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# **Development of a custom microarray platform for nutrigenomics studies in sheep**

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**STREP-EC 2005-2008 “Evaluating physiological and environmental consequences of using organic  
wastes after technological processing in diets for livestock and humans”**

## Background

Nutrients and diet regulate gene expression and are relevant for functional homeostasis - NUTRIGENOMIC

Nutrigenomic studies are based on HTS techniques

Sheep are also a model of dairy ruminants – cows ?

Microarray or equivalent HTS are not yet available for sheep

## **Requirements to apply Nutrigenomic concepts and to develop HTS**

Dietary compounds with nutraceutical activity

Animal model to study their bioactivity:

**Proof of evidence**



# PHYTOCHEMICALS



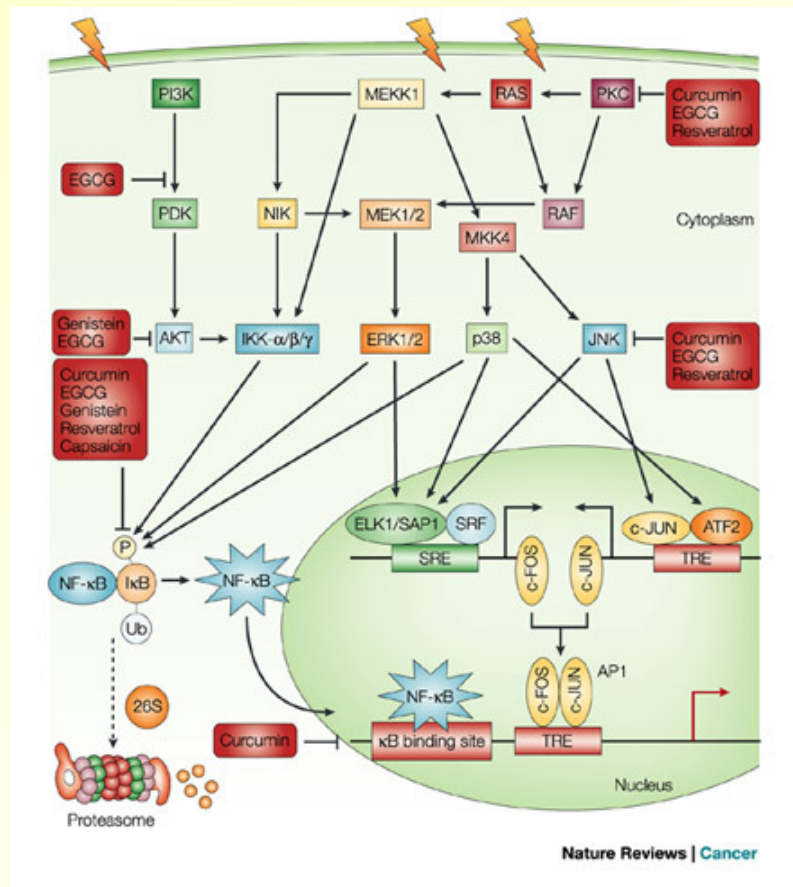
The beneficial role of nutraceuticals in enhancing the endogenous defences, through the enhancement of key enzymes of **antioxidant defences in humans** has been carefully investigated



In the last decade the interest for these bioactive compounds has been extended **also to the livestock**



# Bioactivity of phytochemicals: Beyond the antioxidant activity



From: Cancer chemoprevention with dietary phytochemicals (Young-Joon Surh).

## **Animal model**

**Some metabolic and chronic diseases can be related to environmental and oxidative stress and to proinflammatory conditions**

**Stress related (overcrowding, management, stabulation, ..)**

**Cognitive and Non cognitive stress**

**Laminitis / Lameness**

**Ketosis**

**Milk fever**

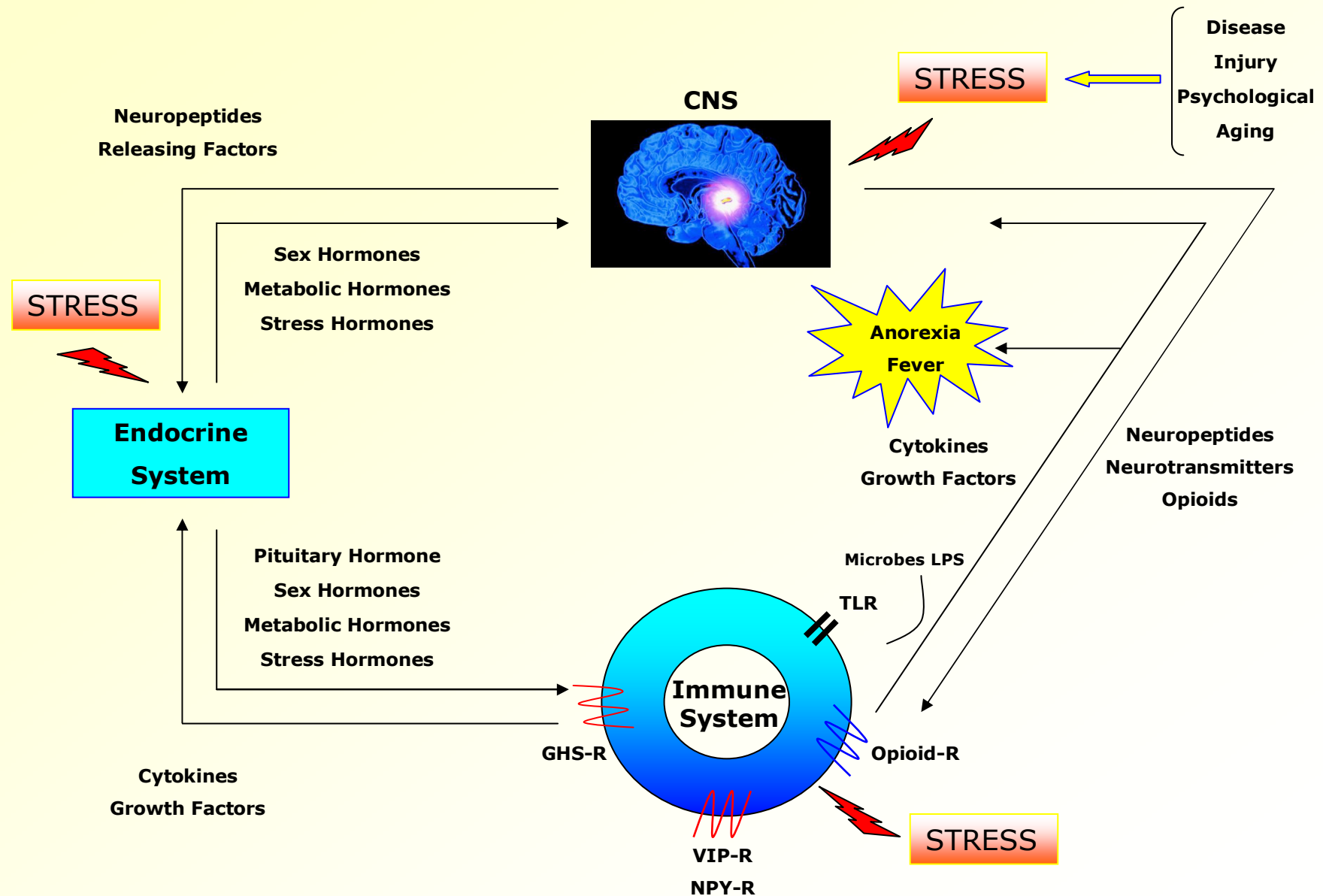
**Dietary imbalance**

**Abomasum displacement**

**Chronic inflammation**

**Peripartum and onset of lactation**

# Neuroendocrine Immuno Loop





## Aims

*Effects of dietary administration of phytochemicals  
on transcriptome modification after ACTH  
challenge in sheep using a custom microarray*







# Materials and methods



# *Phytochemicals*

| Compound                | Part of plant                     | Bioactive molecules                                                | Activity expected                                                                            | Supplier                                   |
|-------------------------|-----------------------------------|--------------------------------------------------------------------|----------------------------------------------------------------------------------------------|--------------------------------------------|
| Echinacea angustifolia  | Flowers of Echinacea angustifolia | Echinacoside                                                       | Stimulation of phagocytosis, Cytokine production, B lymphocyte production, Anti-inflammatory | INDENA (Milan)                             |
| Polinacea™              | Roots of Echinacea angustifolia   | Echinacoside and IDN 5405 – a polyphenol derived from caffeic acid | Stimulation of phagocytosis, Cytokine production, B lymphocyte production, Anti-inflammatory | INDENA (Milan)                             |
| Andrographis panicolata | Leaves                            | Andrographolide                                                    | Anti-inflammatory, Hepatoprotective, Antioxidant, Pro-apoptotic                              | INDENA (Milan)                             |
| Larix decidua           | Sawdust from larch wood           | Larixyl acetate, arabinogalactan,                                  | Anti-inflammatory, Immunestimulating                                                         | Jannach Lärchenholz GmbH, Thalheim, Styria |

## Animals

36 Sardinian sheep not pregnant and lactating randomly allotted to 6 experimental groups of 6 ewes each and fed the same basal diet (complete pelleted diet).





- ✓ **CTR:** negative control, -- ACTH;
- ✓ **ACTH:** positive control, + ACTH ;
- ✓ **Echi:** 3 mg/kg LW *Echinacea angustifolia* + ACTH;
- ✓ **Andro:** 1 mg/kg LW *Andrographis paniculata* + ACTH;
- ✓ **Poli:** 3 mg/kg LW *Polinacea<sup>TM</sup>* + ACTH;
- ✓ **Larch:** 1 g/kg LW *Larix decidua* + ACTH



## Experimental model of stress

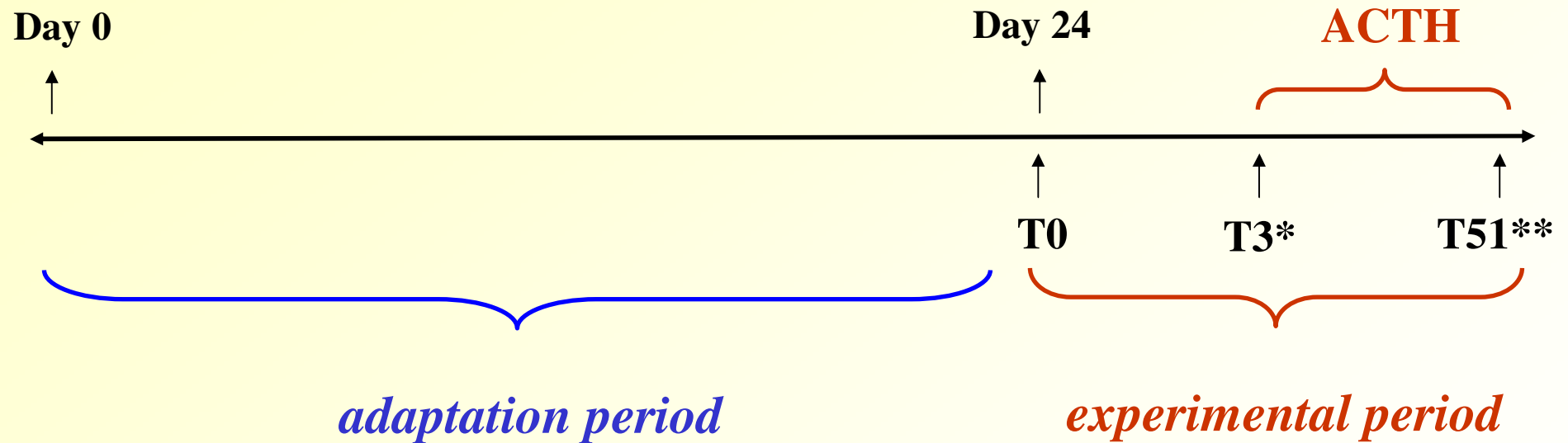
### *Induction of cortisol secretion with ACTH injection*



i.m. injection of 0.5 mg ACTH agonist twice a day for 3 days

Synacthen, Novartis, Varese, Italy - Tetracosactrin acetate

## Scheduled blood samples



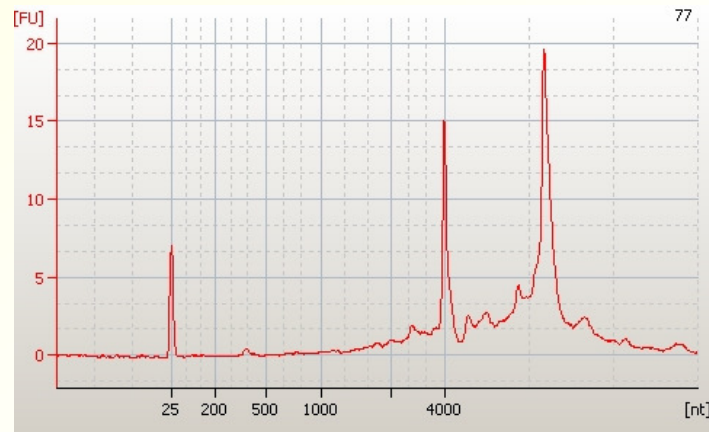
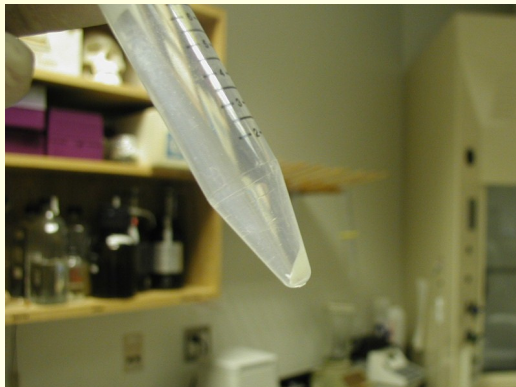
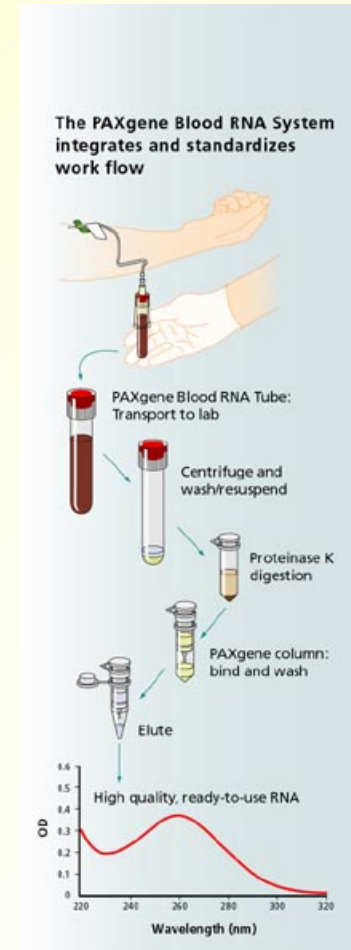
*All the samples were collected at the morning before food administration*

*\* Sampled 3 hours after the beginning of ACTH treatment*

*\*\* Sampled 3 hours after the last ACTH injection (51 hours after the beginning of ACTH treatment)*

# Laboratory protocol

- ✓ mRNA extraction from PAXgene blood RNA kit
- ✓ Pooling mRNA of ewes of the same experimental group (equal quantity for each sheep - total 18 pools: 6 groups x 3 times);



- ✓ Design of probes with Oligowiz software (12.194 Unigenes – NCBI Build #13)
- ✓ Custom array with 12194 Unigenes, 35-40mer probes

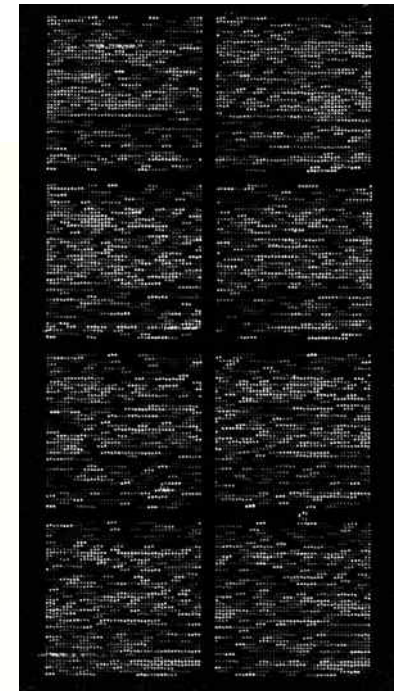
- ✓ Labelling of the target with Cy5 fluorophore



*Combimatrix 90K*

Hyb Clamp for CustomArray™ 4X2K

- ✓ Hybridization;
- ✓ Scanning and image acquisition.





# Statistical analysis

- ✓ Expression values of ACTH and TRT groups related to CTR group expression values: Log10 of the ratio for statistical analysis;
- ✓ **Two-factor (group and time) ANOVA (MeV software v4.1 - TIGR); p-values < 0.001;**
- ✓ Hierarchical clustering (Heat map) analysis of differentially expressed genes (MeV software v 4.1 – TIGR);
- ✓ **Annotation of genes with HomoloGene system (about 50% of the genes present on the array have been annotated);**
- ✓ Data mining with FatiGo+ tool (Babelomics v3.2).

# Results

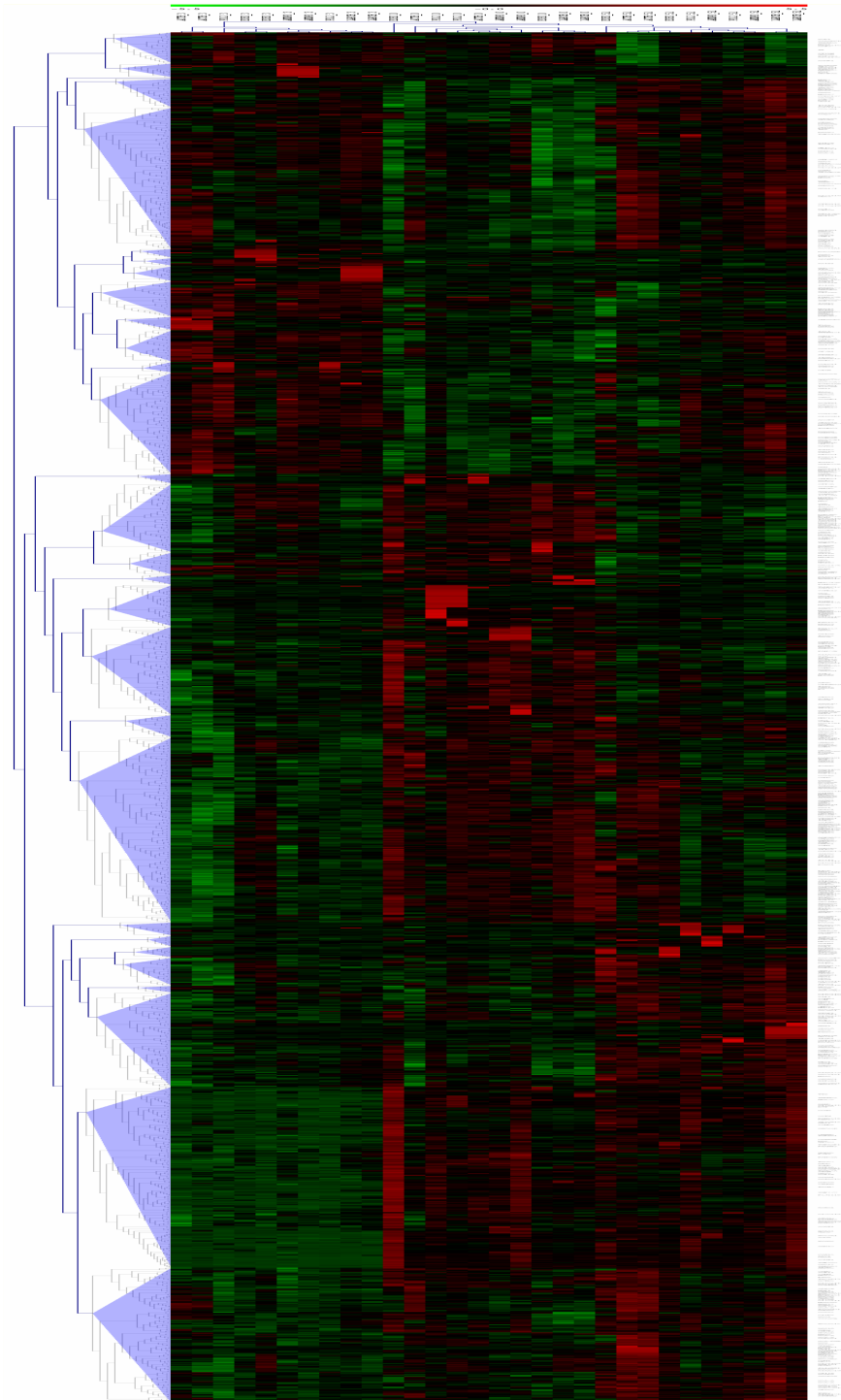
## Differentially expressed genes - ANOVA

|              |       |
|--------------|-------|
| Factor GROUP | → 221 |
|--------------|-------|

|             |       |
|-------------|-------|
| Factor TIME | → 386 |
|-------------|-------|

|                          |      |
|--------------------------|------|
| Interaction GROUP x TIME | → 98 |
|--------------------------|------|

|       |       |
|-------|-------|
| TOTAL | → 695 |
|-------|-------|



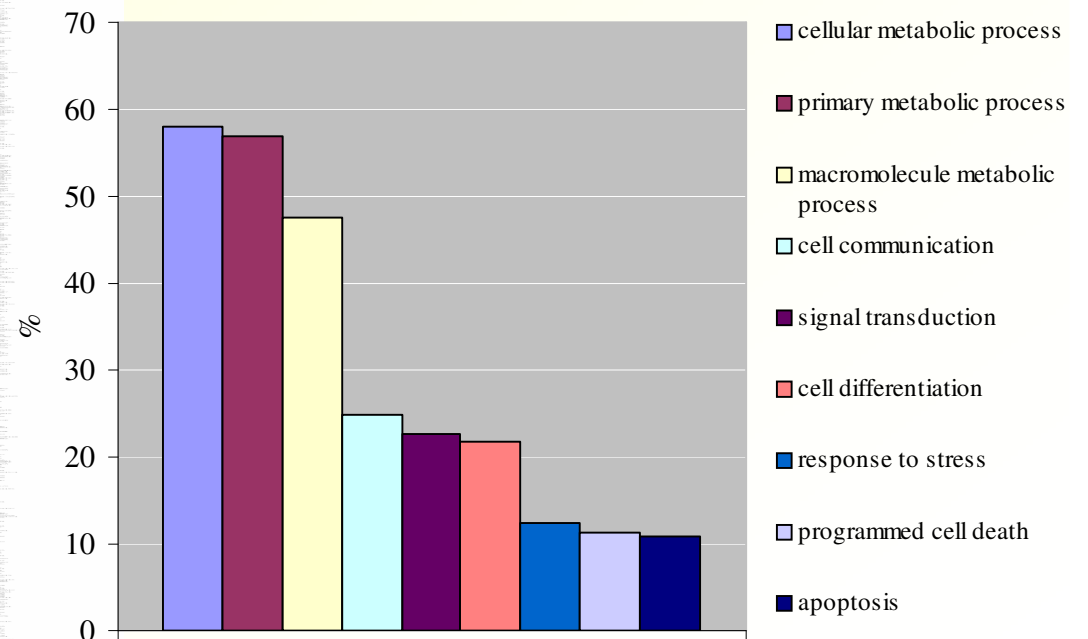
## Hyerarchical clustering analysis

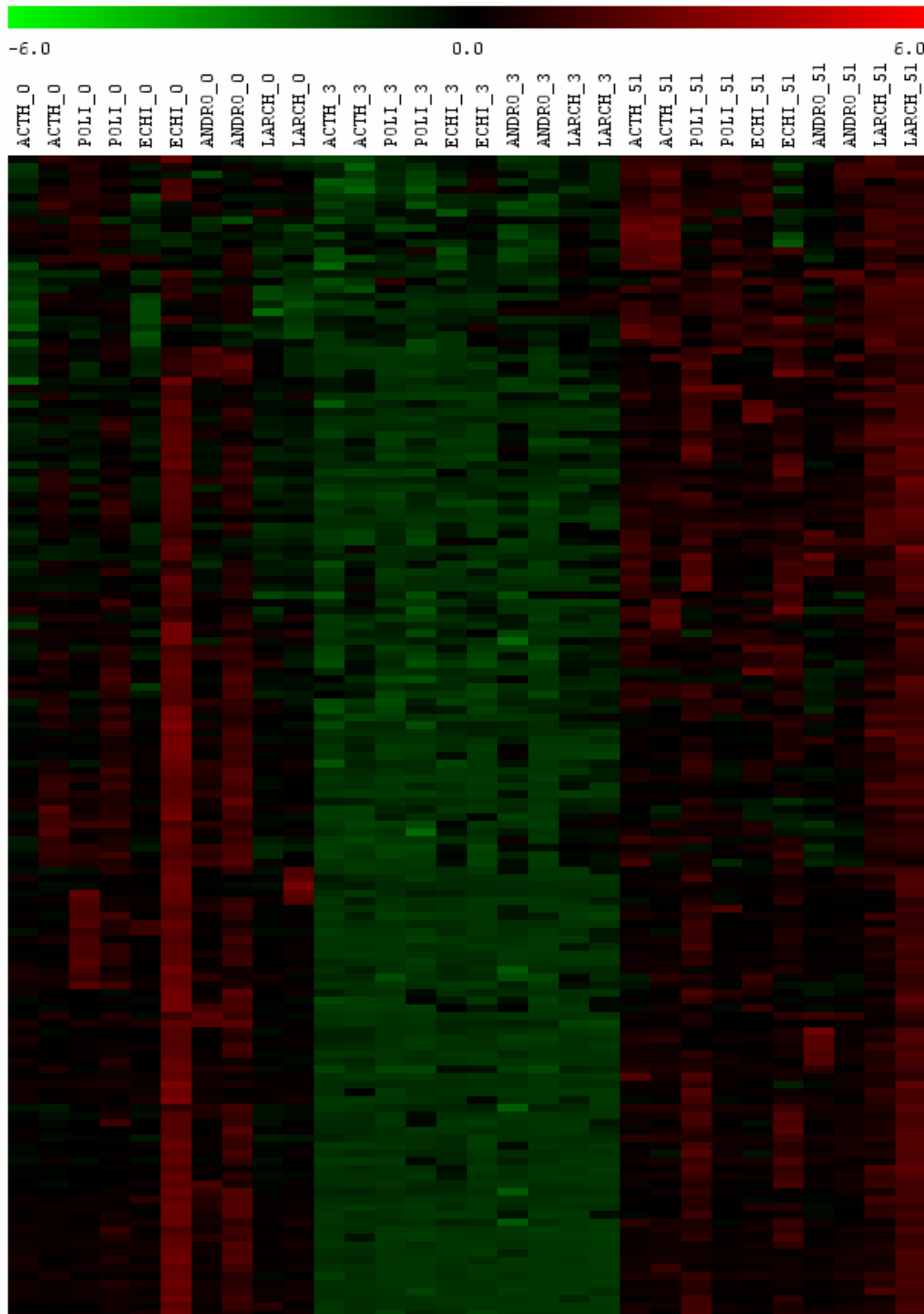
695 differentially expressed genes;

341 annotated genes;

29 gene clusters.

### Biological processes

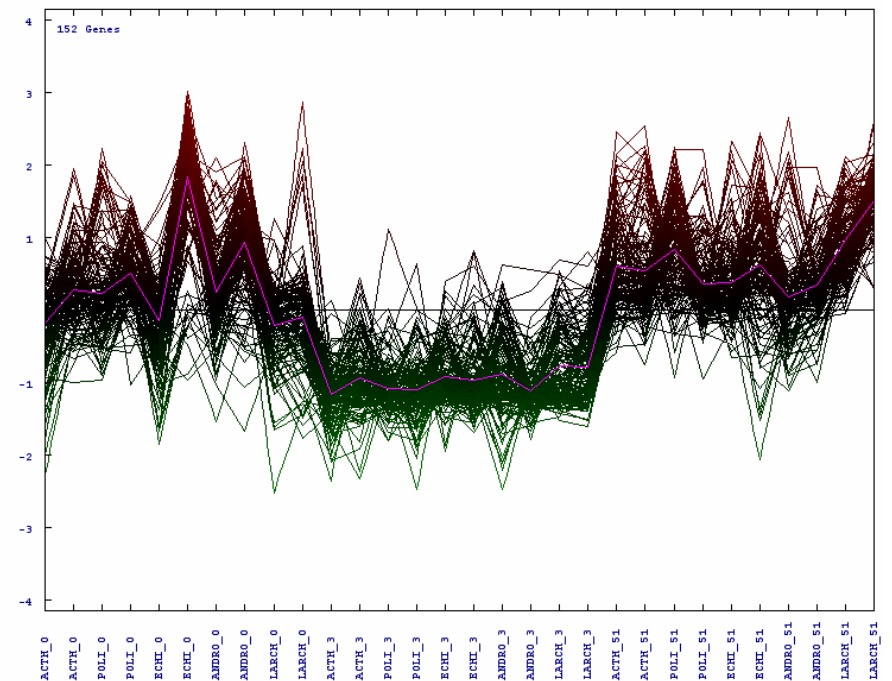




## Evidence of ACTH effect

### Cluster 25:

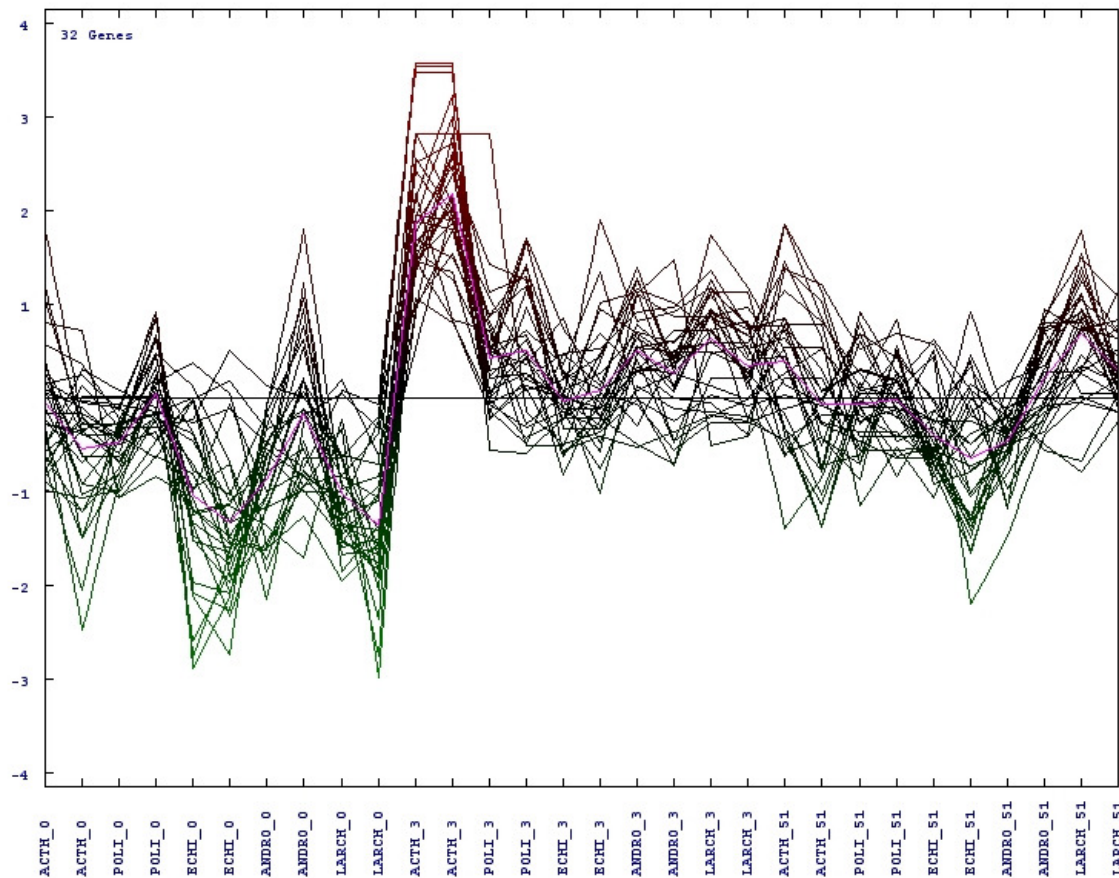
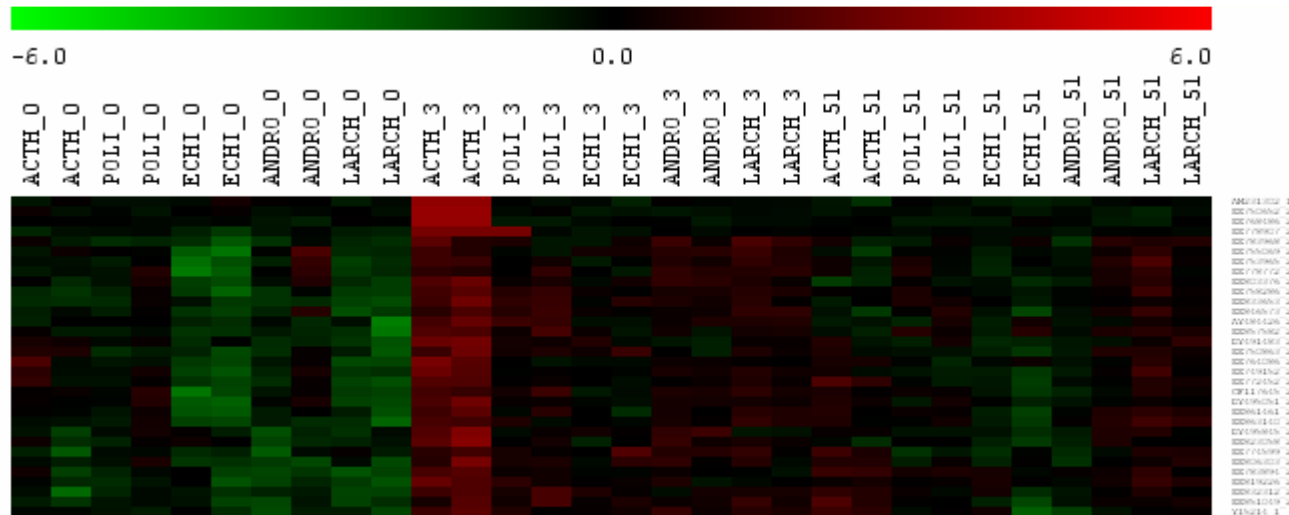
152 (**58 annotated**) genes  
DOWN-regulated in T<sub>3</sub> for  
all the group.



## Cluster 25:

| Biological processes                                                  | %            | Genes                                                                                                                                                     |
|-----------------------------------------------------------------------|--------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------|
| cellular metabolic process                                            |              | RSL1D1 IFNG AGK EPHX2 SUOX DUT LOC730144 CSE1L AIFM1 SUGT1 SCYL1 CREBZF ARV1 MEF2C C14orf126 NSDHL PCF11 TTK CALR ZNF470 RBM5 CCNH RIOK2 NT5C3 NQO2 PSMC5 |
|                                                                       | <b>62.97</b> | TGFBR1                                                                                                                                                    |
| primary metabolic process                                             |              | RSL1D1 IFNG AGK SUOX DUT LOC730144 CSE1L AIFM1 SUGT1 SCYL1 CREBZF ARV1 MEF2C C14orf126 NSDHL PCF11 TTK CALR ZNF470 RBM5 CCNH RIOK2 NT5C3 PSMC5 TGFBR1     |
|                                                                       | <b>58.14</b> |                                                                                                                                                           |
| macromolecule metabolic process                                       |              | RSL1D1 IFNG AGK DUT LOC730144 CSE1L AIFM1 SUGT1 SCYL1 CREBZF MEF2C PCF11 TTK CALR ZNF470 RBM5 CCNH RIOK2 PSMC5 TGFBR1                                     |
|                                                                       | <b>46.51</b> |                                                                                                                                                           |
| nucleobase, nucleoside, nucleotide and nucleic acid metabolic process |              | DUT AIFM1 SCYL1 CREBZF MEF2C PCF11 CALR ZNF470 RBM5 CCNH NT5C3 PSMC5 TGFBR1 IFNG                                                                          |
|                                                                       | <b>32.56</b> |                                                                                                                                                           |
| cell communication                                                    |              | TGFBR1 ARFGEF2 IFNG AGK AIFM1 ZFYVE16 VAC14 GPR125 CEP57 OPTN SNX10                                                                                       |
|                                                                       | <b>25.58</b> |                                                                                                                                                           |
| establishment of localization                                         |              | CALR CEP57 OPTN SNX10 SLC31A2 ARFGEF2 TRAPPC6B IFNG CSE1L ZFYVE16 SEC24D                                                                                  |
|                                                                       | <b>25.58</b> |                                                                                                                                                           |
| transport                                                             |              | CSE1L ZFYVE16 SEC24D CALR CEP57 OPTN SNX10 SLC31A2 ARFGEF2 TRAPPC6B                                                                                       |
|                                                                       | <b>23.26</b> |                                                                                                                                                           |
| signal transduction                                                   |              | TGFBR1 ARFGEF2 IFNG AGK AIFM1 ZFYVE16 VAC14 GPR125 CEP57 OPTN                                                                                             |
|                                                                       | <b>23.26</b> | IFNG BCL2A1 LOC730144 CSE1L AIFM1 CALR CEP57 OPTN PSMC5 TGFBR1                                                                                            |
| cell differentiation                                                  |              |                                                                                                                                                           |
|                                                                       | <b>23.26</b> |                                                                                                                                                           |
| transcription                                                         |              | PSMC5 TGFBR1 IFNG SCYL1 CREBZF MEF2C CALR ZNF470 CCNH                                                                                                     |
|                                                                       | <b>20.93</b> |                                                                                                                                                           |
| RNA biosynthetic process                                              |              | SCYL1 CREBZF MEF2C CALR ZNF470 CCNH PSMC5 IFNG                                                                                                            |
|                                                                       | <b>18.6</b>  |                                                                                                                                                           |
| cell cycle                                                            |              | TTK CALR RBM5 CCNH PSMD2 NCAPG2 SUGT1                                                                                                                     |
|                                                                       | <b>16.28</b> |                                                                                                                                                           |
| programmed cell death                                                 |              | CSE1L,AIFM1,CALR,PSMC5,IFNG,BCL2A1                                                                                                                        |
|                                                                       | <b>13.95</b> |                                                                                                                                                           |
| response to stress                                                    |              | CCNH IFNG EPHX2 LOC730144 AIFM1 ORM2                                                                                                                      |
|                                                                       | <b>13.95</b> |                                                                                                                                                           |
| cellular catabolic process                                            |              | EPHX2,SUOX,AIFM1,C14orf126,PSMC5                                                                                                                          |
|                                                                       | <b>11.63</b> |                                                                                                                                                           |



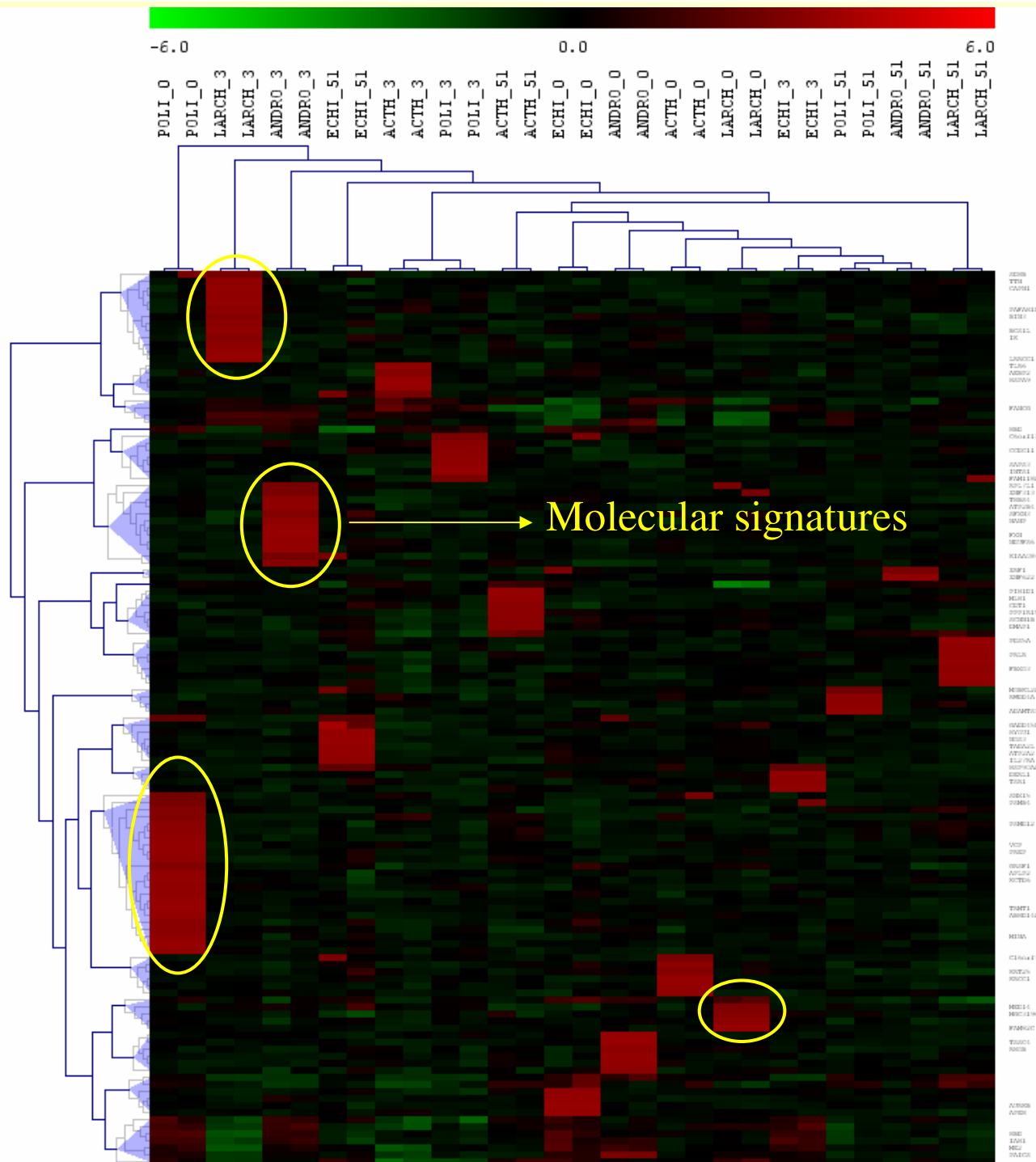


## Evidence of bioactive compounds regulation

Cluster 18:  
32 (19 annotated) genes  
UP-regulated in ACTH\_3

## Cluster 18:

| <b>Biological processes</b>         | <b>%</b>    | <b>Genes</b>                                                      |
|-------------------------------------|-------------|-------------------------------------------------------------------|
| cellular metabolic process          | <b>66.7</b> | ITGB2 HSPA9 B3GAT3 GADD45A EIF5B SF3B1 KIAA0368 MAP2K1 TLR6 TXNIP |
| primary metabolic process           | <b>60.0</b> | ITGB2 HSPA9 B3GAT3 GADD45A EIF5B SF3B1 KIAA0368 MAP2K1 TLR6       |
| macromolecule metabolic process     | <b>60.0</b> | ITGB2 HSPA9 B3GAT3 GADD45A EIF5B SF3B1 KIAA0368 MAP2K1 TLR6       |
| cell differentiation                | <b>40.0</b> | GADD45A,EIF5B,TRAF4,TXNIP,ITGB2,HSPA9                             |
| cell communication                  | <b>33.3</b> | RGS19,ITGB2,TRAF4,MAP2K1,TLR6                                     |
| signal transduction                 | <b>33.3</b> | RGS19,ITGB2,TRAF4,MAP2K1,TLR6                                     |
| response to stress                  | <b>33.3</b> | GADD45A,TLR6,TXNIP,ITGB2,HSPA9                                    |
| cell development                    | <b>33.3</b> | ITGB2,HSPA9,GADD45A,EIF5B,TRAF4                                   |
| apoptosis                           | <b>26.7</b> | ITGB2,HSPA9,GADD45A,TRAF4                                         |
| programmed cell death               | <b>26.7</b> | TRAF4,ITGB2,HSPA9,GADD45A                                         |
| response to chemical stimulus       | <b>26.7</b> | MAP2K1,TXNIP,ITGB2,HSPA9                                          |
| response to external stimulus       | <b>20.0</b> | MAP2K1,TLR6,ITGB2                                                 |
| response to biotic stimulus         | <b>20.0</b> | TLR6,ITGB2,HSPA9                                                  |
| immune response                     | <b>20.0</b> | HLA-A,TLR6,ITGB2                                                  |
| regulation of programmed cell death | <b>13.3</b> | HSPA9,TRAF4                                                       |



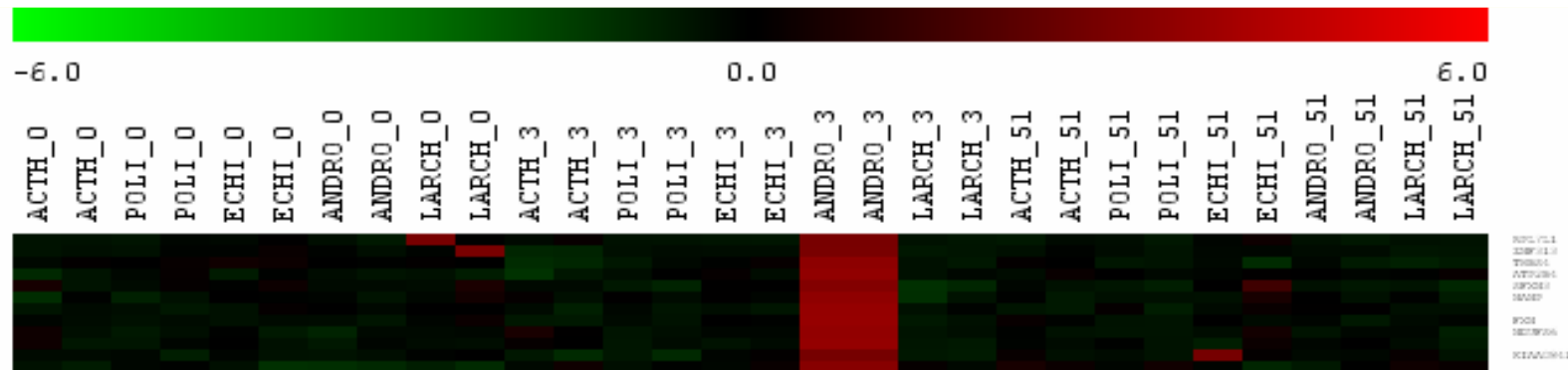
## Molecular signature

Factor GROUP x TIME

98 genes

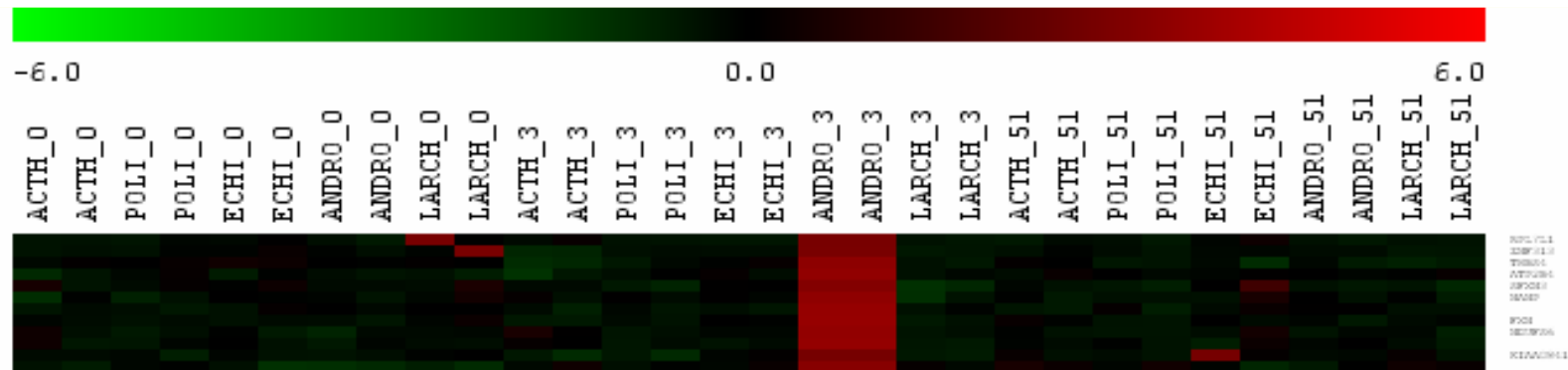
75 annotated genes;

19 clusters



## Molecular signature for *Andrographis paniculata* at T\_3

- RPL7L1 (ribosomal protein L7-like 1);
- ZNF313 (zinc finger protein 313);
- THBS4 (thrombospondin 4);
- ATP2B4 (ATPase, Ca<sup>++</sup> transporting, plasma membrane 4);
- SFXN3 (sideroflexin 3);
- NANP (N-acetylneuraminic acid phosphatase);
- NDUFS6 (NADH-coenzyme Q reductase);
- FXN (frataxin);



## Molecular signature for *Andrographis paniculata* at T\_3

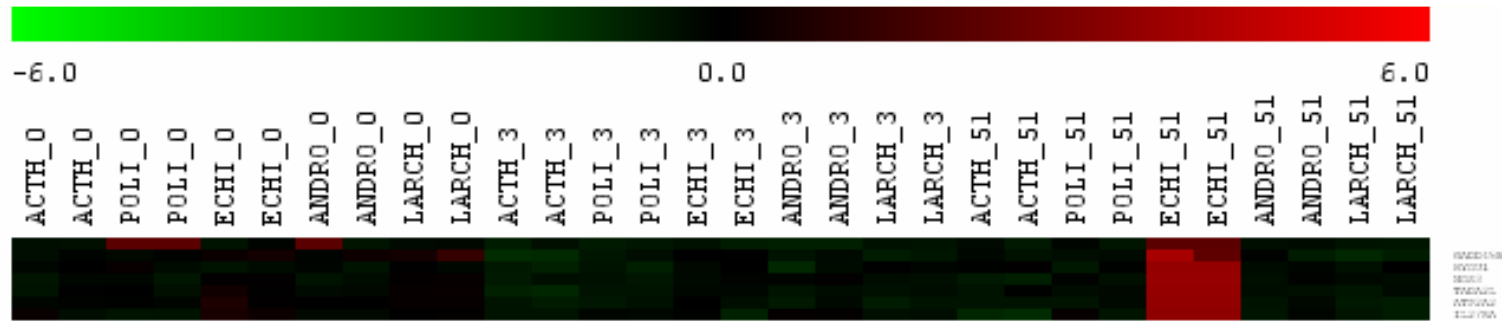
ATP2B4: Ca<sup>++</sup> regulation

SFXN3: Iron transporter

NDUFS6: Oxidoreductase

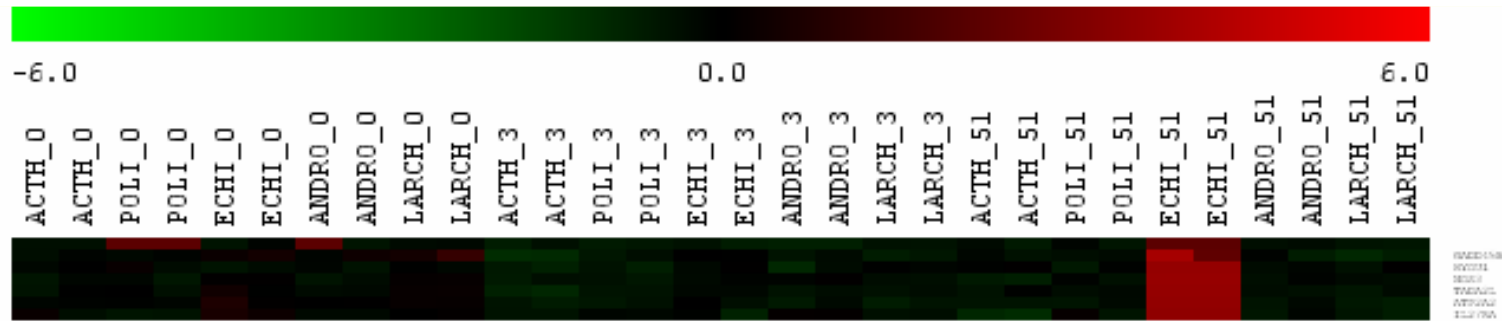
FXN: Prevents mitochondrial damage and ROS production





## Molecular signature for Echinacea angustifolia at T\_51

- GADD45B (growth arrest and DNA-damage-inducible, beta);
- HYOU1 (hypoxia up-regulated 1); HSP70 family
- NOS3 (nitric oxide synthase 3 (endothelial cell);
- ATP2A2 (ATPase, Ca<sup>++</sup> transporting, cardiac muscle, slow twitch 2);
- IL27RA (interleukin 27 receptor, alpha)



## Molecular signature for Echinacea angustifolia at T\_51

GADD45B: Responds to environmental stresses, regulation of growth and apoptosis

HYOU1: Member of HSP70 family

eNOS: deficiency results in hypertension, increased vascular smooth muscle cell proliferation in response to vessel injury, increased leukocyte–endothelial interactions, hypercoagulability and increased diet-induced atherosclerosis.

ATP2A2: Ca++ regulation

IL27RA: Involved in the regulation of Th1-type immune responses.

Involved in innate defense mechanisms

# CONCLUSIONS

- ✓ The custom microarray is available for nutrigenomic applications – need to be updated;
- ✓ Data mining showed that a large percentage of differentially expressed genes belong to biological processes:
  - cell communication
  - signal transduction
  - cell differentiation
  - intracellular signalling cascade
  - response to stress
  - apoptosis

# CONCLUSIONS

- ✓ HCL underlined the effect of ACTH treatment and specific biomarkers of cortisol-mediated stress
- ✓ HCL allowed to study the regulatory effects of compounds administered
- ✓ Tentative molecular signatures of the tested compounds
- ✓ CLINICAL TRIALS ARE REQUIRED TO VALIDATE THE USE OF THESE COMPOUNDS AT A FARM LEVEL



Thank you

