

# Alternative splicing mRNA isoforms linked to subclinical intramammary infection in Holstein cattle

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Problem: **MASTITIS**

# Molecular mechanisms involved in subclinical mastitis pathogenesis

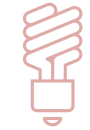


**more insights needed**

*Focus on the*  
**TRANSCRIPTOME**

*Studying*  
**gene  
expression**

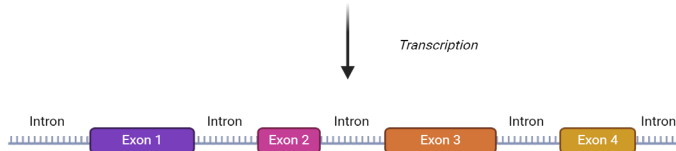
*and even deeper the*  
**mRNA isoforms  
expression**



Gene

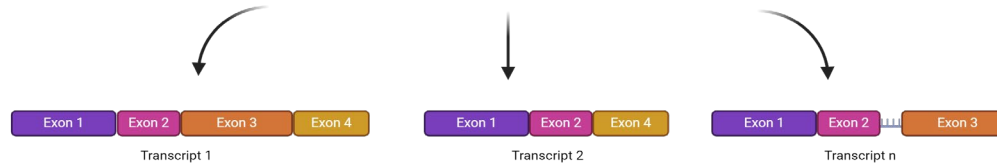


Pre-mRNA

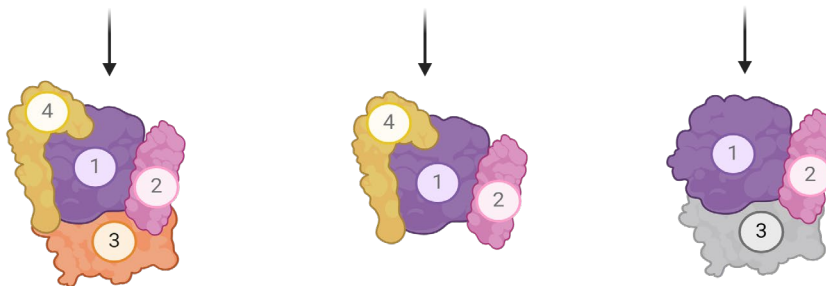


***alternative splicing***

Mature  
mRNA



Protein



- **Alternative splicing** generates **different mature transcripts** from the same pre-mRNA sequence (**mRNA isoforms**)

**Different phenotypic manifestations**

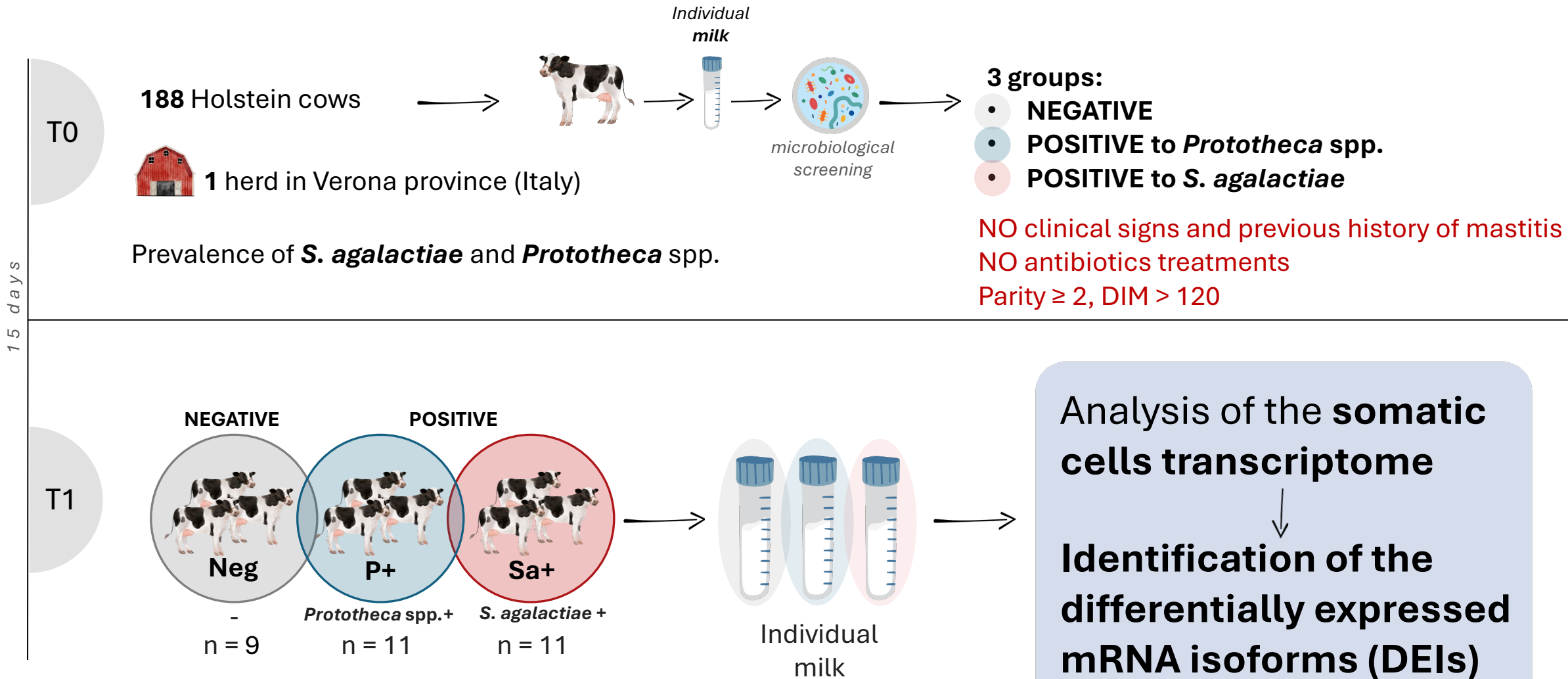
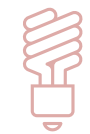
- Process **relevant** to **several diseases**, including bovine mastitis



We aim to study the milk somatic cells mRNA isoforms expression of healthy animals and animals with a subclinical intramammary infection

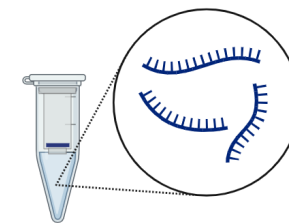
↓  
due to *Streptococcus agalactiae*  
due to *Prototheca* spp.





## WET LAB ANALYSIS

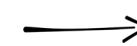
- Somatic cells (**SC**) RNA extraction



- Library preparation



- RNA-sequencing



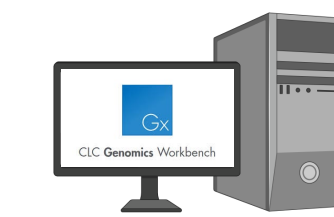
DNBSEQ G400 high  
throughput machine  
(**MGI** Tech CO., Ltd.)





WET LAB  
ANALYSIS

BIOINFORMATIC  
ANALYSIS



QIAGEN CLC Genomics  
Workbench 23.0.5

- Quality control and trimming
- Large Gap Read Mapping → *Bos Taurus* ARS-UCD1.3
- Transcript discovery → produce a **predicted genes** and **transcripts tracks**
- Mapping and alignment → *Bos Taurus* ARS-UCD1.3 + **predicted genes** and **transcripts tracks**

WET LAB ANALYSIS

BIOINFORMATIC ANALYSIS

- Quality control and trimming
- Large Gap Read Mapping
- Transcript discovery
- Mapping and alignment



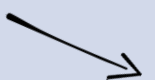
**mRNA isoforms discovery**



1 **Annotated isoforms**



2 **Novel length** isoforms from **annotated genes**



3 **Novel** isoforms from **non-annotated genes**



- Differential mRNA isoforms expression**

Counts normalization

t-test with False Discovery Rate (**FDR**) correction

Comparison:  
**P+ vs Neg**  
**Sa+ vs Neg**

**DIFFERENTIALLY EXPRESSED  
ISOFORMS (DEIs)**

$FDR < 0.05$  and  $|Fold\ Change| > 2$



QIAGEN CLC Genomics  
Workbench 23.0.5

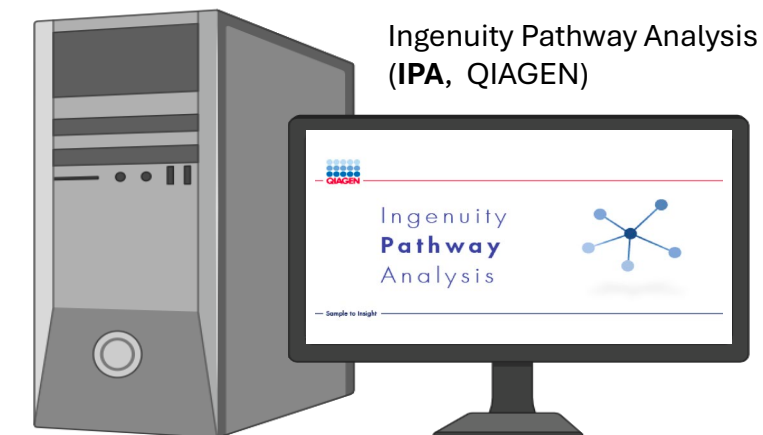




# • Ingenuity Pathway Analysis

- Canonical pathway
- Upstream regulators
- Regulator effect

**Input:** Gene name of the DEIs and their FC

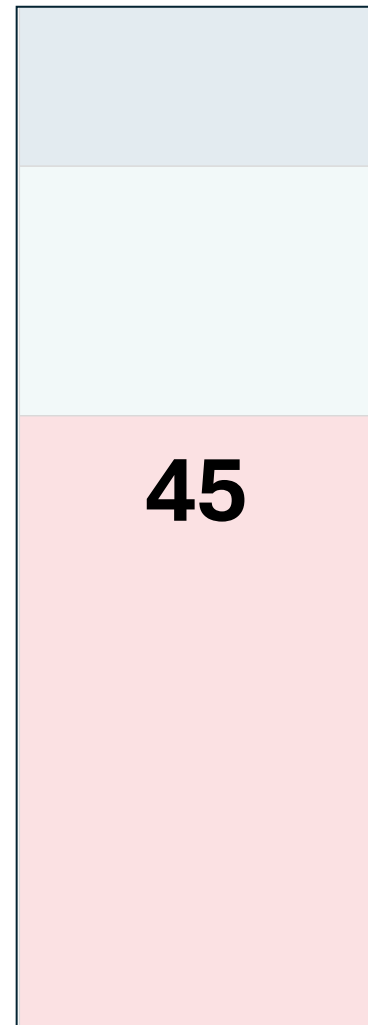


Ingenuity Pathway Analysis (IPA, QIAGEN)



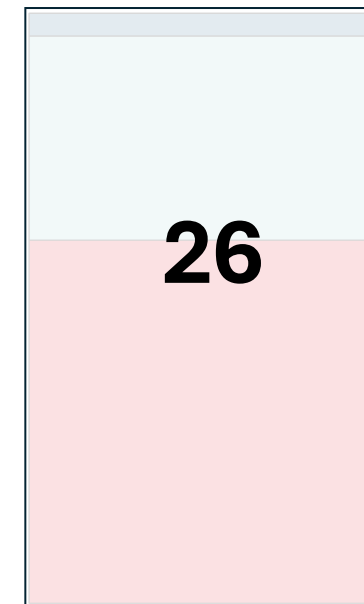
# DIFFERENTIALLY EXPRESSED mRNA ISOFORMS (DEIs)

**P+  
vs  
Neg**



# of DEIs

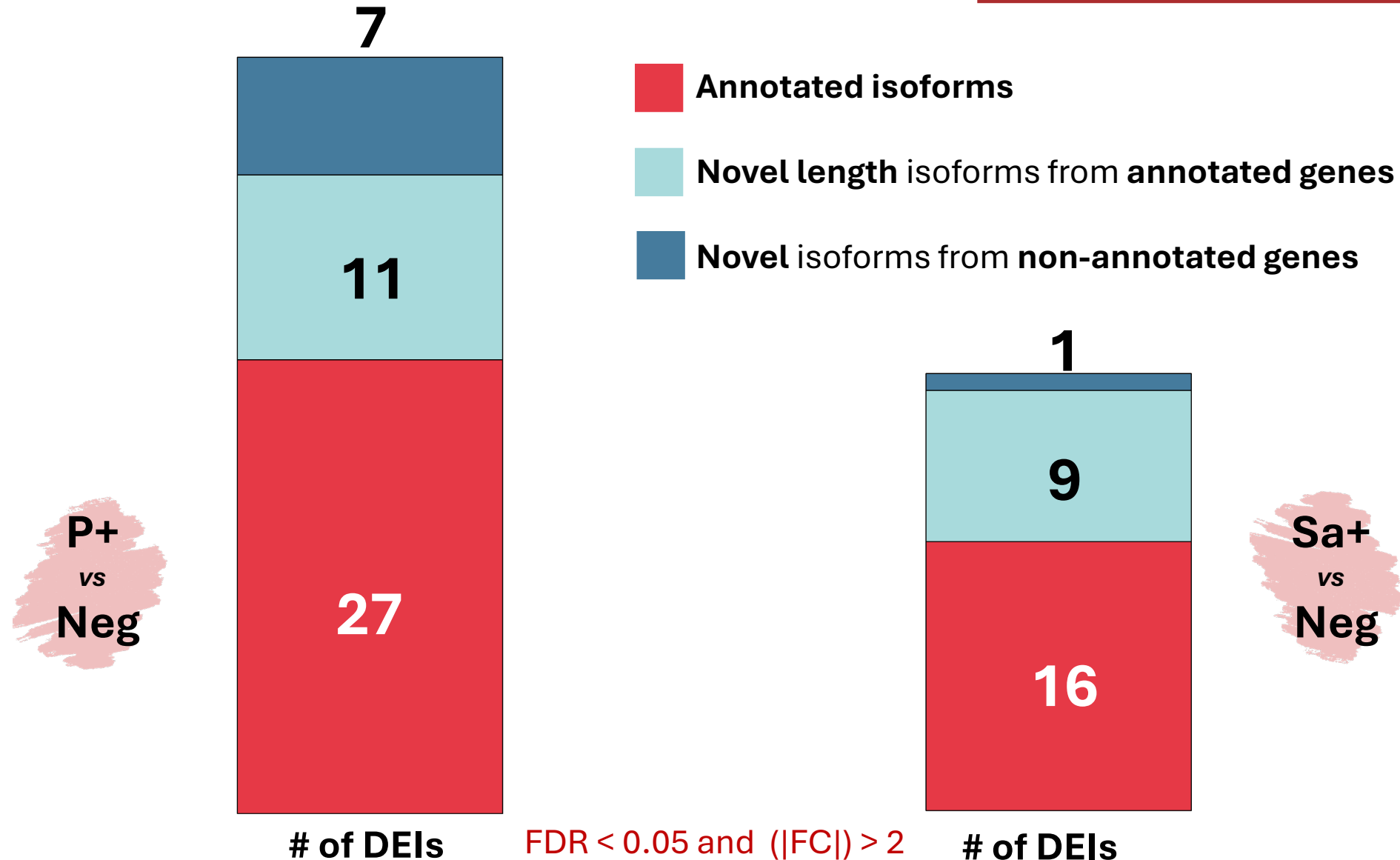
FDR < 0.05 and (|FC|) > 2



# of DEIs

**Sa+  
vs  
Neg**







# DIFFERENTIAL EXPRESSION ANALYSIS

**P+ vs Neg**



## PDE4C-201

*Phosphodiesterase 4C*

Annotated transcript

**2 splice variants**



## BOLA-DOA\_6

*Major histocompatibility complex,  
class II, DO alpha*

Novel length isoform from  
annotated genes

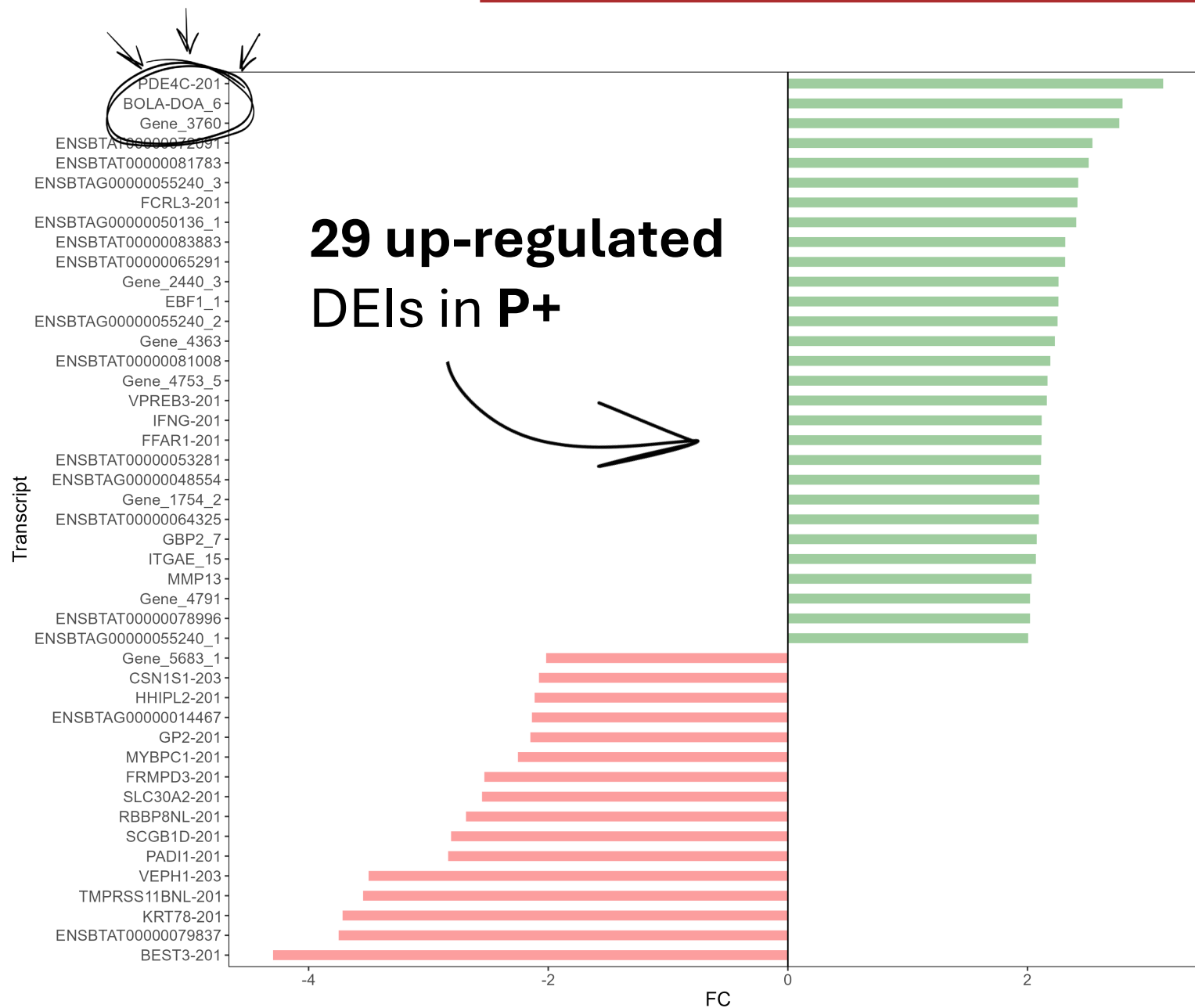
**12 splice variants**



## Gene\_3760

Novel transcript

**1 splice variant**





# DIFFERENTIAL EXPRESSION ANALYSIS

P+ vs Neg



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## **BEST3-201**

Bestrophin 3

Annotated transcript

1 splice variant



## **ENSBTAT00000079837**

Annotated transcript

2 splice variant

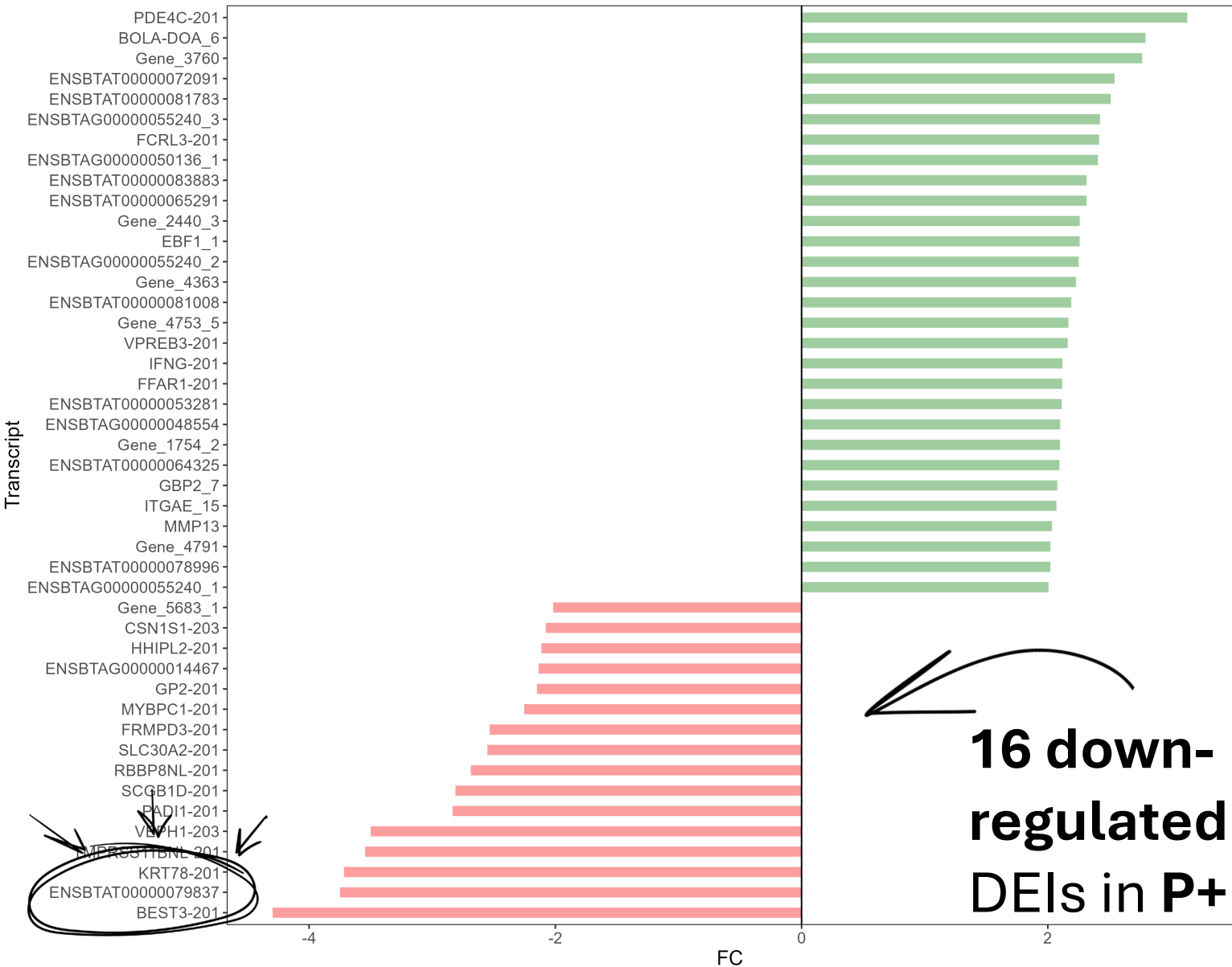


## **KRT78-201**

Keratin 78

Annotated transcript

1 splice variant





# DIFFERENTIAL EXPRESSION ANALYSIS

Sa+ vs Neg

## *HIPK3\_13*

Homeodomain interacting protein kinase 3

Novel length isoform from annotated genes

13 splice variants

## *TRAF2\_5*

TNF receptor associated factor 2

Novel length isoform from annotated genes

17 splice variants

## *PDE4C-201*

Phosphodiesterase 4C

Annotated transcript

2 splice variants

Like in P+!





# DIFFERENTIAL EXPRESSION ANALYSIS

Sa+ vs Neg



## NTSR-201

Neurotensin receptor 2

Annotated transcript

1 splice variants



## GSTM1-201\_2

Glutathione S-transferase M1

Novel length isoform from annotated genes

1 splice variants

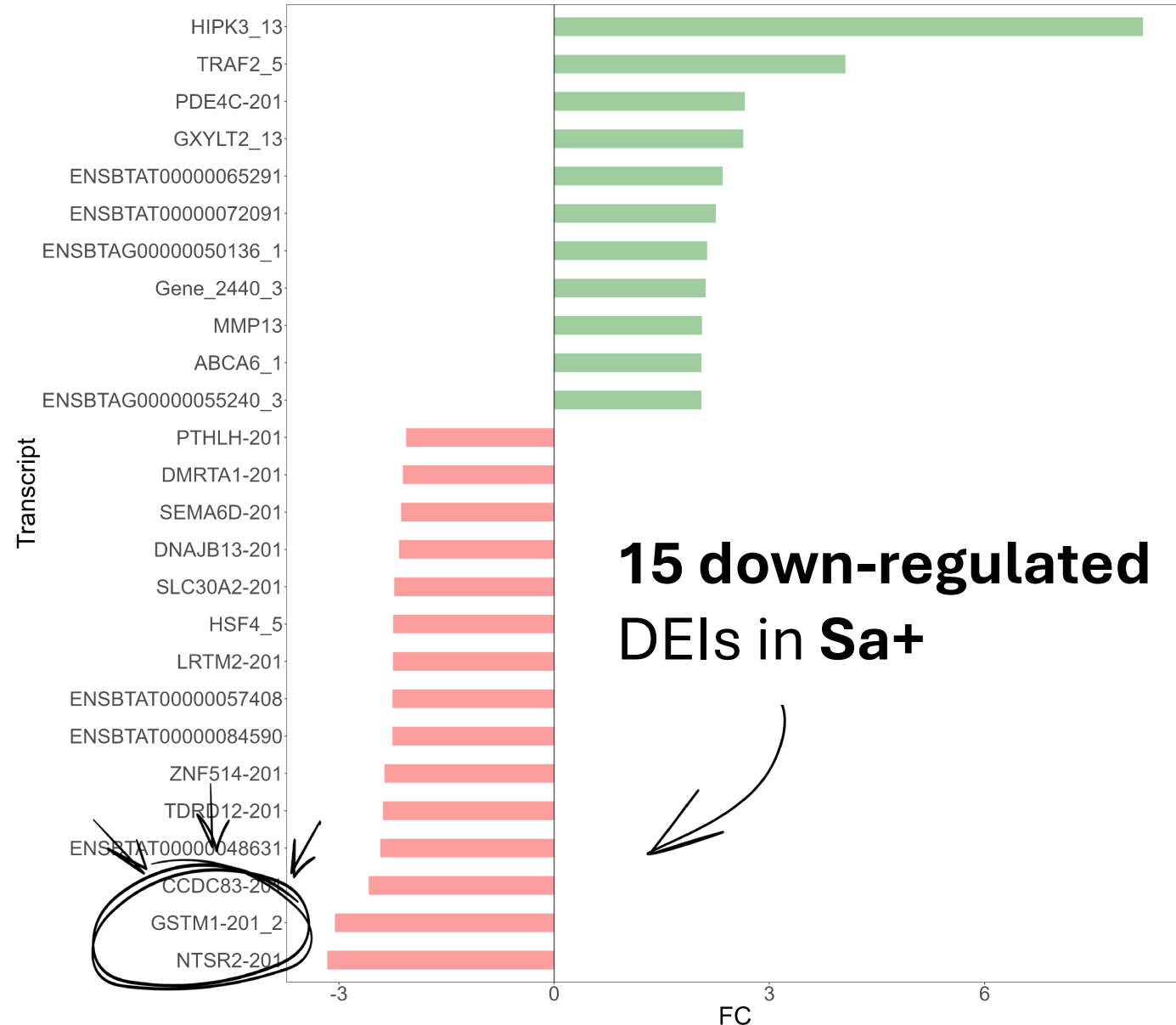


## CCDC83-201

Coiled-coil domain containing 83

Annotated transcript

1 splice variants

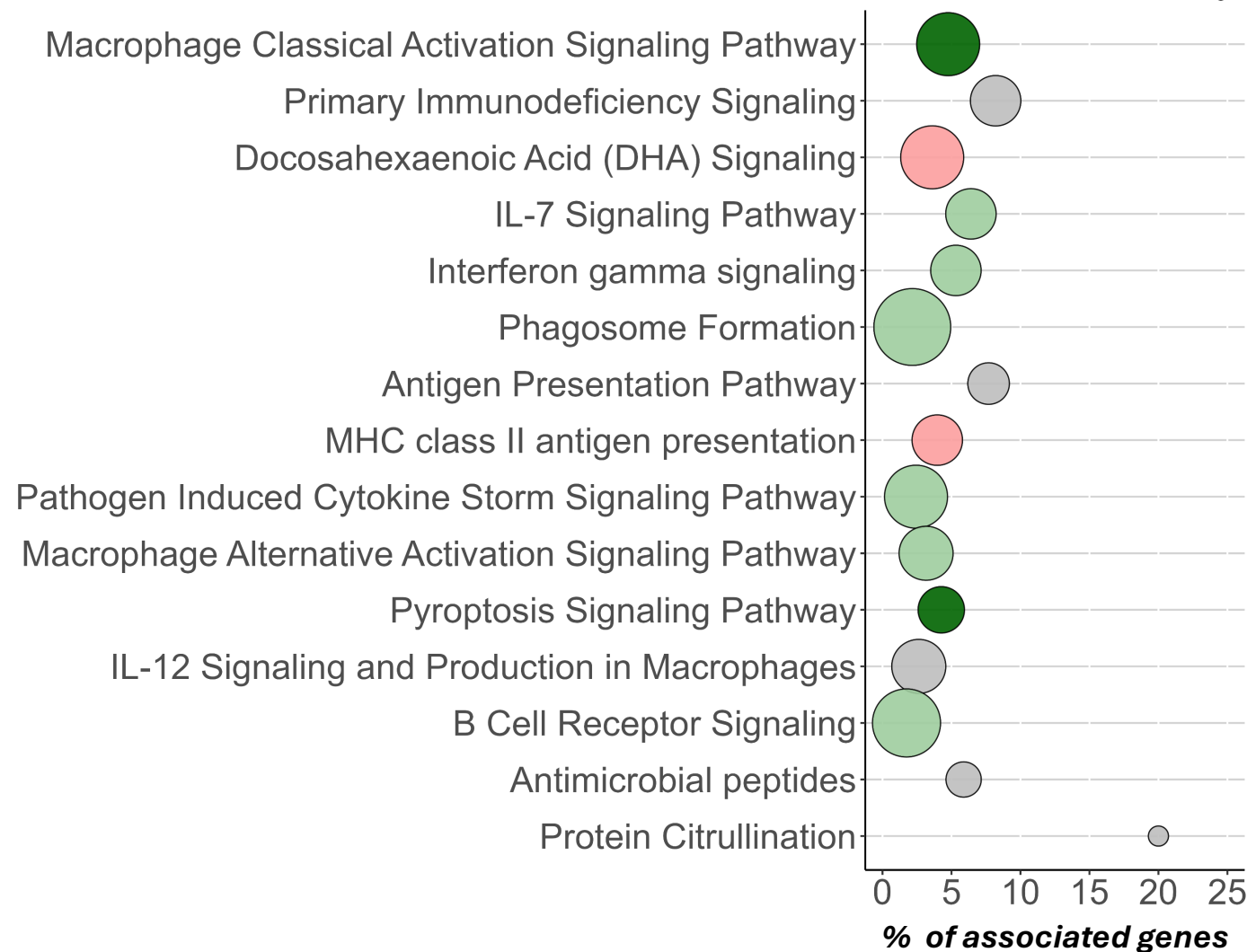


# FUNCTIONAL ANALYSIS

P+ vs Neg

- A total of **50** enriched pathways (FDR < 0.05)
- **Pathways related to immune response!**

## Canonical Pathway



● activation ● activation trend ● inhibition trend ● not predictable

5 10 15

Overlap



# FUNCTIONAL ANALYSIS

Sa+ vs Neg



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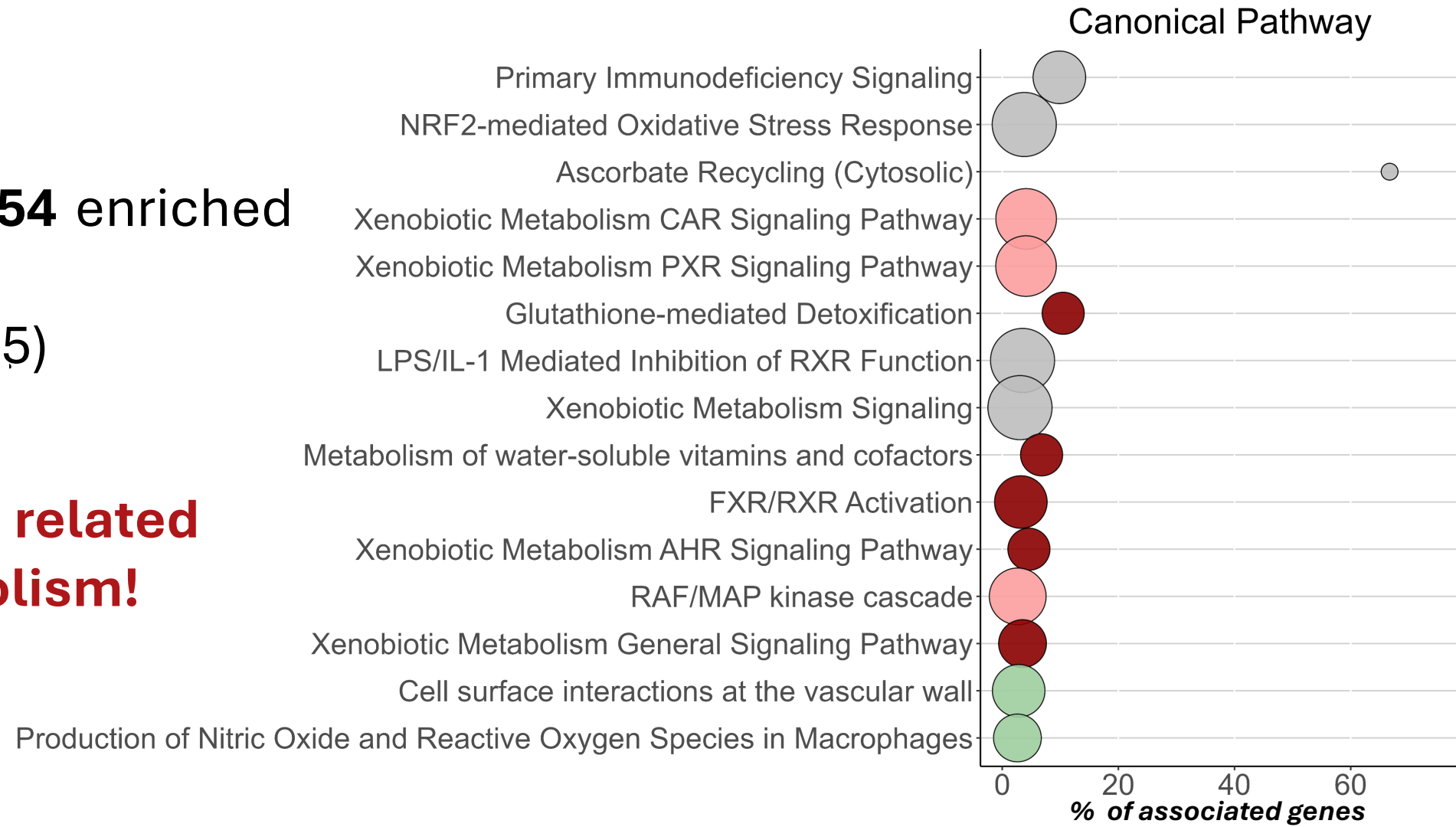
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- A total of **54** enriched pathways (FDR < 0.05)

- **Pathways related to metabolism!**



● activation trend ● inhibition ● inhibition trend ● not predictable

2 4 6 8  
Overlap

# To conclude..

- The differential expression analysis identified **multiple isoforms differentially expressed** between positive and negative groups, many of which are linked to genes related to **immune and inflammatory responses**. Some DEIs were **shared**, while others were **unique to specific comparison**
- In ***Prototheca* spp.** infection we found enriched **pathways** predominantly related to **immune response**, while in ***S. agalactiae*** infection predominantly related to **metabolism** and **detoxification process**

These **differences** were likely due **to the specific** effects of the two distinct pathogens on **the host immune response**

- Identification of **functional variants (SNPs and INDELs)** within the expressed regions **leading to splice site events** potentially **responsible for alternative splicing** of the detected **DEIs**
- **Validation** of these results in an independent population

**This research could offer new potential targets for the development of new screening tools for mastitis, as well for the selection of more resistant animals**



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NextGenerationEU



Ministero  
dell'Università  
e della Ricerca



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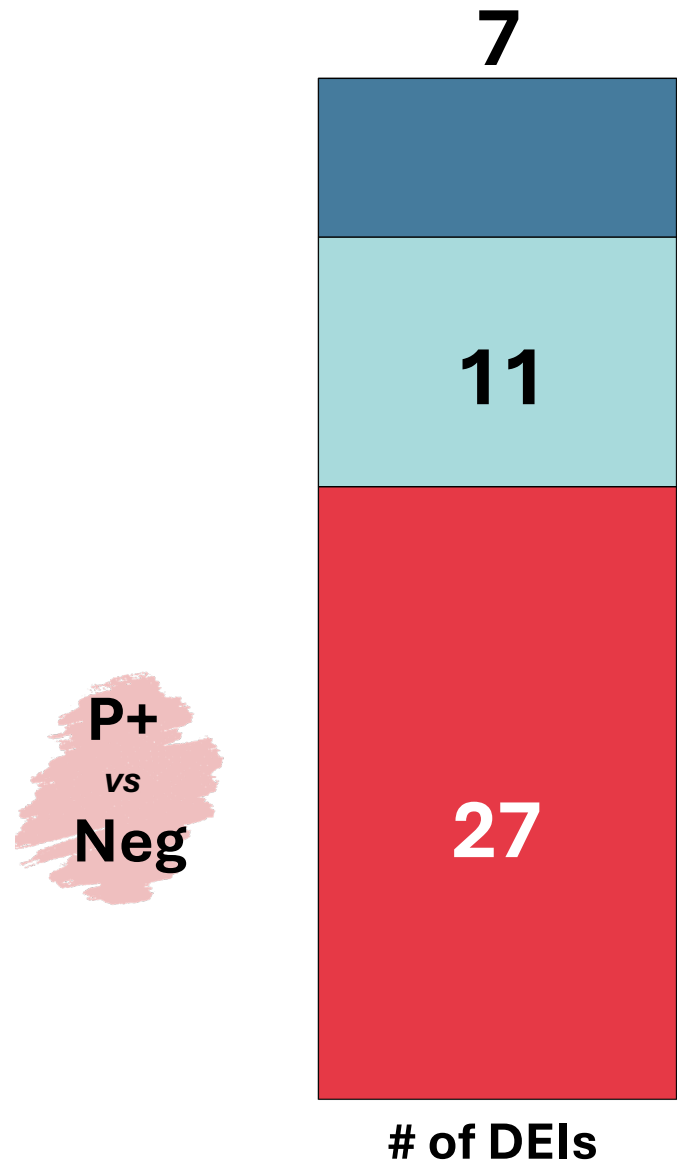
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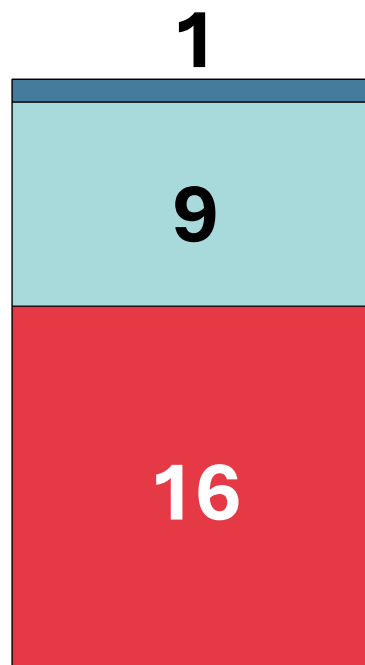


FDR < 0.05 and (|FC|) > 2

- **45** DE transcripts derived from **43** single genes
- **24** transcripts with at least two annotated isoforms



# Sa+ vs Neg



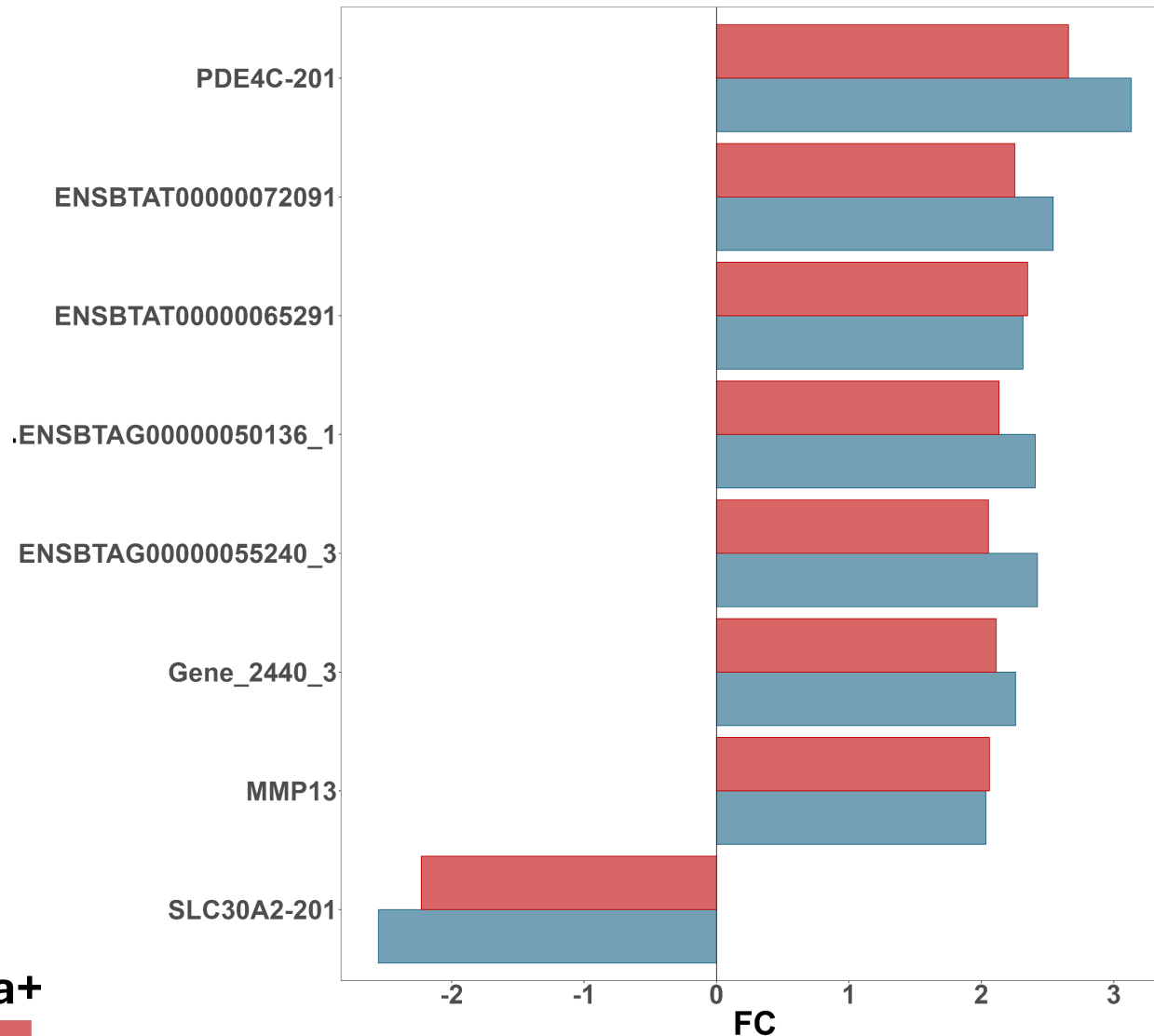
# of DEIs

- All the 26 transcripts derived from **different genes**
- **13** transcripts with at least two annotated isoforms

- 8 DEIs were **shared** between the two comparisons
- All of them showed the **same expression pattern**

P+ Sa+

FC



| Pathway                                                    | -Log(P) | z      | trend            | overlap | Ratio% | Genes                                                                                                            | Down       | No change  | Up          |
|------------------------------------------------------------|---------|--------|------------------|---------|--------|------------------------------------------------------------------------------------------------------------------|------------|------------|-------------|
| <b>Macrophage Classical Activation Signaling Pathway</b>   | 4.09    | 3      | activation       | 9       | 4.76   | CXCL10, CXCL11, CXCL9, GBP2, GBP4, HLA-DOA, HLA-DOB, IFNG, TNFSF11                                               | 0/189 (0%) | 0/189 (0%) | 9/189 (5%)  |
| <b>Primary Immunodeficiency Signaling</b>                  | 3.56    | 0      | no predictable   | 5       | 8.2    | IGHA1, IGHG1, IGLL1/IGLL5, JCHAIN, PTPRC                                                                         | 0/61 (0%)  | 0/61 (0%)  | 5/61 (8%)   |
| <b>Docosaehaenoic Acid (DHA) Signaling</b>                 | 3.19    | -0.333 | inhibition trend | 9       | 3.6    | ADGRF1, ALOX15, FABP3, FFAR1, PDPK1, PLCG2, PRKAA2, RPS6KB2, SPTBN2                                              | 6/250 (2%) | 0/250 (0%) | 3/250 (1%)  |
| <b>IL-7 Signaling Pathway</b>                              | 3.06    | 1.342  | activation trend | 5       | 6.41   | EBF1, IFNG, IGHG1, PAX5, PDPK1                                                                                   | 0/78 (0%)  | 0/78 (0%)  | 5/78 (6%)   |
| <b>Interferon gamma signaling</b>                          | 2.7     | 1.342  | activation trend | 5       | 5.32   | GBP1, GBP2, GBP4, IFNG, IRF6                                                                                     | 1/94 (1%)  | 0/94 (0%)  | 4/94 (4%)   |
| <b>Phagosome Formation</b>                                 | 2.58    | 0.535  | activation trend | 15      | 2.16   | ADGRF1, ADGRV1, COLEC12, FFAR1, GPRC5C, IGHG1, IGHG1, ITGA1, ITGAE, JCHAIN, MAPK4, PLCG2, PROKR1, RPS6KB2, S1PR3 | 6/696 (1%) | 0/696 (0%) | 9/696 (1%)  |
| <b>Antigen Presentation Pathway</b>                        | 2.23    | 0      | no predictable   | 3       | 7.69   | HLA-DOA, HLA-DOB, IFNG                                                                                           | 0/39 (0%)  | 0/39 (0%)  | 3/39 (8%)   |
| <b>MHC class II antigen presentation</b>                   | 2.16    | -0.447 | inhibition trend | 5       | 3.97   | CTSV, HLA-DOA, HLA-DOB, SEC23A, SPTBN2                                                                           | 3/126 (2%) | 0/126 (0%) | 2/126 (2%)  |
| <b>Pathogen Induced Cytokine Storm Signaling Pathway</b>   | 2.05    | 1.667  | activation trend | 9       | 2.43   | CCL28, CXCL10, CXCL11, CXCL9, HLA-DOA, HLA-DOB, IFNG, MAPK4, TNFSF11                                             | 2/371 (1%) | 0/371 (0%) | 7/371 (2%)  |
| <b>Macrophage Alternative Activation Signaling Pathway</b> | 2.02    | 1.633  | activation trend | 6       | 3.16   | ALOX15, FFAR1, HLA-DOA, HLA-DOB, IGHG1, JCHAIN                                                                   | 1/190 (1%) | 0/190 (0%) | 5/190 (3%)  |
| <b>Pyroptosis Signaling Pathway</b>                        | 1.92    | 2      | activation       | 4       | 4.26   | GBP1, GBP2, GBP4, GZMA                                                                                           | 0/94 (0%)  | 0/94 (0%)  | 4/94 (4%)   |
| <b>IL-12 Signaling and Production in Macrophages</b>       | 1.67    | 0      | no predictable   | 6       | 2.63   | ALOX15, IFNG, IGHG1, JCHAIN, PRKAA2, TNFSF11                                                                     | 2/228 (1%) | 0/228 (0%) | 4/228 (2%)  |
| <b>B Cell Receptor Signaling</b>                           | 1.41    | 1.342  | activation trend | 11      | 1.74   | CD22, CD79B, EBF1, IGHG1, IGHG1, JCHAIN, PAX5, PDPK1, PLCG2, PTPRC, RPS6KB2                                      | 1/634 (0%) | 0/634 (0%) | 10/634 (2%) |
| <b>Antimicrobial peptides</b>                              | 1.39    | 0      | no predictable   | 2       | 5.88   | GNLY, HTN1                                                                                                       | 1/34 (3%)  | 0/34 (0%)  | 1/34 (3%)   |
| <b>Protein Citrullination</b>                              | 1.33    | 0      | no predictable   | 1       | 20     | PADI1                                                                                                            | 1/5 (20%)  | 0/5 (0%)   | 0/5 (0%)    |

| Pathway                                                                      | -Log(P) | z      | trend            | overlap | Ratio% | Genes                                                       | Down       | No change  | Up         |
|------------------------------------------------------------------------------|---------|--------|------------------|---------|--------|-------------------------------------------------------------|------------|------------|------------|
| <b>Primary Immunodeficiency Signaling</b>                                    | 4.94    | 0      | no predictable   | 6       | 9.84   | IGHA1,IGHG4,IGLC2,IGLL1/IGLL5,JAK3,PTPRC                    | 0/61 (0%)  | 0/61 (0%)  | 6/61 (10%) |
| <b>NRF2-mediated Oxidative Stress Response</b>                               | 3.77    | 0      | no predictable   | 9       | 3.8    | ABCC4,AOX1,DNAJB13,DNAJB14,GSTM1,GSTM4,GSTO1,GSTO2,MAP2K1   | 8/237 (3%) | 0/237 (0%) | 1/237 (0%) |
| <b>Ascorbate Recycling (Cytosolic)</b>                                       | 3.69    | 0      | no predictable   | 2       | 66.7   | GSTO1,GSTO2                                                 | 2/3 (67%)  | 0/3 (0%)   | 0/3 (0%)   |
| <b>Xenobiotic Metabolism CAR Signaling Pathway</b>                           | 3.65    | -1.89  | inhibition trend | 8       | 4.12   | GSTM1,GSTM4,GSTO1,GSTO2,MAP2K1,PPP2R2A,SULT1C4,WSCD2        | 8/194 (4%) | 0/194 (0%) | 0/194 (0%) |
| <b>Xenobiotic Metabolism PXR Signaling Pathway</b>                           | 3.64    | -1.89  | inhibition trend | 8       | 4.1    | CAMK2A,GSTM1,GSTM4,GSTO1,GSTO2,PPP1R14B,SULT1C4,WSCD2       | 8/195 (4%) | 0/195 (0%) | 0/195 (0%) |
| <b>Glutathione-mediated Detoxification</b>                                   | 3.57    | -2     | inhibition       | 4       | 10.5   | GSTM1,GSTM4,GSTO1,GSTO2                                     | 4/38 (11%) | 0/38 (0%)  | 0/38 (0%)  |
| <b>LPS/IL-1 Mediated Inhibition of RXR Function</b>                          | 3.51    | 0      | no predictable   | 9       | 3.5    | ABCC4,GSTM1,GSTM4,GSTO1,GSTO2,IL1RL2,SULT1C4,TRAF2,WSCD2    | 7/257 (3%) | 0/257 (0%) | 2/257 (1%) |
| <b>Xenobiotic Metabolism Signaling</b>                                       | 3.09    | 0      | no predictable   | 9       | 3.06   | CAMK2A,GSTM1,GSTM4,GSTO1,GSTO2,MAP2K1,PPP2R2A,SULT1C4,WSCD2 | 9/294 (3%) | 0/294 (0%) | 0/294 (0%) |
| <b>Metabolism of water-soluble vitamins and cofactors</b>                    | 2.84    | -2     | inhibition       | 4       | 6.78   | AOX1,GSTO1,GSTO2,MOCOS                                      | 4/59 (7%)  | 0/59 (0%)  | 0/59 (0%)  |
| <b>FXR/RXR Activation</b>                                                    | 2.32    | -2.449 | inhibition       | 6       | 3.21   | GSTM1,GSTM4,GSTO1,GSTO2,PRKAA2,TNFSF8                       | 5/187 (3%) | 0/187 (0%) | 1/187 (1%) |
| <b>Xenobiotic Metabolism AHR Signaling Pathway</b>                           | 2.23    | -2     | inhibition       | 4       | 4.6    | GSTM1,GSTM4,GSTO1,GSTO2                                     | 4/87 (5%)  | 0/87 (0%)  | 0/87 (0%)  |
| <b>RAF/MAP kinase cascade</b>                                                | 2.2     | -0.378 | inhibition trend | 7       | 2.67   | CAMK2A,EREG,JAK3,MAP2K1,NF1,RASAL1,TEK                      | 4/262 (2%) | 0/262 (0%) | 3/262 (1%) |
| <b>Xenobiotic Metabolism General Signaling Pathway</b>                       | 2.16    | -2.236 | inhibition       | 5       | 3.5    | GSTM1,GSTM4,GSTO1,GSTO2,MAP2K1                              | 5/143 (3%) | 0/143 (0%) | 0/143 (0%) |
| <b>Cell surface interactions at the vascular wall</b>                        | 2.07    | 0.816  | activation trend | 6       | 2.84   | CD47,IGHA1,IGLC2,IGLL1/IGLL5,SDC2,TEK                       | 2/211 (1%) | 0/211 (0%) | 4/211 (2%) |
| <b>Production of Nitric Oxide and Reactive Oxygen Species in Macrophages</b> | 1.66    | 1.342  | activation trend | 5       | 2.62   | JAK3,MAP2K1,NCF1,PPP1R14B,PPP2R2A                           | 3/191 (2%) | 0/191 (0%) | 2/191 (1%) |