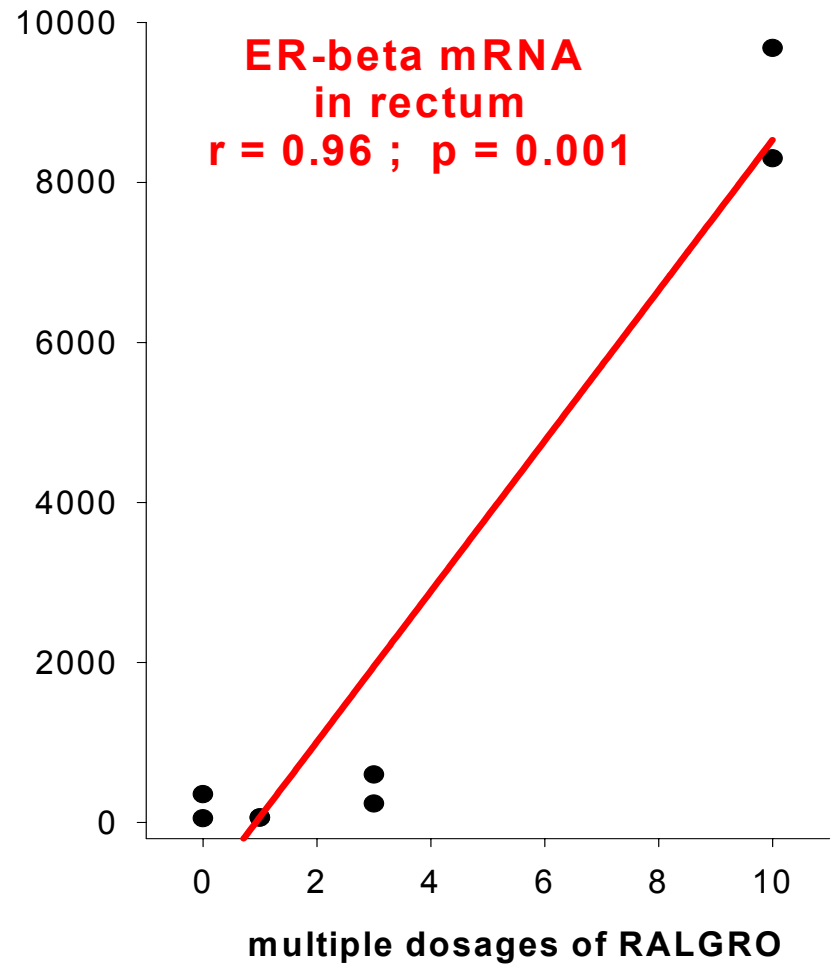
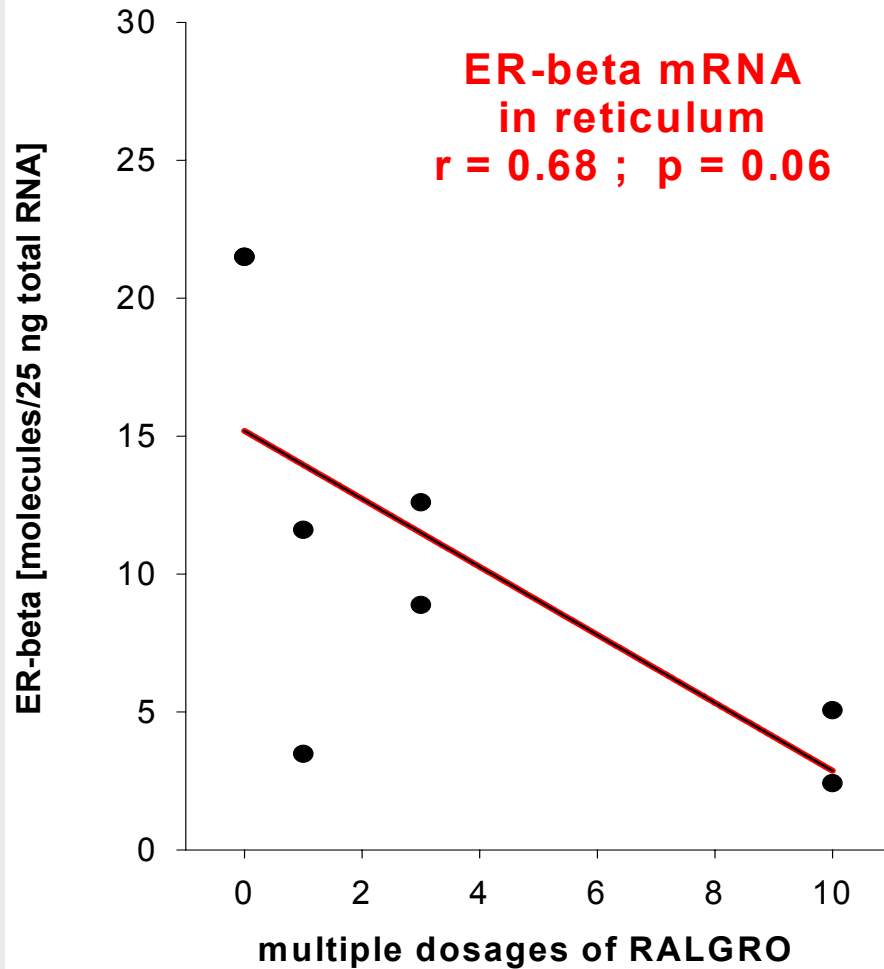
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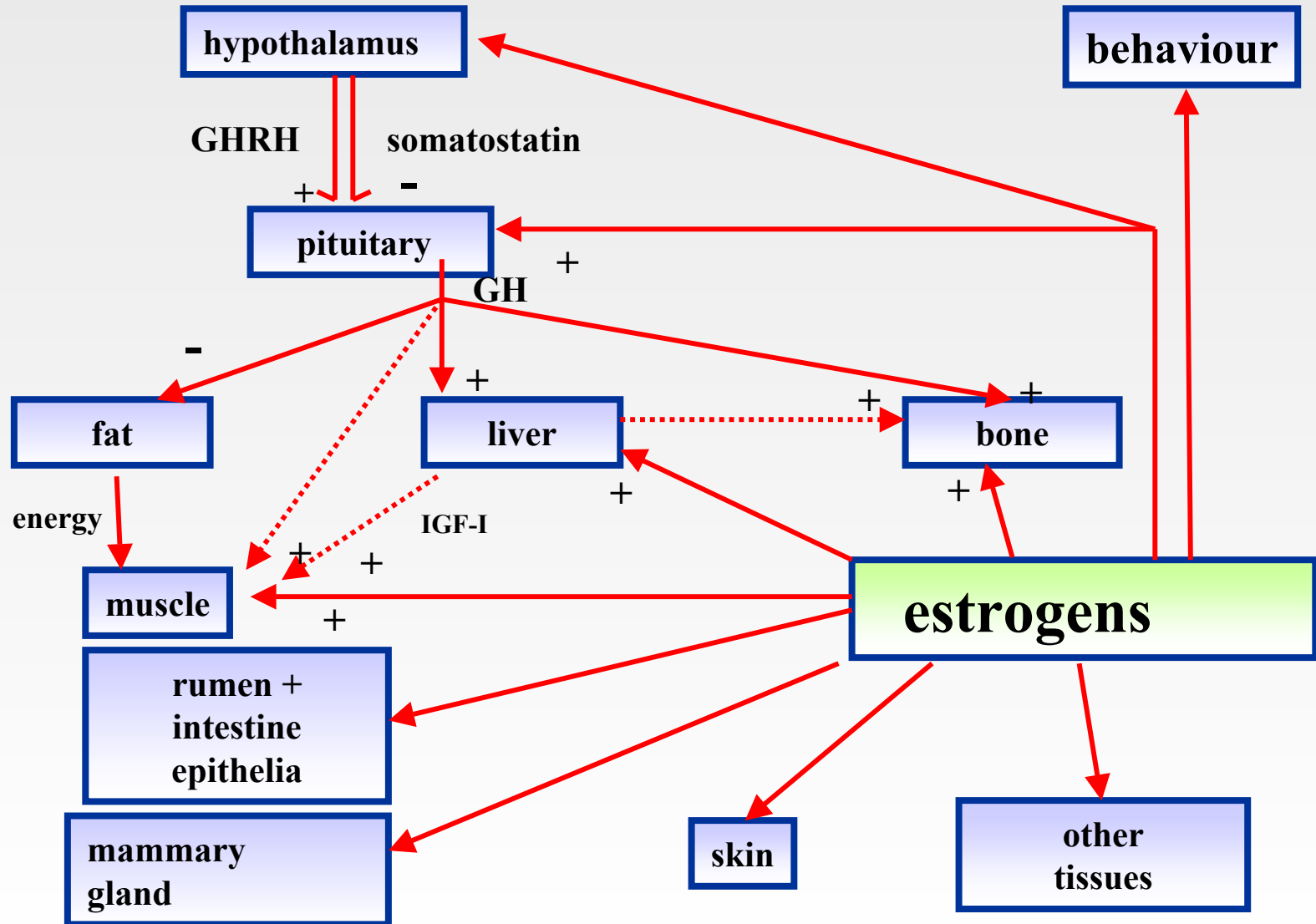
ER α mRNA expression in bovine tissues



ER β expression in bovine gut compartments



Secondary features of estrogens







Molecular physiology

mRNA quantification via qRT-PCR

Physiology Weihenstephan

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⇒ **Androgenic activity of crop fungicides**
gene expression assay in prostate cell lines

⇒ Mammary gland immunology
pro-inflammatory signals of somatic cells and epithelia

⇒ Embryo-maternal communication



Screening of androgenic activity

Situation

Androgenic and anti-androgenic side activities of herbicides, fungicides etc. are rarely investigated

Task

Development of a selective bioassay for analysis of androgenic activities

Answer

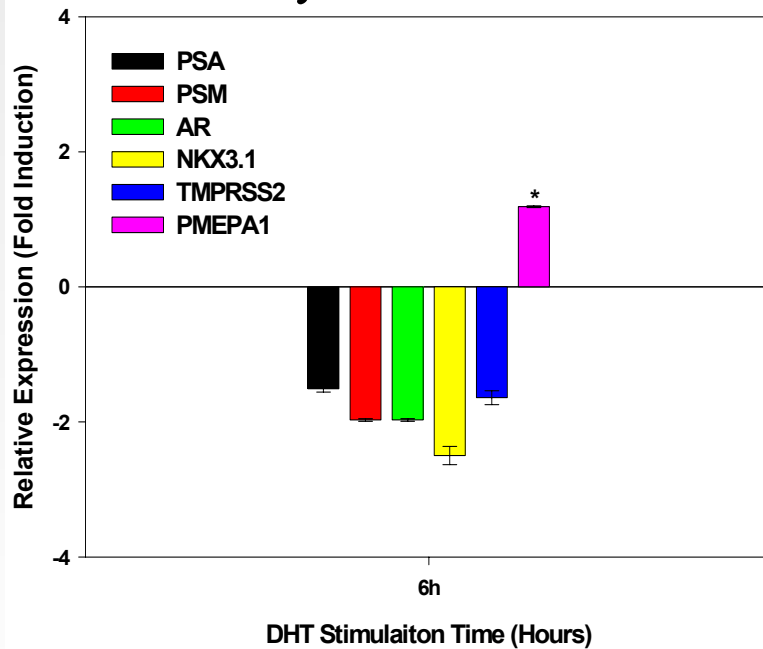
Gene expression assay

Characterisation of relevant substances
and biosamples (food, urine)

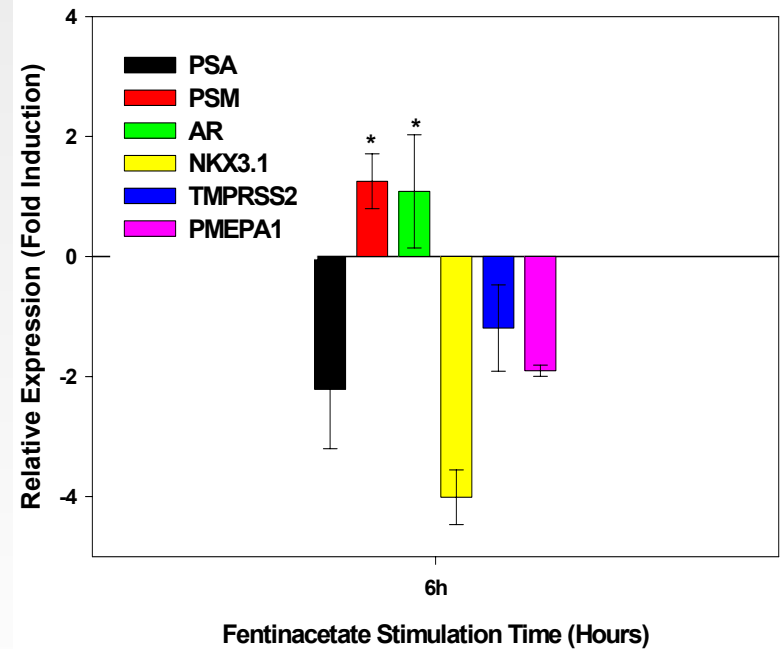
based on androgen regulated genes
in human prostata cell lines

Gene expression assay – prostate cells

Dihydrotestosterone



Fentineacetate



(Hartel et al., 2002)

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Physiology Weihenstephan

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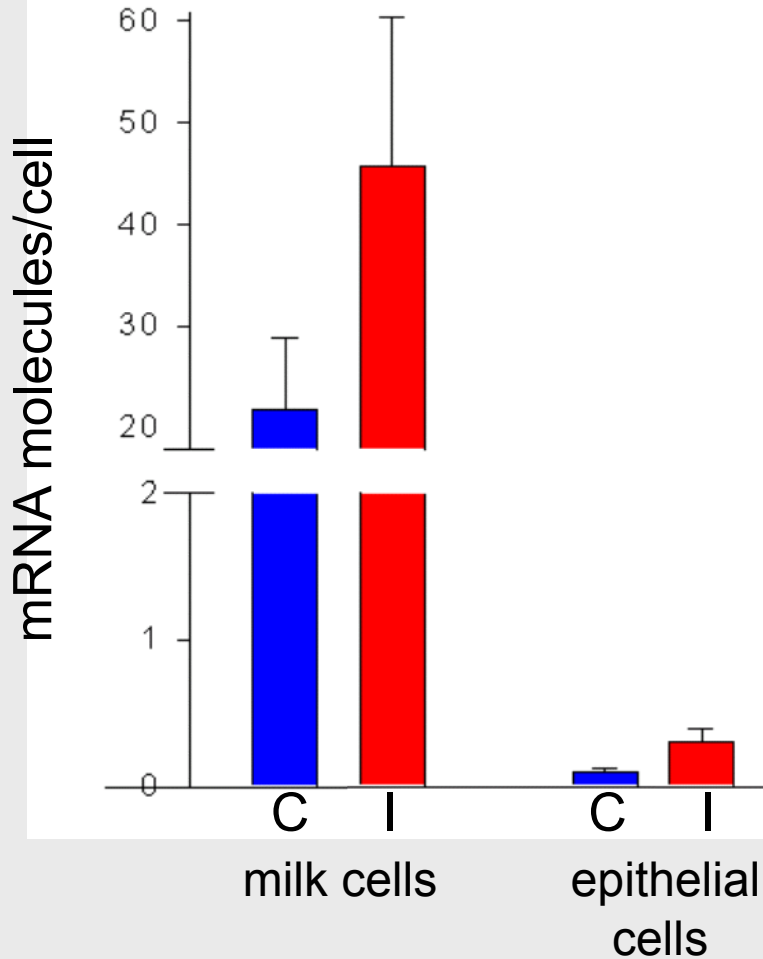
⇒ **Mammary gland immunology**
pro-inflammatory signals of somatic cells and epithelia

⇒ Embryo-maternal communication



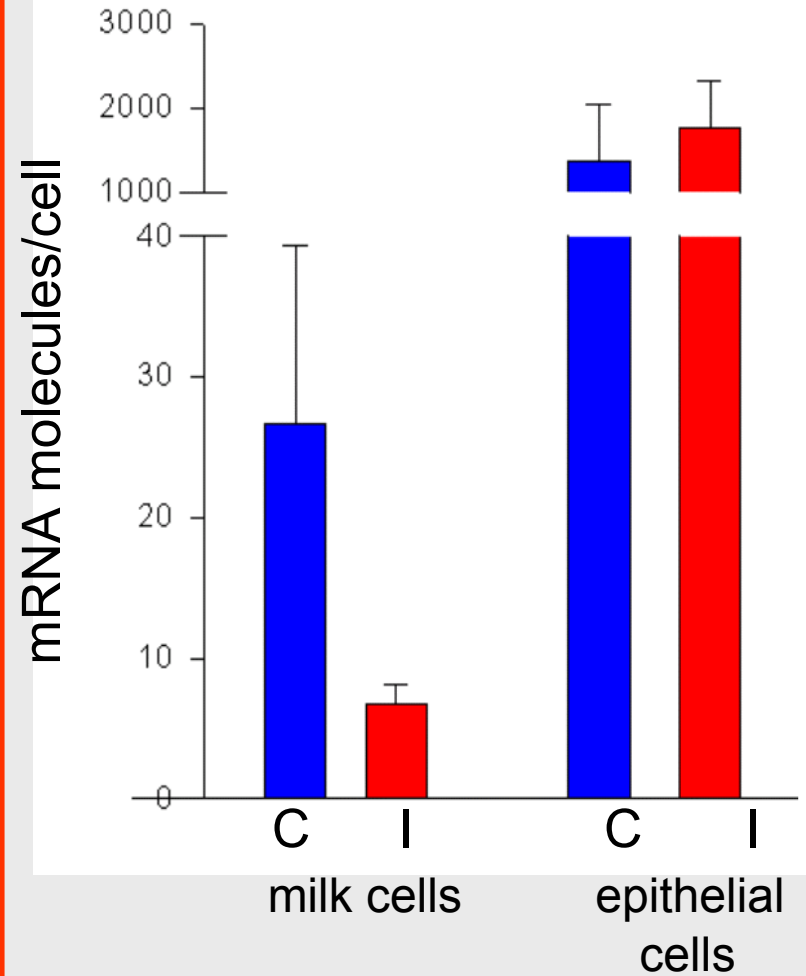
TNF α -mRNA

(tumor necrosis factor α , cytokine)



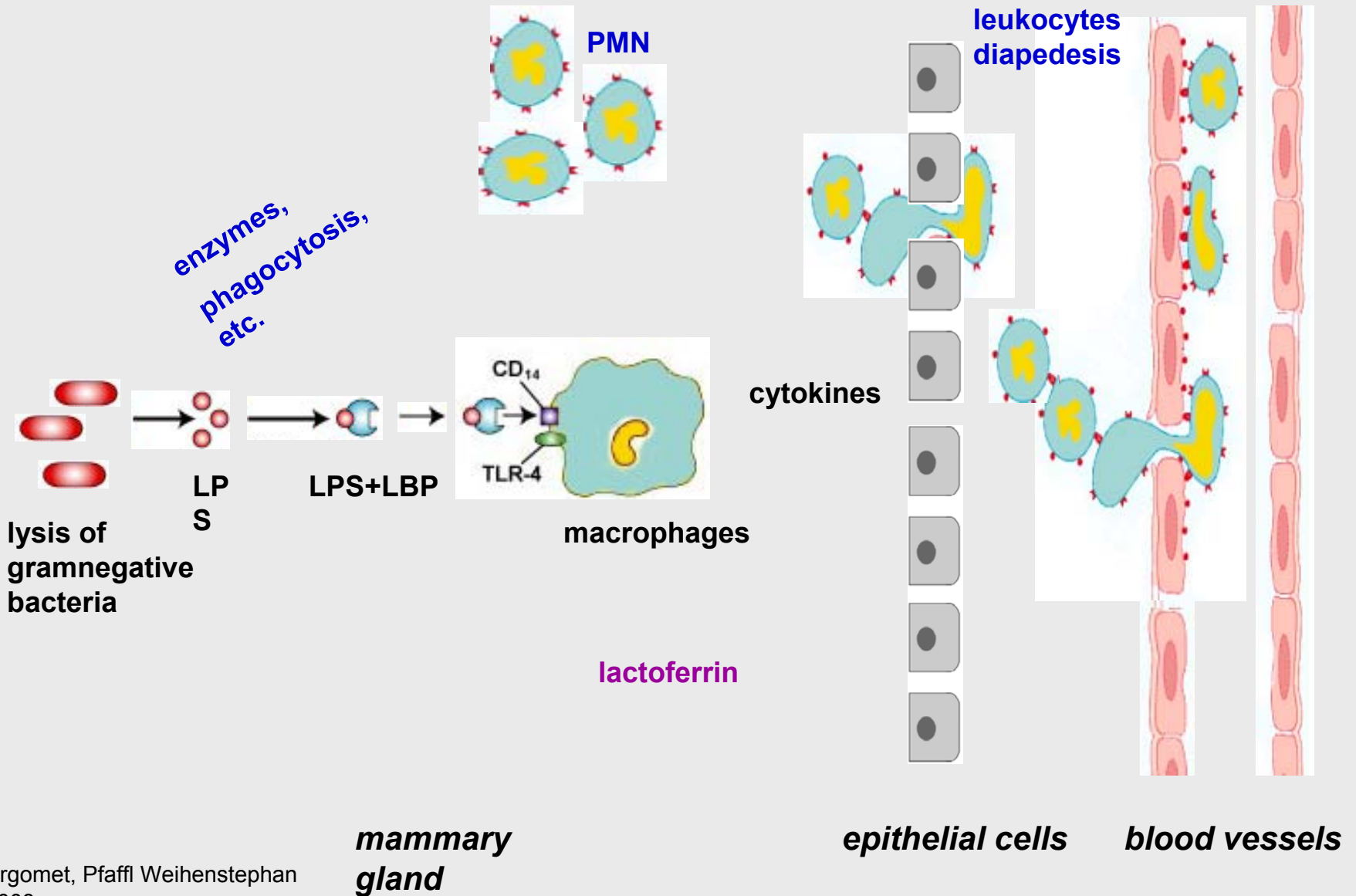
C: control I: infected

Lactoferrin-mRNA

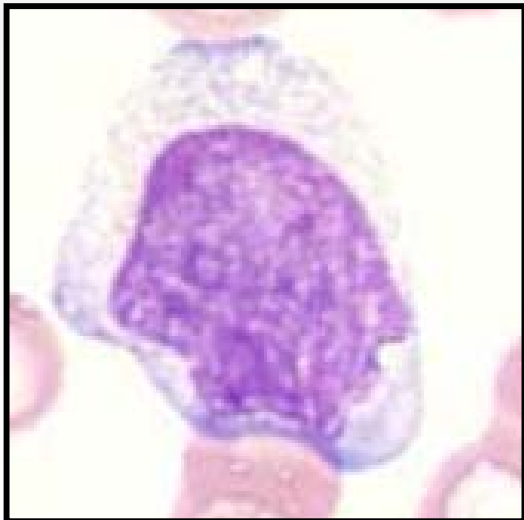
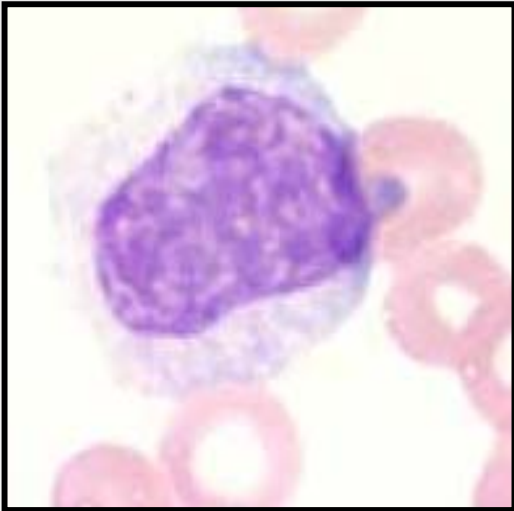


Wittmann et al. 2001, FML Weihenstephan

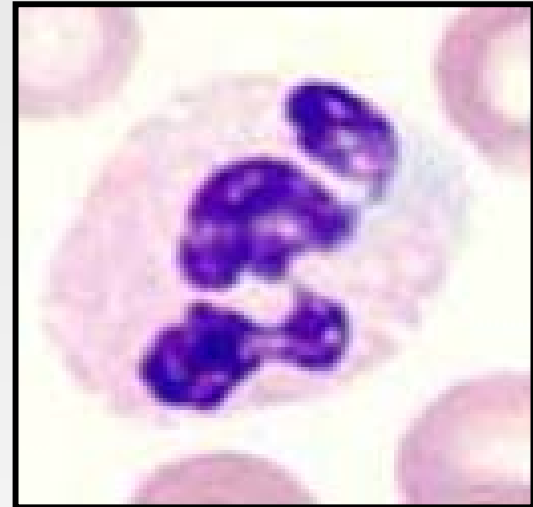
Activation of immune defence via E. coli lipopolysaccharide (LPS)



Macrophages



Granulocytes





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Conclusion: qPCR application potential 2004

Validity

quantification range: 1-100 to 10^6 - 10^8 molecules

good precision (and accuracy) also for low abundance analytes

DNA analytes – gene quantification

new option for clinical diagnostics

pathogen identification

biotechnology

forensic analysis etc.

RNA analytes – transcriptomics

allows a more precise view to regulatory processes on the transcription level during development and reactive physiology

Conclusions: qPCR limitations and challenges 2004

- ⇒ qPCR will hardly see mRNA splicing variants
- ⇒ method development and validation for every analyte
- ⇒ costs
- ⇒ interpretation of multitude data
- ⇒ corresponding data on protein and metabolism level

Perspectives: qPCR 2004

- ⇒ Miniaturisation, high throughput etc.
 - competitive to array technology ?
- ⇒ automated, improved instrumentation
- ⇒ fast assay systems
 - clinical diagnostics
 - food hygiene
- ⇒ functional genome analysis in microorganisms, plants, animals and humans

