Dietary bioactive compounds: the transcriptional responses of hepatic cultured cells reveal possible hypolipidemic strategies to prevent metabolic syndrome

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PATHWAY-27 overview

- PATHWAY-27 focuses on the role and mechanisms of action of 3 bioactives, as ingredients for the enrichment of 3 different food matrices (dairy, bakery and egg products) to determine how they affect physiologically-relevant primary and secondary endpoints for Metabolic Syndrome.

\[ \text{In vitro} \quad \text{In vivo} \]
### PATHWAY-27 in vivo studies

<table>
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<th>Step</th>
<th>Description</th>
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| **Step 1** | • Formulation of bioactive enriched food (BEF)  
• 3 different food in each category, each one enriched with the bioactives alone or in combination (15 BEF in total) |
| **Step 2** | • Selection of the best BEF in each category, based on technological and sensory characteristics  
• Each selected BEF (milkshake – dairy; biscuits – bakery; pancakes – egg based) enriched with the bioactives alone or in combination |
| **Step 3** | • Pilot intervention studies to select the best enrichment in each BEF, based on the effectiveness on primary and secondary end-points (mainly blood TG and HDL-C) for the MetS |
| **Step 4** | • Large intervention study (double blind, randomized vs placebo) to evidence the effects of the BEF selected in the pilot studies  
• on primary and secondary end-points for the MetS  
• on other biomarkers related to advanced omics techniques (epigenetics, nutrigenetics, metabolomics).  
• Evaluation of BEF digestibility and bioactive bioavailability in pigs |
The aim of the in vitro studies is to understand the protective role and mechanism of action of each bioactive compound, alone and in combination, their cross-talk at the cellular level and their role in the aetiology and development of the MetS.

Two cell models:
- adipocytes
- hepatocytes
PATHWAY-27 bioactives

- Docosahexaenoic acid (DHA), a long-chain PUFA
- β-glucans (BG)
- Anthocyanins (AC)

Five different bioactive combinations:
- DHA alone
- BG alone
- AC alone
- DHA+BG
- DHA+AC

- DHA
- Propionate (PRO), a SCFA derived from BG colonic fermentation
- Protocatechuic acid (PCA), the main metabolite of anthocyanins

Five different bioactive combinations:
- DHA alone
- PRO alone
- PCA alone
- DHA+PRO
- DHA+PCA
Aim of the study

- The aim of the study was to evaluate the effects of the bioactives on the transcriptome of hepatic cells.
- The bioactives were supplemented to HepG2 cells for 6 or 24 h at the following concentrations:
  - 50 μM DHA
  - 70 μM PRO
  - 20 μM PCA
  - 50 μM DHA + 70 μM PRO
  - 50 μM DHA + 20 μM PCA
- Concentrations used were within the physiological range, and possible cytotoxicity was excluded in previous experiments.
Methods – cell culture

**HepG2 cells**

- seeded in 6-well plates at 1 x 10^6 cells/mL concentration
- at 70% confluence, supplemented with bioactives for 6 or 24 h
- scraped-off in PBS

Total RNA was isolated using Direct-zol™ RNA MiniPrep (Zymo Research Corporation).
Quantity and quality of RNA were assessed
Replicates from the same experiment were pooled together, and diluted to 40 ng/µL.
Three independent experiments were performed
Methods - RNA sequencing

- The RNA integrity was determined using Fragment Analyzer (AATI, High Sensitivity Analysis Kit) according manufacturer’s instructions.
- mRNA enrichment, cDNA synthesis, library construction and amplification were performed using Illumina TruSeq RNA Library Prep kit v2 on a Biomek4000 Liquid Handling Automated Workstation (BeckmanCoulter).
- Library quantification was performed using KAPA Library Quantification Kit according manufacturer’s instructions.
- All DNA purification steps were performed using AMPureXP SPRI beads (Agencourt, BeckmanCoulter) according manufacturer’s instructions.
- Sequencing was performed on an Illumina HiSeq1500 instrument according to manufacturer’s instructions. All samples were sequenced in 50bp reads to a depth of approximately 15 mill. Reads were aligned to human genome build hg19 using STAR aligner (Dobin et al 2012).
- Differential expression analyses were performed using iRNA-seq pipeline (Madsen et al. 2015).
- All downstream analyses were performed using R bioinformatics platform.
**Methods - qPCR**

- **reversed transcription** to cDNA of 1 μg RNA (QuantiTect reverse transcription kit - Qiagen).

- **quantitative real-time PCR** (qPCR) using the Rotor-Gene 6000 (Corbett Research) detection system, according to QuantiTect SYBR Green RT-PCR kit (Qiagen).

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<th>Target genes</th>
<th>Reference genes</th>
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<tr>
<td>3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR); low density lipoprotein receptor (LDLR); srebf-1 sterol regulatory element binding transcription factor 1 (SREBF1); srebf-2 sterol regulatory element binding transcription factor 2 (SREBF2)</td>
<td>β-actin (ACTB); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); hydroxymethylbilane synthase (HMBS); succinate dehydrogenase complex, subunit A (SDHA)</td>
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- Data analysis by the DataAssist Software (Applied Biosystems).

- Results expressed as the mean fold change of relative expression compared to control cells, normalized to one.
Results – 6 h treatment

Very limited amount of significantly regulated genes (Padj =< 0.05)
All samples are closely related (PCA analysis)
# of significantly regulated genes (Padj < 0.05)

- 11 by DHA (4 induced, 7 repressed)
- 0 by PRO
- 0 by PCA
- 5 by DHA + PRO (1 induced, 4 repressed)
- 23 by DHA + PCA (7 induced, 16 repressed)
Results – 24 h treatment

Higher amount of significantly regulated genes (Padj <= 0.05) PCA analysis does not show clusterization
# of significantly regulated genes (Padj <= 0.05)

- 56 by DHA (15 induced, 41 repressed)
- 2 by PRO (2 induced)
- 0 by PCA
- 116 by DHA + PRO (37 induced, 79 repressed)
- 97 by DHA + PCA (30 induced, 47 repressed)

The most of genes regulated by DHA alone were also regulated by DHA + PRO (47 out of 56) and DHA + PCA (44 out of 56)
Among the 56 genes regulated by DHA:

- 28 belong to the lipid and lipoprotein metabolism pathway, and 25 are also regulated by both DHA + PRO and DHA + PCA.
- The combinations DHA + PRO and DHA + PCA regulate additional 13 and 10 genes of the pathway, respectively. 

http://pathcards.genecards.org/Pathway/16887

- 4 belong to the glucose and fructose metabolism pathway.
- The combinations DHA + PRO and DHA + PCA regulate additional 6 and 3 genes of the pathway, respectively.

http://pathcards.genecards.org/Pathway/16154
Conclusion

- The treatment with the bioactives does not alter the cell transcriptome drastically
- Effect on transcription is time dependent
- Considering supplementation with single bioactives, DHA is the one inducing the bigger alteration of the cell transcriptome
- The most of regulated genes belong to the “metabolism superpathway”, mainly lipid, cholesterol and lipoprotein metabolism
- PRO and PCA alone has no or very limited effect on transcription, but exert additive effect when supplemented with DHA after 24 h supplementation
One of the mechanisms of action of DHA is in the regulation of gene expression.

Although many genes and pathways have been reported to be regulated by DHA, our results confirm its ability to regulate mainly genes involved in lipid metabolism.

PATHWAY-27 addresses possible synergies between different bioactives. Combination of DHA with other bioactives could reduce DHA effective dose.
A “DHA signature” and the additive effects of PRO and PCA to DHA supplementation have been also verified investigating hepatocyte metabolome (by NMR)

*In vivo* (pilot intervention studies) foods enriched with DHA + AC or DHA + BG have been identified as the most active on MetS endpoints

http://pathway27.eu/
THANK YOU FOR YOUR KIND ATTENTION!

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