

A combination of LCPUFAs regulates the expression of miRNA-146a-5p in a murine asthma model and human alveolar cells

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ABSTRACT

Background: LCPUFAs are suggestive of having beneficial effects on inflammatory diseases such as asthma. However, little is known about the modulative capacity of omega-(n)-3 and n-6 LCPUFAs within the epigenetic regulation of inflammatory processes.

Objective: The aim of this study was to investigate whether a specific combined LCPUFA supplementation restores disease-dysregulated miRNA-profiles in asthmatic mice. In addition, we determined the effect of the LCPUFA supplementation on the interaction of the most regulated miRNA expression and oxygenase activity *in vitro*.

Methods: Sequencing of miRNA was performed by NGS from lung tissue of asthmatic and control mice with normal diet, as well as of LCPUFA supplemented asthmatic mice. Network analysis and evaluation of the biological targets of the miRNAs were performed by DIANA- miRPath v.3 webserver software, TargetScanMouse 7.2, and tool String v.10, respectively. Expression of hsa-miRNA-146a-5p and activity of COX-2 and 5-LO in LCPUFA-treated A549 cells were assessed by qPCR and flow cytometry, respectively.

Results: In total, 62 miRNAs were dysregulated significantly in murine allergic asthma. The LCPUFA combination restored 21 of these dysregulated miRNAs, of which eight (mmu-miR-146a-5p, -30a-3p, -139-5p, -669p-5p, -145a-5p, -669a-5p, -342-3p and -15b-5p) were even normalized compared to the control levels. Interestingly, six of the eight rescued miRNAs are functionally implicated in TGF- β signaling, ECM-receptor interaction and fatty acid biosynthesis. Furthermore, *in vitro* experiments demonstrated that upregulation of hsa-miRNA-146a-5p is accompanied by a reduction of COX-2 and 5-LO activity. Moreover, transfection experiments revealed that LCPUFAs inhibit 5-LO activity in the presence and absence of anti-miR-146a-5p.

Conclusion: Our results demonstrate the modulative capacity of LCPUFAs on dysregulated miRNA expression in asthma. In addition, we pointed out the high regulative potential of LCPUFAs on 5-LO regulation and provided evidence that miR-146a partly controls the regulation of 5-LO.

1. Introduction

Asthma is a chronic respiratory disease characterized by eosinophilic and polymorphonuclear inflammation, airway hyperresponsiveness and airway remodeling [1,2]. Long-chain polyunsaturated fatty acid (LCPUFA) supplementations have been discussed extensively to

have beneficial effects as supportive treatment of asthma [3–5]. LCPUFA can be endogenously converted into either pro-inflammatory eicosanoids or specialized pro-resolving mediators (SPMs) by cyclooxygenases (COX) and lipoxygenases (LO). Whereas SPMs, such as resolvins, maresins and protectins are mostly biosynthesized from essential n-3 LCPUFA-species, like eicosapentaenoic acid (EPA),

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docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), pro-inflammatory eicosanoids are biosynthesized from arachidonic acid (AA). Pro-inflammatory eicosanoids, such as (cysteinyl)-leukotrienes, have been shown to impair bronchoconstriction and promote inflammatory processes in asthma [6,7]. In contrast, SPMs are involved in the control of self-limited inflammation by initiating and promoting the resolution processes of inflammation [8–12].

However, until today, little is known about the impact of LCPUFAs on miRNA expression, although there has been evidence that miRNA expression is altered in chronic diseases, such as asthma [13–15]. Since miRNAs are small 22–24 nt non-coding RNA sequences targeting 3' untranslated regions (UTRs) of mRNAs, they are capable of destabilizing and degrading mRNA transcripts. Thus, miRNAs regulate approximately 60% of the genome post-transcriptionally [16]. Dysregulated miRNA in asthma were already reviewed by Tsitsiou et al. [13], Collison et al. [14] and Rebane et al. [15] who described an upregulation of miR-21, -106a, -126, -145, -146a, -155, -221 and -222 and a downregulation of let-7, miR-20b and miR-133a. In view of this, restoration of miRNA homeostasis could ameliorate the clinical outcome in allergic asthma. Furthermore, previous investigations have shown an interrelation between epigenetic regulation and SPMs, like resolvin (Rv) D1. For instance, RvD1 was shown to regulate several miRNAs involved in self-limited acute inflammation by controlling pro-inflammatory mediators and proteins [17]. Consequently, miRNAs are of special interest to obtain a better understanding of the inflammatory process in asthma and subsequently, to evolve new therapeutic options or to be biomarker for therapy success.

In a previous study, we already showed that a specific combined LCPUFA supplementation could restore disease-specific altered fatty acid profiles in asthmatic mice [18]. To work out and to identify the supportive and therapeutic potential of this LCPUFA combination on miRNA expression, asthmatic mice were supplemented with the specific combination of LCPUFAs. miRNA expression analysis was performed subsequently by Next Generation Sequencing (NGS) and qPCR. Additionally, we determined the effect of the specific LCPUFA supplementation on the interaction of the most regulated miRNA and of the COX-2 and 5-LO activity *in vitro*.

2. Material & methods

2.1. Asthma mouse model

Female C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, Delaware, USA) at the age of 6–8 weeks. They were maintained in individually ventilated stainless-steel cages with an alternating light/dark cycle and fed with mouse lab chow *ad libitum* (Sniff, Soest, Germany). All animal procedures were performed according to protocols approved by the German Animal Subjects Committee (Gen.Nr.FK/1036). Mice were sensitized for ten consecutive days (day (d)1–d10) with house dust mite (HDM) extract (40 µg/day, or PBS as control) to induce a manifest allergic asthma (see Fig. 1). After sensitization, the mice received either no supplementation (Ctrl and HDM group) or a specific LCPUFA combination (LCPUFA group) for 24 days from d11 to d34.

During the last three days (d32 to d34) of supplementation, sensitization with HDM was boosted for further three daily doses (recall model [19]). On d35, 24 h after the last HDM and LCPUFA administration, lung tissue (n = 4–5 animals per group) was chopped and transferred to 5 mL tubes (Nunc™ Low Profile 5.0 mL Externally-Threaded Universal Tube, Thermo Scientific, Dreieich, Germany) pre-filled with 2 mL of RNeasy lysis buffer (Qiagen, Hilden, Germany). After collection, samples were stored immediately on ice according to the manufacturer's recommendations before they were stored at 4 °C for a maximum of 4 weeks until RNA isolation for NGS.

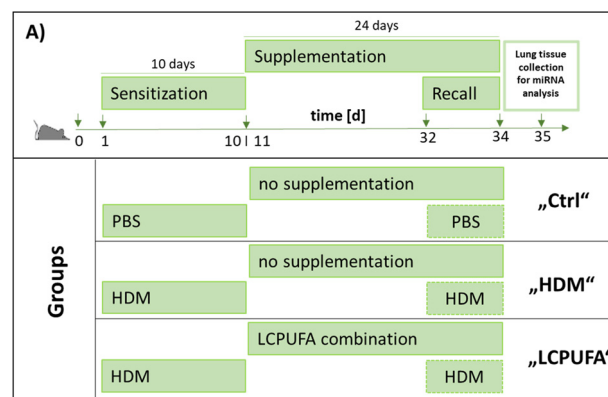


Fig. 1. Schematic design of experiments to prove the effects of LCPUFA supplementation on miRNA expression in allergic asthma. C57BL/6 mice were sensitized with house dust mite (HDM) extract (or PBS as Control) for 10 consecutive days (d) to induce manifest allergic asthma. Upon d11, mice received either no or LCPUFA supplementation until d34. During the final three days (d32–d34), three further daily doses of HDM (or PBS as Control) were administered to mice (as described in a recall model). Lung tissue collection for miRNA analysis was done on d35, 24 h after the last LCPUFA and HDM administration.

2.2. Oral gavage

Daily doses of the oil blend contained 1000 mg/kg EPA, 229.6 mg/kg DHA, 246.0 mg/kg GLA and 200.9 mg/kg SDA and were emulsified with 0.5% (w:v) gum arabic solution (Carl Roth, Karlsruhe, Germany) to gain a total volume of 200 µL and to assure defined administration. Emulsions were administered with a feeding needle (Robert Helwig GmbH, Berlin, Germany). The blend was freshly mixed on a daily basis and homogenized prior to administration by an ultrasonic homogenizer (Sonopuls, Bandelin, Berlin, Germany).

2.3. In vitro cell culture

The human lung carcinoma cell line A549 was cultured in T75 flasks (Greiner bio-one, Frickenhausen, Germany) with Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Darmstadt) containing 10% fetal calf serum (FCS) (Sigma-Aldrich, Taufkirchen, Germany) and 1% Penicillin-Streptomycin (Life Technologies, Darmstadt, Germany). Cells were seeded at 2×10^6 cells per flask at 37 °C in a humidified atmosphere which contained 5% CO₂. For *in vitro* experiments, cells were plated in 6-Well plates at 5×10^5 cells/mL. After 24 h adherence time, the combination of LCPUFAs was conjugated to BSA (Bovine Serum Albumin, 99 mg/mL (w:v) in PBS; Sigma-Aldrich, Taufkirchen, Germany) in a molecular ratio of 1:2.7 at 50 °C for exactly 3 min as described by Tigistu-Sahle et al. [20]. Fatty acid stock mix was diluted with 100% ethanol (Sigma Aldrich, Taufkirchen, Germany). The final concentrations in cell cultures were 0.1 pmol/cell of EPA and DHA and 0.05 pmol/cell of stearidonic and γ -linoleic acid (all purchased from Sigma Aldrich, Taufkirchen, Germany). Fatty acid solutions were added immediately after conjugation to a total amount of 5×10^5 cells/well in FCS-reduced media (0.5% FCS). Controls were exposed to FCS-reduced media with ethanol (vehicle) and FCS-reduced media only, respectively. After 24 h of incubation, cells were washed with PBS and stimulated with a pro-inflammatory cytokine mix (CM) containing 400 U/mL IFN- γ , 50 U/mL IL-1 β and 20 ng/mL TNF- α (all Peprotech, Hamburg, Germany). After 4 h or 24 h of stimulation, cells were detached using Accutase™ cell detachment solution (Sigma-Aldrich Chemie, Taufkirchen, Germany) and washed with PBS. For miRNA determination, cell pellets were resolved instantly either in QIAzol Lysis Reagent (Qiagen, Hilden, Germany) for miRNA isolation or in Stain Buffer for flow cytometry. Cell viability was assessed using Trypan Blue (1:10 (v:v), Trypan Blue

0.4%, Clorox HealthCare, Oakland, CA, USA) and XTT-assay (AppliChem, Darmstadt, Germany) according to the manufacturer's protocol. There was no reduction in cell viability observed under any experimental condition (data not shown).

2.4. Knock-down of hsa-miR-146a-5p in A549 cells

A549 cells were reversely transfected with anti-hsa-miR-146a-5p using Lipofectamine RNAiMAX (Invitrogen, Karlsruhe, Germany) prior to the incubation with fatty acids and prior to the stimulation with a cytokine mix. Both siRNA and Lipofectamine (Invitrogen, Karlsruhe, Germany) were diluted with Opti-MEM® media (Thermo Fisher, Dreieich, Germany) according to the protocol and then mixed 1:2 (v:v). After 5 min of incubation at RT, 250 µL of the final siRNA-lipid complex (25 pmol siRNA) were added to each well followed by 1 mL cell suspension containing 500.000 cells/mL (reverse transfection). For a significant knock-down, cells were incubated with the siRNA-lipid complex at 5% CO₂ and RT for 72 h. Successful transfection was checked by miRVana™ miRNA Inhibitor let7c positive control and miRVana™ negative control (both Thermo Fisher, Dreieich, Germany). Anti-miR-let-7c inhibitor activity was checked by qPCR detecting HMGA-2 mRNA. Results of qPCR revealed a significantly increased biosynthesis of HMGA-2 upon anti-miR-let7c transfection which representatively confirmed an efficient transfection of the introduced anti-miRNAs (miRVana-Control: 0.48 ± 0.05 RQ; anti-miR-let-7c: 1.00 ± 0.03 ; $p < 0.001$, data not shown). Therefore, t-RNA was isolated from lung cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and subsequently transcribed into cDNA by QuantiTect Reverse Transcription-Kit (Qiagen, Hilden, Germany). Following the reverse transcription, cDNA was amplified for qPCR via Fast-Advanced Master Mix-Kit (Thermo Fisher Scientific, Dreieich, Germany) and with HMGA-2 probes (Cat. No. 4331182, Thermo Fisher Scientific, Dreieich, Germany). Furthermore, qPCR was conducted using StepOnePlus Real-Time PCR Systems and StepOnePlus™ software (Thermo Fisher Scientific, Dreieich, Germany). Following conditions were used: Uracil-N-glycosylase (UNG) incubation at 50 °C for 2 min and polymerase activation at 95 °C for 2 min followed by 40 cycles of 1 s at 95 °C (denaturation) and 20 s at 60 °C (annealing). Samples had RNA Integrity Numbers (RINs) > 8.5 with a mean of 8.82 ± 1.18 (SEM). All samples were measured as triplicates against non-template controls.

2.5. miRNA isolation

Total RNA, including miRNA, was isolated using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Prior to extraction, max. 30 µg of macrodissected lung tissue was lysed and homogenized in QIAzol Lysis Reagent (Qiagen, Hilden, Germany) using a GentleMACS (Miltenyi Biotech, Bergisch Gladbach, Germany). A549 cells were dissolved directly in QIAzol Lysis Reagent. After homogenization with QIAshredder (Qiagen, Hilden, Germany), chloroform was added to murine or human lung cell suspensions. Samples were centrifuged and ethanol was added to the upper aqueous phase containing RNA. Finally, samples were applied to RNeasy mini spin columns and washed several times to guarantee the elution of high-quality RNA in RNase-free water. RNA concentration as well as 260/280- and 260/230-ratios were determined with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Concentration of RNA was assessed by using Nanodrop Lite spectrometry (Thermo Scientific, Dreieich, Germany) and RNA quality was assessed by the RINs using the Agilent RNA 6000 Nano Kit and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Carla, CA, USA). All murine samples had RINs > 8.5 with a mean of 9.27 ± 0.69 (SEM) and cell culture samples had RINs with a mean of 9.99 ± 0.01 (SEM). Upon quality control, RNA was frozen at -80 °C and later either arranged for library preparation or TaqMan qPCR.

2.6. Library preparation

miRNA libraries were generated with the NEXTflex Small RNA-Seq Kit v3 (BIOO Scientific, Austin, TX, USA) using 1 µg of total RNA, the Human Brain (Ambion, Thermo Fisher, Dreieich, Germany) and the miRNA control (included in the Kit). In the first step adenylated adaptors were ligated at 3', followed by 4N adapter ligation at 5'. Thereafter, qPCR was conducted using M-MuLV Reverse Transcriptase and cDNA was amplified using universal primers and barcode primers for each sample followed by gel-free size selection and clean-up. DNA concentration was determined using the Qubit dsDNA Assay Kit, Qubit assay tubes and the Qubit 3.0 fluorometer (all from Thermo Fisher Scientific, Dreieich, Germany). DNA quality was validated with the Agilent High Sensitivity DNA Reagents and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Carla, CA, USA) using High-Sensitivity DNA chips. Libraries were frozen at -20 °C until miRNA Sequencing.

2.7. miRNA sequencing and analysis

Next generation sequencing (NGS) was performed with the MiSeq Reagent Kit v3, the PhiX Sequencing Control v3 and the MiSeq™ Desktop Sequencer (all Illumina Inc., San Diego, USA). The results were processed according to Bioo Scientific Small RNA analysis pipeline. Cutadapt V1.14 was used to remove adapter sequences and random bases and to discard too short reads. Subsequently, bowtie 2 (V2.2.9; <http://www.bowtie-bio.sourceforge.net>) was used to align readouts to murine mature miRNAs from miRBase (mature.fs, v21, June 2014; <http://www.mirbase.org>), and finally miRUtils (v1.0.0; <http://www.mirutils.sourceforge.net>) was used to process the aligned files which were provided for further analyses.

Differential expression analysis was performed in R version 3.2.3 (<https://cran.r-project.org/>). Coverage files were converted into raw counts matrices. After NGS analysis, we obtained ~8 Mio reads with an average read count per sample of 324,026 and an average of 323 reads per miRNA per sample (Supplementary Fig. 1A). Overall, we detected 1001 miRNAs of which 104 had 5 or more reads in each sample and 317 miRNAs were detected with 5 or more reads in at least 50% of the samples. No *a-priori* filtering for sparse read count or normalization has been applied, since the implemented pipeline accounts for low reads internally. To identify technical outliers, we performed hierarchical cluster analysis of log2-transformed raw count data based on the Euclidean distance between samples and the Ward algorithm implemented in R using the normalized Read counts (function *counts (Reads, normalize = T)*) miRNAs or the top 100 miRNAs based on variance. Of the analyzed 13 samples, 1 sample (S55, Control) was excluded (Supplementary Fig. 1B). For comparison between two groups we loaded the respective raw read counts into DESeq2 using the "DESeqDataSetFromMatrix" function. Differential expression was estimated using the function "DESeq" based on the developer's recommendation. Since the groups showed no differences (ANOVA *p*-values > 0.1) with respect to RNA quality and read-depth, no additional corrections were to be included. Fdr correction was applied for each miRNA passing DESeq-quality thresholds. And miRNAs were considered to be differentially expressed with $p_{adj} < 0.05$ and not-differentially expressed with $p_{adj} > 0.1$. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE135597 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135597>).

Identified miRNAs were subjected to functional enrichment analysis (KEGG pathways) using DIANA- miRPath v.3 webserver software [21]. Network analysis was done using the igraph package in R. For visualization and sub-network identification we set the correlation threshold to $cor > 0.5$. Subnetworks identified were subjected to DIANA functional enrichment analysis as described above. To further evaluate biological targets of the miRNAs and to determine sites that match the

seed region of mmu-miR-146a-5p, mmu-miR-139-5p, mmu-miR-669p-5p, mmu-miR-15b-5p, mmu-miR-342-3p and mmu-miR-669a-5p, we used TargetScanMouse 7.2 which considers matches to mouse 3' UTRs and their orthologs, as defined by University of California, Santa Cruz (UCSC) whole-genome alignments [22]. This statistical model predicts the effects of miRNAs binding to canonical sites and scores the sum of contribution of 14 different features of the miRNA, miRNA site, and mRNA by creating a total context score (TCS). The more negative the score, the greater the repression [23]. Targets of the miRNAs related to (1) cytokine-mediated signaling pathway and TGF-beta signaling pathway (2), fatty acid and steroid metabolic processes, and (3) extracellular matrix interaction. These were grouped and network analysis was conducted using the software tool String v.10 (<https://string-db.org/>) with standard settings which have been employed to visualize networks of target protein-protein interaction of the mmu-miR-146a-5p-subcluster [24]. In addition, network analysis was performed for miR-146a-5p targets which were also targets of at least one of the other miRNAs.

2.8. miRNA validation and quantification by qPCR

NGS based expression levels of mmu-miRNA-146a-5p as wells as hsa-miRNA-146a-5p levels *in vitro* were validated and assessed by TaqMan qPCR (TaqMan® Advanced miRNA Assays; Thermo Fisher, Dreieich, Germany), respectively. Therefore, appropriate probes targeting either human or murine miR-146a-5p were purchased from Applied Biosystems (mature miRNA Sequence: UGAGAACUGAAUUC AUGGGUU, Stem-loop Accession Number: [MI0000477](#), Thermo Fisher, Dreieich, Germany).

Preparation of cDNA was done by extending 5 ng t-RNA at the 3' end of mature transcripts through poly (A) addition. Thereafter, the 5' end was lengthened by adaptor ligation and followed by a universal reverse transcription and amplification using TaqMan® Advanced miRNA cDNA Synthesis Kit and a thermal cycler (GeneAmp Cyclor PCR Systems 9700 v3.12., Thermo Fisher, Dreieich, Germany). The thermal cycler program of RT cycles was programmed according to manufacturer's description. Undiluted cDNA was stored at -20°C . Ahead of the sample quantification, TaqMan PCRs were conducted with four potential murine (miR-16-5p, miR-26a-5p, miR-24-3p, miR-191-5p) and three human (miR-16-5p, miR-25-3p and miR-93-5p) endogenous controls (all TaqMan Advanced miRNA Assay, Thermo Fisher, Dreieich, Germany). A suitable endogenous control for murine and human lung cells under experimental conditions was selected based on variance and abundance over samples. Experiments done in fourfold determination revealed that mmu- and hsa-miR-16-5p was expressed relatively constant and moderately abundant in murine and human lung cells. Thus, miR-16-5p was used as endogenous control. For qPCR 5 μL of 1:10 (v:v, in TE-buffer) diluted cDNA were introduced to oligonucleotides. qPCR was conducted using StepOnePlus Real-Time PCR Systems and the StepOnePlus™ software (Thermo Fisher Scientific, Dreieich, Germany). Evaluation was done by $2^{-\Delta\Delta\text{Ct}}$ method in Expression Suite software v1.1 (Fisher Scientific, Dreieich, Germany), Excel (Microsoft Office, Redmond, Washington) and GraphPad Prism 5 (La Jolla, California).

2.9. Flow cytometry

Detached cells were washed with staining buffer (PBS containing 1% FCS), lysed (1x BD Lysing Solution (BD Biosciences, Heidelberg, Germany)) and permeabilized (1x BD Lysing Solution (BD Biosciences, Heidelberg, Germany) with 0.2% Saponin (Sigma-Aldrich, Taufkirchen, Germany)). Intracellular staining was carried out using the PE-conjugated mouse anti-human COX-2 monoclonal antibody clone AS67 (BD Biosciences, Heidelberg, Germany) and FITC-conjugated rabbit anti-human 5-LO antibody (Biozol, Eching, Germany). Accordingly, cells were washed with Stain Buffer and resuspended in PBS. Measurement of COX-2 and 5-LO was conducted using the FACSVerse flow cytometer

(BD Biosciences, Heidelberg, Germany) and the FACSuite software v1.0.6 (BD Biosciences, Heidelberg, Germany). Time points of measurements for COX-2 (4 h) and 5-LO (24 h) were chosen according to the time points of maximal activity of these enzymes known from literature [25,26] and own experiments (data not shown).

2.10. Statistics

miRNA expression levels which were determined by qPCR are presented as Relative Quantification (RQ) of gene expression whereas the rest of the data are presented as mean $\pm$ standard error of the mean (SEM). All experiments were performed at least three times. If not otherwise indicated, differences between groups were determined using the one-way ANOVA (multiple groups) with Bonferroni *post-hoc* analysis. Calculations were performed using R version 3.2.3 (<https://cran.r-project.org/>), GraphPad Prism 5 software (GraphPad software, La Jolla, California) and Excel (Microsoft Office, München, Germany). Statistical significance was defined as $p < 0.05$. Significant p values are indicated as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3. Results

3.1. LCPUFA supplementation restored 21 dysregulated miRNAs in asthma

To determine the impact of the LCPUFA combination on dysregulated miRNA in asthma, we assessed miRNA expression by NGS. A total of 62 miRNAs were expressed significantly (all Ctrl vs. HDM $p_{\text{adj}} < 0.05$) different in asthmatic mice compared to control mice (Fig. 2A and B red circle); 32 of the 62 dysregulated miRNAs were downregulated (Sup. Table 1A) and 30 were upregulated (Sup. Table 1B), respectively. LCPUFA supplementation restored (all Ctrl vs. LCPUFA $p_{\text{adj}} > 0.1$) the expression levels of 21 dysregulated miRNAs (Fig. 2B, light green circle). Thereof eight miRNAs were restored back to control levels and nominally significantly different between the untreated and LCPUFA-treated asthmatic mice (Table 1 and Fig. 2B, dark green circle, Ctrl vs. HDM $p_{\text{adj}} < 0.05$; Ctrl vs. LCPUFA $p_{\text{adj}} > 0.1$ and HDM vs. LCPUFA $p_{\text{nom}} < 0.05$). These eight miRNAs were namely: mmu-miR-146a-5p, mmu-miR-30a-3p, mmu-miR-139-5p, mmu-miR-669p-5p, mmu-miR-145a-5p, mmu-miR-669a-5p, mmu-miR-342-3p and mmu-miR-15b-5p. Of these eight miRNAs mmu-miRNA146a-5p survived correction for multiple testing (HDM vs. LCPUFA $p_{\text{adj.}} = 0.005$, Fig. 2C) being rescued from 7.14 ± 0.10 to control-level of 6.70 ± 0.17 log2(normalized counts). The expression levels of mmu-miRNA146a-5p were confirmed using TaqMan qPCR and relative expression correlated significantly ($p < 0.05$) with the normalized RNASeq reads.

3.2. Functional analysis revealed impact of asthma-dysregulated and LCPUFA-restored miRNAs on biological processes

To identify the contribution of miRNAs to biological processes, co-regulated network-analysis of dysregulated miRNAs in asthma was performed and targets of the individual miRNAs within clusters were tested for KEGG-pathway enrichment. Network analyses revealed three different (Fig. 3) clusters marked here in turquoise and blue (upregulated) and green (downregulated). Interestingly, six of the eight rescued miRNAs were coregulated in the turquoise cluster, targets of which were functionally implicated in TGF- β signaling ($p = 6.24 \times 10^{-7}$), ECM-receptor interaction ($p = 2.13 \times 10^{-5}$) and fatty acid biosynthesis ($p = 4.49 \times 10^{-4}$). Moreover, mmu-miR-146a-5p is a central coregulator of eleven other dysregulated miRNAs, five of which were likewise (nominally) restored upon LCPUFA treatment: mmu-miR-139-5p, mmu-miR-669p-5p, mmu-miR-15b-5p, mmu-miR-342-3p and mmu-miR-669a-5p. The mmu-miR-146a-5p-cluster was confirmed to contribute to cytokine-cytokine interactions ($p = 1.27 \times 10^{-5}$), steroid biosynthesis ($p = 6.53 \times 10^{-6}$) and ECM-receptor interaction ($p =$

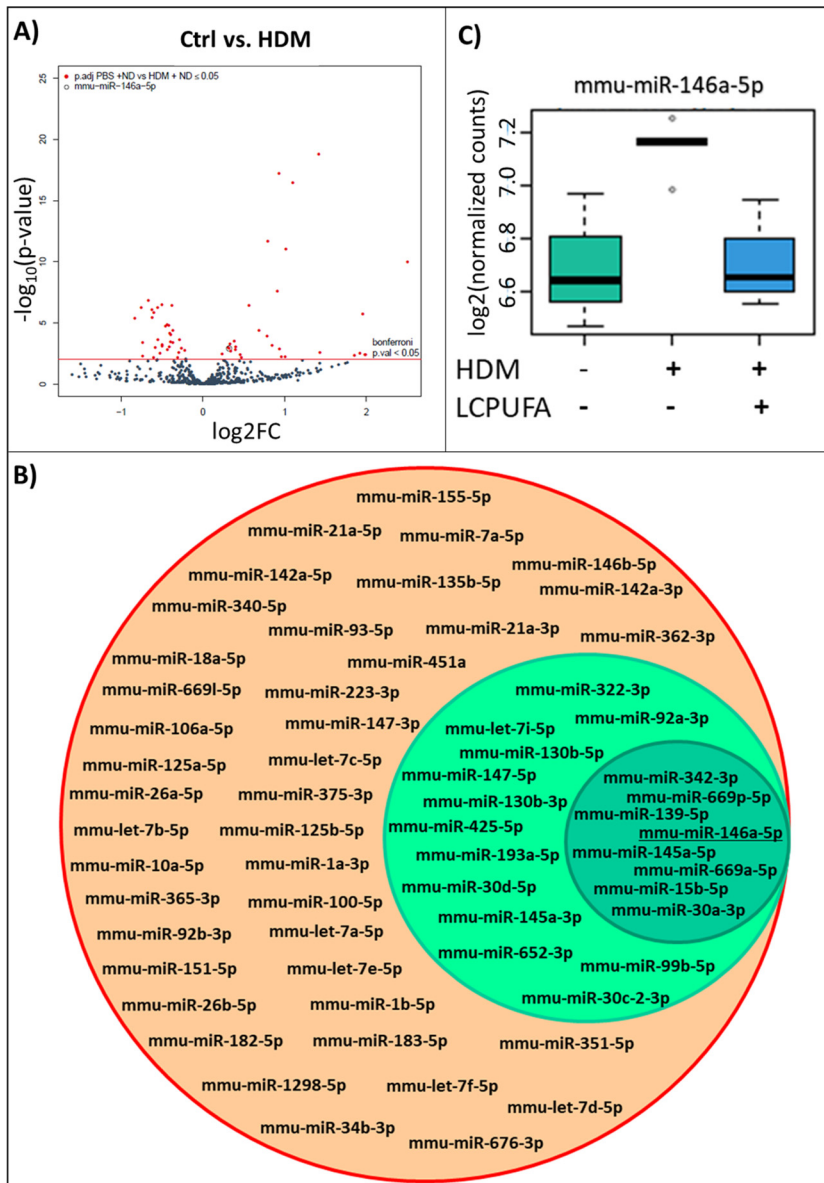


Fig. 2. A) Sixty-two miRNAs were dysregulated (marked red) in HDM-induced asthmatic mice as depicted in a volcano plot comparing control versus asthmatic (HDM) mice. B) Of all 62 dysregulated miRNAs (red circle; Ctrl vs. HDM $p_{adj} < 0.05$), 21 could be restored by the LCPUFA combination (light green circle; Ctrl vs. HDM $p_{adj} < 0.05$ and Ctrl vs. LCPUFA $p_{adj} > 0.1$) and 8 thereof even reached control levels (dark green circle; Ctrl vs. HDM $p_{adj} < 0.05$; Ctrl vs. LCPUFA $p_{adj} > 0.1$ and HDM vs. LCPUFA $p_{nom} < 0.05$). C) Particularly, mmu-miR-146a-5p survived multiple testing and was significantly rescued by the LCPUFA combination (Ctrl vs. HDM $p_{adj} < 0.05$; Ctrl vs. LCPUFA $p_{adj} > 0.1$ and HDM vs. LCPUFA $p_{adj} < 0.05$). Results are depicted as $\log_2$ (normalized counts); $n = 3-5$. Thick bars correspond to the median, boxes to the interquartile range and whiskers to 2.5 x IQR.

1.27×10^{-5}).

Biological target analyses of miR-146a-5p by TargetScanMouse 7.2 revealed 166 transcripts with conserved sites including targets for cytokine-cytokine interactions and ECM receptor interactions such as IRAK1, TNF receptor associated factor (TRAF6), Interphotoreceptor

matrix proteoglycan 1 (IMPG1), as well as for targets of the fatty acid and steroid biosynthesis, such as the Androgen receptor (AR) and Lysosomal acid lipase/cholesteryl ester hydrolase (LIPA). Targeting mRNA of the prostaglandin F2 receptor inhibitor (PTGFRN) showed further a connection to the prostaglandin biosynthesis. In addition,

Table 1

Eight miRNAs in asthmatic mice were significantly restored and reached control levels after LCPUFA supplementation. Results are expressed as $\log_2FC$ with their respective p -values ($n = 3-5$). In addition, these results are ordered according to their significance of restoration (p_{nom} and p_{adj} of HDM vs. LCPUFA). Normalization of miRNA expression was considered to be significant when differences for Ctrl vs. HDM indicated $p_{adj} < 0.05$, Ctrl vs. LCPUFA indicated $p_{adj} > 0.1$ and HDM vs. LCPUFA indicated $p_{nom} < 0.05$.

miRNA	Ctrl vs. HDM		Ctrl vs. LCPUFA		HDM vs. LCPUFA		
	$\log_2FC$	P_{adj}	$\log_2FC$	P_{adj}	$\log_2FC$	P_{nom}	P_{adj}
mmu-miR-146a-5p	0,320	1,10E-02	0,000	0,999	-0,321	6,50E-06	0,005
mmu-miR-30a-3p	-0,525	2,41E-02	-0,272	0,378	0,262	2,66E-04	0,066
mmu-miR-139-5p	0,471	4,38E-02	0,032	0,972	-0,439	7,87E-03	0,590
mmu-miR-669p-5p	1,008	3,67E-02	0,512	0,410	-0,490	1,31E-02	0,590
mmu-miR-145a-5p	-0,368	4,73E-03	-0,147	0,385	0,226	2,73E-02	0,736
mmu-miR-669a-5p	1,433	2,03E-02	1,008	0,157	-0,428	2,86E-02	0,736
mmu-miR-342-3p	0,401	9,61E-03	0,193	0,320	-0,206	3,13E-02	0,781
mmu-miR-15b-5p	0,308	1,03E-02	0,160	0,135	-0,145	4,08E-02	0,805

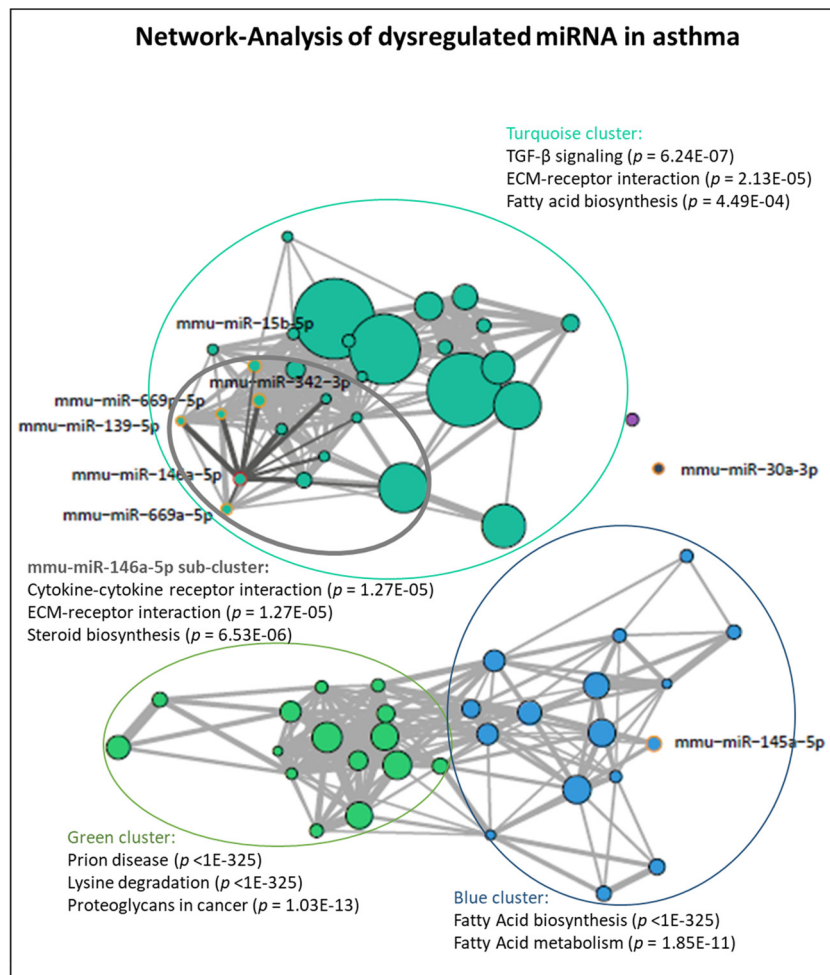


Fig. 3. Network Analysis of asthma-dysregulated miRNAs revealed three main clusters (turquoise, green and blue). The eight miRNAs which were restored and even reached control levels after LCPUFA supplementation in asthmatic mice were highlighted by orange and red circles. A correlation network analysis determined a coregulatory network of mmu-miR-146a-5p and eleven other miRNAs. Following network analysis which was conducted with igraph and KEGG-pathway analysis by miRPath v.3 [21] was utilized to identify pathways in which miRNAs might be involved in and was established to determine predicted or experimentally validated miRNA targets.

Smad4 and vasorin (VASN), a transforming growth factor β -binding protein, could be detected as conserved sites for TGF- β signaling (Suppl. Fig. 2).

Networks analysis by STRING confirmed the protein-protein interactions of miR-146a-5p, miR-15b-5p, miR-139-5p and miR-669a-5p at the protein level in common pathways after LCPUFA treatment in the murine asthma model. Further, the analysis revealed that LCPUFAs influence the regulation of miR-146a-5p, miR-15b-5p, miR-139-5p, miR-342-3p and miR-669a-5p related proteins for cytokine-mediated signaling pathways and TGF- β signaling pathway, for fatty acid and steroid metabolic processes, and for extracellular matrix interaction (Suppl. Fig. 2A–C). Of the 166 miR-146a-5p targets, 36 were also targets for at least one of the other miRNAs, 10 were targets of two miRNAs and KLF7 was a target of miR-146a-5p, miR-139-5p, miR-342-3p and miR-669a-5p. Of these miR-146a-5p targets, two were influenced by miR-15b-5p, 17 by miR-139-5p, 11 by miR-342-3p and 29 by miR-669a-5p (Suppl. Fig. 3A, B). Interestingly, network analysis of these targets revealed significant interactions in the regulation of RNA metabolic processes (Suppl. Fig. 3C).

3.3. LCPUFAs regulate hsa-miR-146a-5p in stimulated A549 cells

To dissect the specific functions of LCPUFAs and miR-146a-5p in inflammatory processes dependent on oxygenase's, we designed an *in vitro* model using lung epithelial cells. Therefore, we cultivated A549-lung epithelial cells and incubated them 24 h with a combination of LCPUFAs prior to stimulation with a cytokine mixture (CM) including IFN- γ , IL-1 β and TNF- α for 4 or 24 h (Fig. 4A). Subsequently, we determined miRNA expression and COX-2 and 5-LO activity.

In vitro experiments showed a significant upregulation of hsa-miR-146a-5p upon cytokine stimulation after 24 h ($p < 0.001$, Fig. 4B + C). Interestingly, prior incubation with LCPUFAs led to an increase of hsa-miR-146a-5p in CM-stimulated cells after 4 h ($p < 0.05$, Fig. 4B). In contrast, LCPUFA pre-treated cells which were stimulated for 24 h revealed a decrease of hsa-miR-146a-5p expression by 2.70-fold compared to CM-stimulated cells ($p < 0.001$). Of note, incubation with vehicle only (100% ethanol) did not alter miRNA expression (data not shown).

3.4. COX-2 and 5-LO activity were both reduced by LCPUFA

After 4 h of stimulation, COX-2 expression was increased by $20.92 \pm 1.11\%$ ($p < 0.05$, set as 100%) in cells stimulated with CM compared to controls (Fig. 4D). The increased COX-2 expression was reduced by prior incubation with the LCPUFA combination from 100% to $87.82 \pm 1.02\%$ ($p < 0.001$, Fig. 4D). 5-LO was significantly increased by $81.66 \pm 6.34\%$ ($p < 0.001$, set as 100%) in all cells after 24 h of stimulation and highly significantly downregulated from 100% to $41.61 \pm 4.83\%$ ($p < 0.001$) by pre-incubation with LCPUFA (Fig. 4E).

3.5. Hsa-miR-146a-5p negatively regulates 5-LO activity in stimulated A549 cells

In view of the considerable reduction of 5-LO compared to COX-2 (Figure 4D + E) and the downregulation of miRNA-146a-5p after 24 h stimulation in LCPUFA-pretreated A549 cells (Fig. 4B) compared to non-LCPUFA pretreated but CM-stimulated cells, we consequently

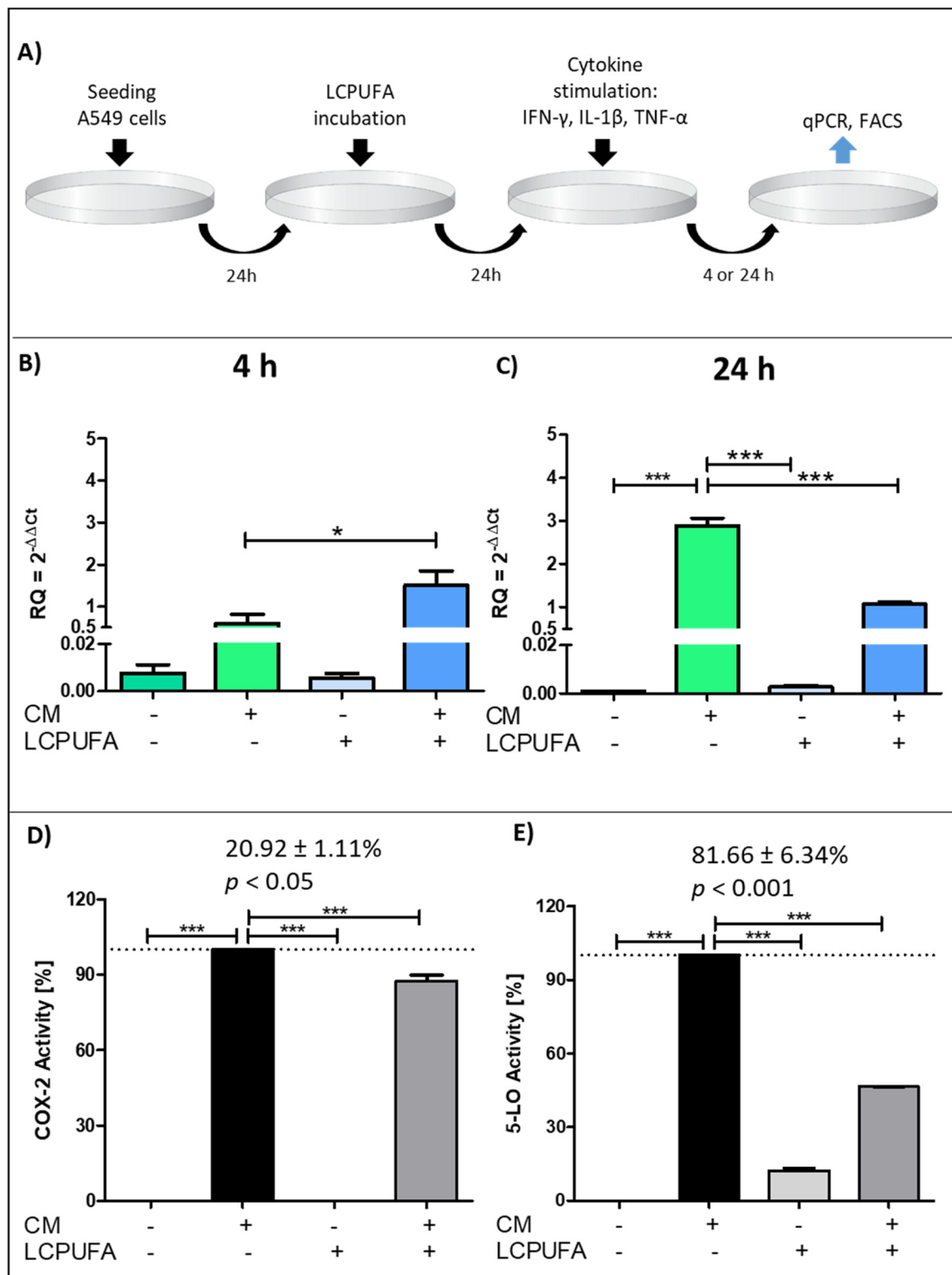


Fig. 4. A) Design of *in vitro* experiments. qPCR or FACS measurements were conducted with A549 cells which were stimulated with a cytokine mixture containing IFN- γ , IL-1 β and TNF- α either with or without pre- LCPUFA incubation. Expression of hsa-miR-146a-5p determined in A549 lung cells after B) 4 h and C) 24 h of cytokine stimulation with or without pre- incubation with LCPUFA by TaqMan qPCR. D) COX-2 and E) 5-LO activity upon cytokine stimulation with or without pre- LCPUFA incubation. MiRNA expression is illustrated as Relative Quantification (RQ) = $2^{-\Delta\Delta Ct}$. COX-2 and 5-LO activity is shown as Relative Activity (%). Results are expressed as mean $\pm$ SEM; n = 3. Differences were considered as statistically significant at p-values * $p < 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ were tested by One-Way ANOVA with Dunnett's *post-hoc* test against positive control (CM).

focused on the role of LCPUFAs and miRNA-146a-5p on 5-LO. Therefore, we conducted transfection experiments using anti-hsa-miR-146a-5p to inhibit the action of hsa-miR-146a-5p and (i) to identify the role

of miR-146a-5p on 5-LO and (ii) to identify the role of LCPUFAs on 5-LO in the presence and absence of anti-hsa-miR-146a-5p. Thus, we observed a significant increase of 5-LO in CM-treated cells in the

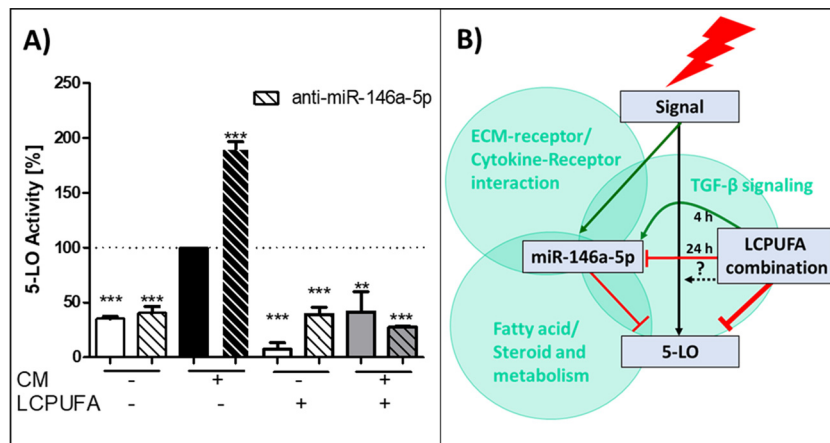


Fig. 5. Inhibition of hsa-miR-146a-5p by transfection of anti-miR-146a-5p in A549 cells. **A)** 5-LO activity was assessed by FACS measurement upon stimulation with or without pre-incubation with the combination of LCPUFAs under inhibition or no inhibition of miR-146a-5p. Results are shown as Relative Activity (%) and expressed as mean $\pm$ SEM; $n = 3$. Differences were considered as statistically significant at p -values $* p < 0.05$, $* p < 0.05$, $** p < 0.01$, $*** p < 0.001$ were tested by One-Way ANOVA with Dunnett's *post-hoc* test against the positive control with sole cytokine stimulation (CM without anti-miR-146a-5p). **B)** Inflammatory signals induce miR-146a-5p expression which we have shown to subsequently repress 5-LO activity in alveolar epithelial cells. A pre-incubation with the specific combined LCPUFA supplementation is capable of decreasing miR146a-5p in inflammatory conditions and repressing 5-LO activity after 24 h of inflammation. However, LCPUFAs did inhibit 5-LO activity in the presence and absence of miR-146a-5p pointing out that LCPUFAs likely act also *via* other pathways than only those miR146a-5p is acting.

presence of anti-hsa-miR-146a-5p from 100% to $190.10 \pm 6.31\%$ ($p < 0.001$) confirming the negative regulation of hsa-miR-146a-5p on 5-LO activity (Fig. 5A). Interestingly, pre-incubation with the LCPUFA combination prevented an increase of 5-LO activity regardless of the absence (100% to $41.30 \pm 18.61\%$, $p < 0.01$) or presence of anti-hsa-miR-146a-5p (100% to $27.47 \pm 1.03\%$, $p < 0.001$).

4. Discussion

Previous studies have revealed that miRNAs influence the progression and maintenance of various diseases such as asthma and allergy. [13–15]. Hence, there is an increasing interest to develop new therapeutic approaches and to adjust disease-specific dysregulated miRNAs.

In our study, the initial determination of altered miRNA-profiles in asthmatic mice by NGS revealed a significant dysregulation of 62 miRNAs in murine HDM-induced asthma. Dysregulated miRNAs in asthma have been described previously by Tsietsiou et al. [13], Collison et al. [14] and Rebane et al. [15]: The analysis of the most frequently dysregulated miRNAs in murine allergic asthma disclosed an upregulation of miR-21, -106a, -126, -145, -146, -155, -221 and -222 and a downregulation of let-7, miR-20b and miR-133a. According to these findings, our results confirmed a significant upregulation of miR-21, -106, -145, -146 and -155. In addition, our studies confirmed a significant downregulation of all miR-let-7a-f except of let-7i which was significantly upregulated.

To investigate whether LCPUFAs influence miRNA expression and whether they rescue dysregulated miRNAs, we supplemented asthmatic mice with a specific dietary LCPUFA combination. Thereby, we demonstrated that the expression level of 21 dysregulated miRNAs was nominally restored by the LCPUFA supplementation. Remarkably, eight of them even reached control levels in the asthmatic mice. To the best of our knowledge, this is the first report which demonstrates a recovery of asthma-dysregulated miRNAs by LCPUFAs.

A major limitation of the NGS approach was the low read depth. Thus, only high abundant miRNAs were measured. In view of this, we were not able to identify subtle changes or low abundant miRNAs. However, the upregulation of mmu-miR-146a-5p in murine asthma was the most significant finding rescued by the LCPUFA supplementation. In allergic asthma, Li et al. [27] showed that the production of IgE antibodies was significantly elevated in mice overexpressing miRNA-146a, while no differences were shown in the affinity maturation of IgM and IgG. Furthermore, the authors showed that miR-146a enhances Ig class switching and secretion of IgE in B cells by upregulating 14-3-3 σ expression which would suggest miR-146a being a potential target for asthma therapy [27]. Previous studies have described an involvement of miRNA-146a in TLR-signaling and a significant increase after

macrophages and dendritic cells have been treated with LPS and pro-inflammatory cytokines, respectively [28,29].

This explains an upregulation of mmu-miR-146a-5p in mice after being sensitized with HDM extract since HDM is recognized *via* TLR-4. In addition, these findings elucidate the association of HDM-induced allergic asthma and cytokine-cytokine receptor interactions and ECM-receptor interactions revealed in KEGG pathway analysis. In line with this, biological target analyses revealed transcripts with conserved sites bind to mRNA for IRAK1 and TRAF, which are associated with cell activation and cytokine production. The latter were previously described as targets for miRNA-146a in the regulation of human dendritic cell apoptosis and cytokine production [30,31]. In regard of ECM-receptor interactions data analysis showed a connection to IMPG1 and to vasolin a vascular type I membrane protein with a fibronectin type III-like motif at the extracellular domain [32,33]. Interestingly, vasorin directly binds to transforming growth factor (TGF)- β and attenuates TGF- β signaling *in vitro* [33]. This fits well to another finding of the KEGG pathway analysis that showed an interaction of miRNA-146a with the TGF- β signaling. Indeed, biological targets analysis predicts SMAD4 as target for miRNA-146a. In allergic asthma, TGF- β is important in suppressing T cells but also mediates repair responses that lead to unwanted remodeling of tissues [34]. The close interaction of miRNA-146a and the TGF- β pathway via Smad4 has already been demonstrated for fibrotic and tissue repair processes during wound healing and in skeletal muscle after acute contusion and might also be involved in allergic asthma processes [35,36]. Moreover, regulation on PTGFRN, an inhibitor of prostaglandin signaling, points to a joint effect of miR-146a on lipid mediator signaling [37].

In a recent publication, Chen et al. demonstrated that miR-146a is involved in the regulation of vertebrate LCPUFA biosynthesis and reduces significantly elongation indexes of 20:3n-6/18:3n-6, 20:4n-3/18:4n-3 and 22:5n-3/20:5n-3 [38]. Recchiuti et al. [17] showed that RvD1, a DHA-derived SPM, upregulated a set of miRNAs by binding to its appropriate GPCR ALX/fpr2 in a GPCR-dependent manner indicating that SPMs are indeed able to regulate miRNAs [17]. In particular, they hypothesized that the miRNA-NF- κ B axis is a key component in the RvD1-GPCR downstream signaling pathways. With regard to miR-146a, Taganov et al. [29] revealed that NF- κ B plays a critical role in the induction of miR-146a transcription by LPS, TNF- α , and IL-1 β by promoter analysis of the miR-146a gene. In addition, trials with 3'UTR luciferase reporters determined that TRAF6 and IRAK1 mRNAs represent potential molecular targets of miR-146a [29]. These findings were confirmed by Paik et al. showing that miR-146a downregulates NF- κ B activity *via* targeting TRAF6 [39]. Consequently, miR-146a seems to play a critical role in regulating and fine-tuning Toll-like receptor- and cytokine receptor-induced signaling in order to prevent

exuberant inflammatory responses.

Furthermore, our computational target and networks analysis revealed a close interaction of miR-146a-5p with miR-15b-5p, miR-139-5p, miR-342-3p and miR-669a-5p and confirmed targets in the common pathways after LCPUFA treatment in the murine asthma model. Indeed, interactions of some of these miRNAs together with miR-146a in inflammatory pathways, TGF- β -signaling, and fatty acid biosynthesis have been demonstrated in the literature [40–43]. Interestingly, our network analysis revealed significant interactions in the regulation of RNA metabolic processes.

For LCPUFAs it might be conceivable that they either have only indirect effects on miRNA transcription by being precursors for lipid mediators or that they also activate transcription factors involved in miRNA transcription *via* receptors such as Peroxisome Proliferator-Activated Receptors (PPARs) [44]. However, the mechanism by which LCPUFAs or their endogenous biosynthesized lipid mediators regulate miR-146a in inflammatory processes are not yet fully understood.

To examine the role of miR-146a-5p on the rescuing effect of LCPUFAs, we determined COX-2 and 5-LO activity in cytokine-stimulated lung epithelial cells in the presence or absence of the LCPUFA combination. Our trials showed an increased expression of hsa-miR146a-5p only after 24 h but not after 4 h in lung cells being stimulated with the cytokine mixture. Interestingly, the expression of hsa-miR-146a-5p continued to be increased upon pre-treatment with LCPUFAs after 4 h, while COX-2 was downregulated significantly. Thus, the upregulation of hsa-miR-146a-5p after 4 h of stimulation might have led to the reduction of COX-2 by negative regulation as it was already suggested by Cornett et al. [45]. After 24 h of stimulation, the expression of hsa-miR-146a-5p was downregulated in stimulated lung cells pre-incubated with LCPUFA, while 5-LO activity was decreased significantly. The downregulation of hsa-miR-146a-5p at 24 h was in line with our *in vivo* trials where we demonstrated reduced mmu-miR-146a-5p in asthmatic mice supplemented with LCPUFAs. An explanation for these findings might be a time-dependent biosynthesis of miRNAs as well as lipid mediators upon an inflammatory event [46,47]. Thus, 24 h after cytokine stimulation or HDM administration, SPMs might be endogenously biosynthesized (results will be published elsewhere) and promote resolution processes in contrast to 4 h after stimulation.

Comparing COX-2 and 5-LO activity, the effect of LCPUFAs was much higher on 5-LO activity with a reduction of nearly 60% compared to COX-2 activity, which was reduced by only 15%. Thus, we speculate that LCPUFA supplementations and hsa-miR-146a-5p have a greater impact on 5-LO than on COX-2. Hence, we investigated the impact of LCPUFA and hsa-miR146a-5p expression on 5-LO in the absence of hsa-miR-146a-5p in knock-down trials further. As expected, we observed an additional upregulation of 5-LO upon cytokine stimulation in hsa-miR-146a-5p-negative cells compared to hsa-miR-146a-5p-positive cells. Thus, we presumed that the upregulation of hsa-miR-146a-5p by cytokines likely prevented an excessive 5-LO activation. These findings are in line with Iacona et al. [48] who showed that miR-146a represses endogenous FLAP expression in lung cancer cells. As Iacona et al. [48] described, 5-LO and its activating protein (FLAP) work together in the first two conversion steps of LT production. Additionally, they showed by reporter assays that the regulation of 5-LO occurs through a direct interaction between the FLAP 3' UTR and miR-146a [48]. Interestingly, LCPUFA combination did downregulate 5-LO activity significantly in both hsa-miR-146a-5p-negative and -positive cells implicating that they regulate 5-LO not only *via* miR-146a-5p expression but also *via* other predominant mechanism.

In the context of asthma, the regulatory potential of miR-146a expression has been described as a two-edged sword: On the one hand miR-146a is shown to elevate IgE-production [27], to promote the apoptosis of bronchial smooth muscle cells [49] and to modulate inflammatory mediator expression in airway smooth muscle cells [50]. On the other hand, miR-146a was demonstrated to downregulate NF- κ B

activity [39] and to repress endogenous FLAP expression [48]. Recently, Wang et al showed the anti-inflammatory role of miR-146a on allergic effects rhinitis by inhibition of the toll-like receptor 4 (TLR4)/TRAF6/NF- κ B signaling pathway [51]. However, our results point towards a time-dependent induction of miR-146a-5p transcription by inflammatory signals and a significant influence of LCPUFA supplementations.

5. Conclusion

Our study revealed that LCPUFAs are capable of restoring asthma-dysregulated miRNAs in HDM-induced asthmatic mice. In particular, mmu-miR-146a-5p was significantly rescued in asthmatic mice confirming the pro-resolving capacities of the LCPUFA combination at miRNA level. Pathway analysis revealed that miR-146a-5p is involved in cytokine-cytokine and ECM-receptor interactions, TGF- β signaling and fatty acid and steroid biosynthesis. Moreover, we demonstrated in cell culture trials (see Fig. 5B), that hsa-miR-146a-5p regulates negatively 5-LO activity. However, we revealed that LCPUFA combination downregulates 5-LO activity in the presence and absence of hsa-miR-146a-5p implicating that LCPUFAs or their endogenous derived lipid mediators also act *via* other pathways. Taken together, our results demonstrate the modulative capacity of LCPUFAs on miRNAs in asthma, especially on miR-146a-5p. We further provided evidence of a miR-146a-5p dependent and independent regulation of 5-LO activity triggered by LCPUFAs.

Contributions

DF, RS and CB were involved in the conception and design of the study. DF performed the murine experiments. DF, CK, TS and CD contributed to the establishment of measurements and to the conductance of experiments in cell culture. GG prepared samples for NGS and qPCR. GG and DF conducted and evaluated qPCR runs. GG and HK conducted NGS runs. CK, HK and RPD contributed to the processing of the results from NGS. AGC performed differential expression analysis from processed NGS reads. DF, TS, RPD, OE, AGC, CB and RS interpreted the data. DF, AGC, SPJ, CB and RS wrote the manuscript. SPJ proofread and spell-checked the manuscript. DF, CK, TS, CD, RPD, OE, GG, SPJ, SZ, HK, CB, AGC and RS contributed to the critical revision and the final approval.

Declaration of Competing Interest

The authors do not have any competing interests to declare. The research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Appendix A. Supplementary data

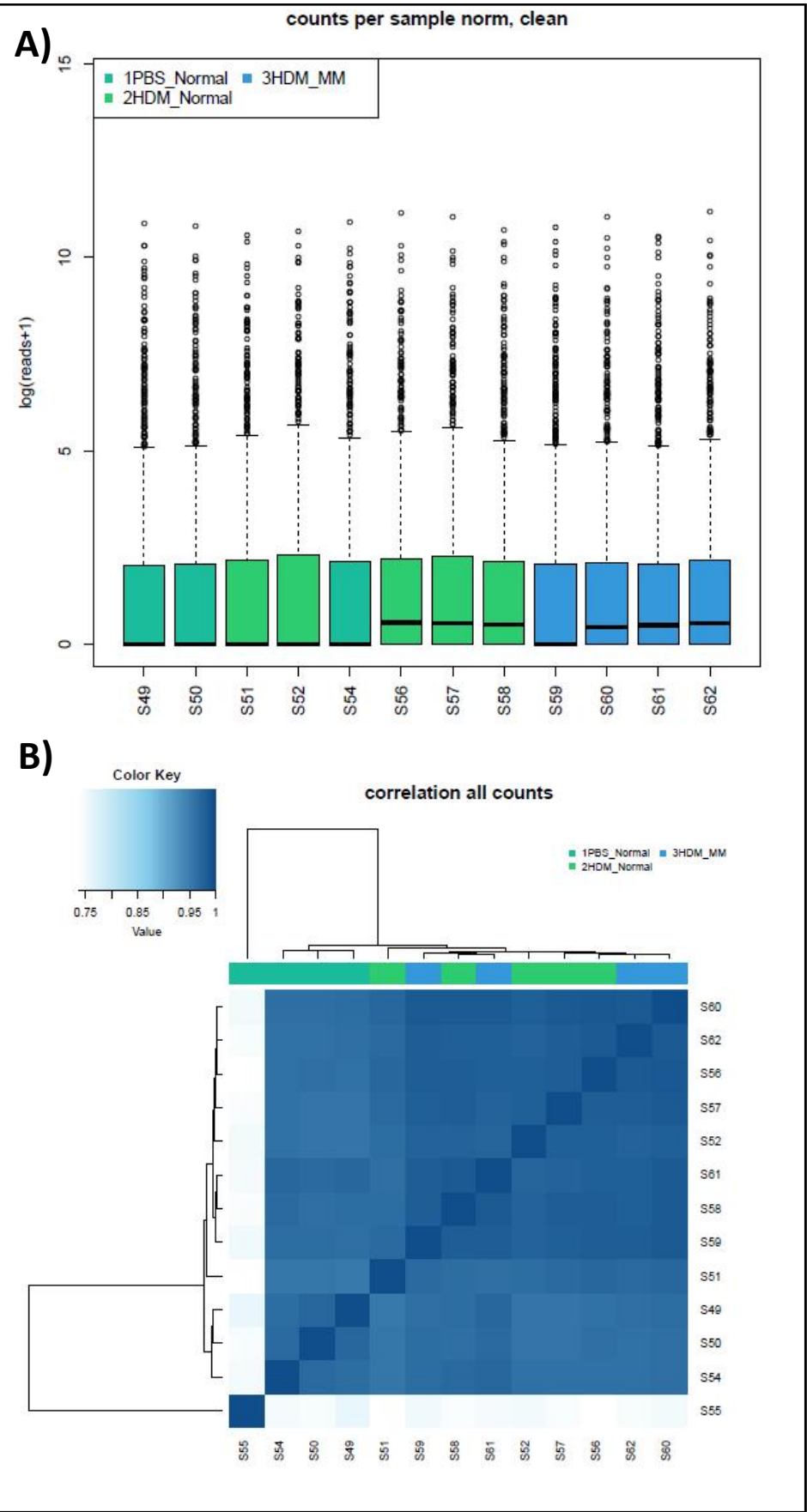
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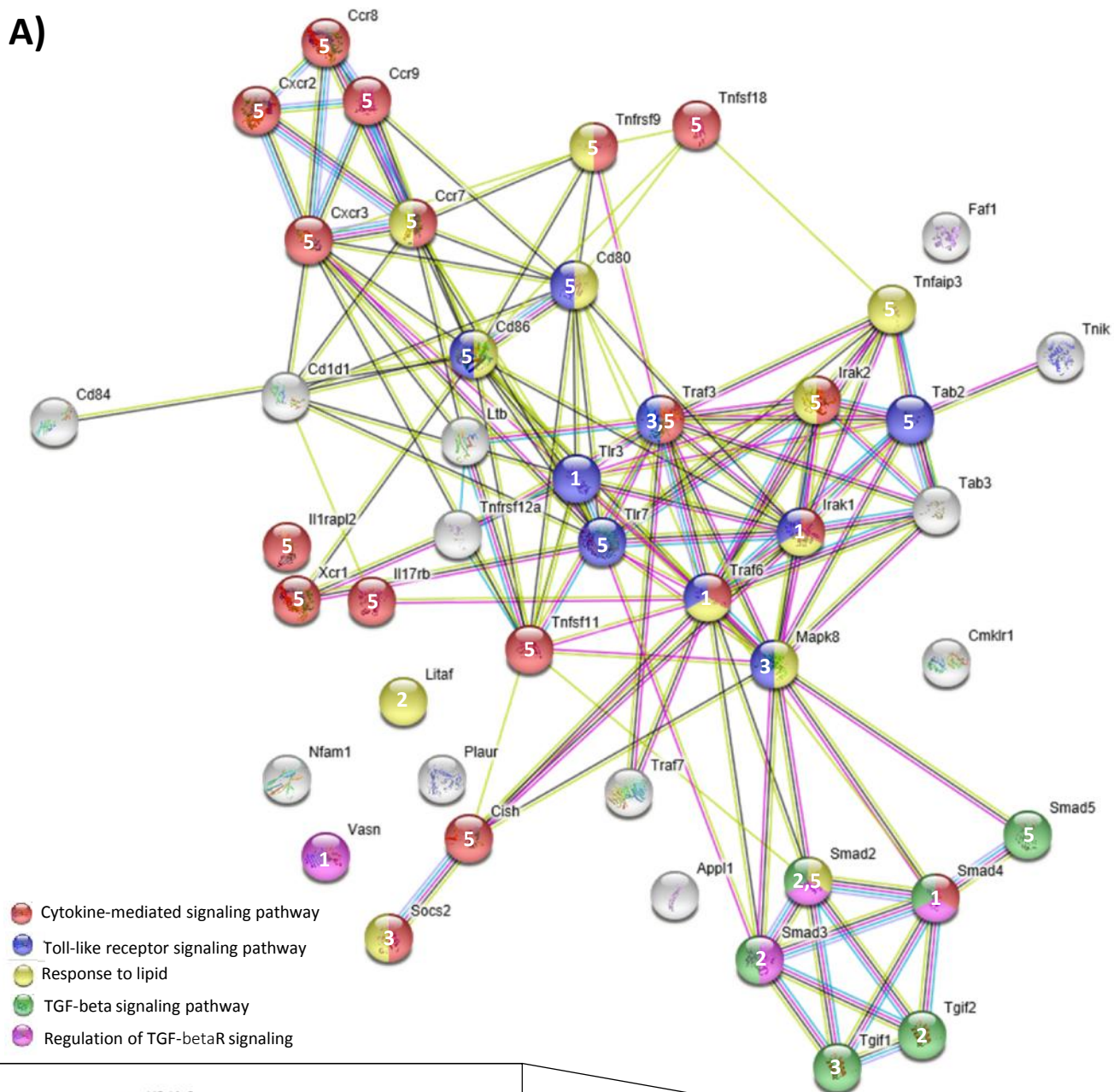
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Supplementary Figure 1

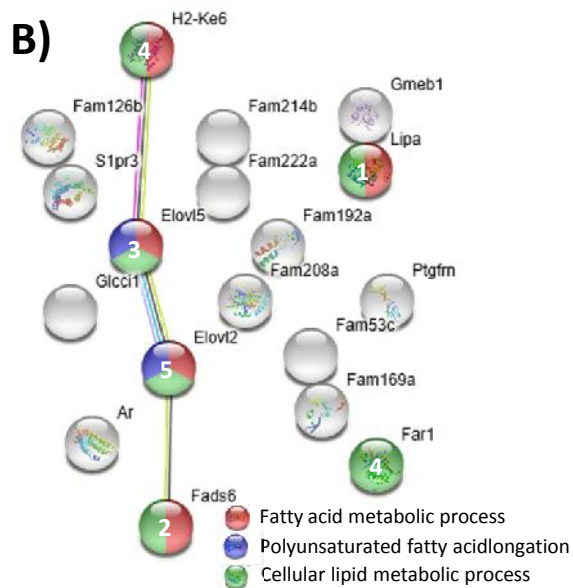


Supplementary Figure 2

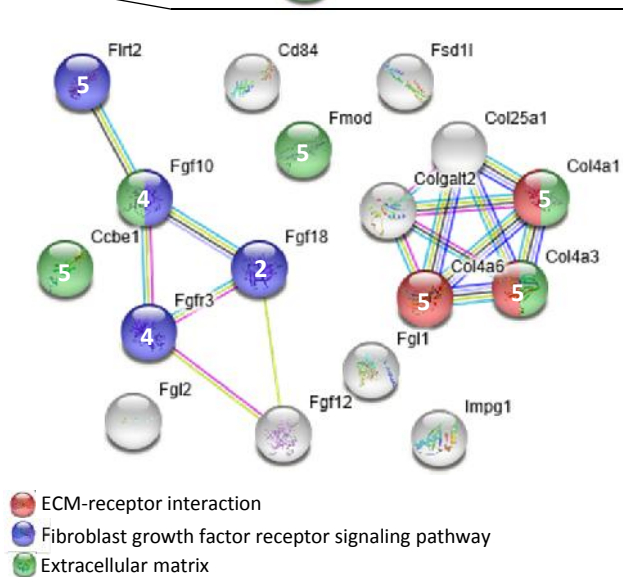
A)



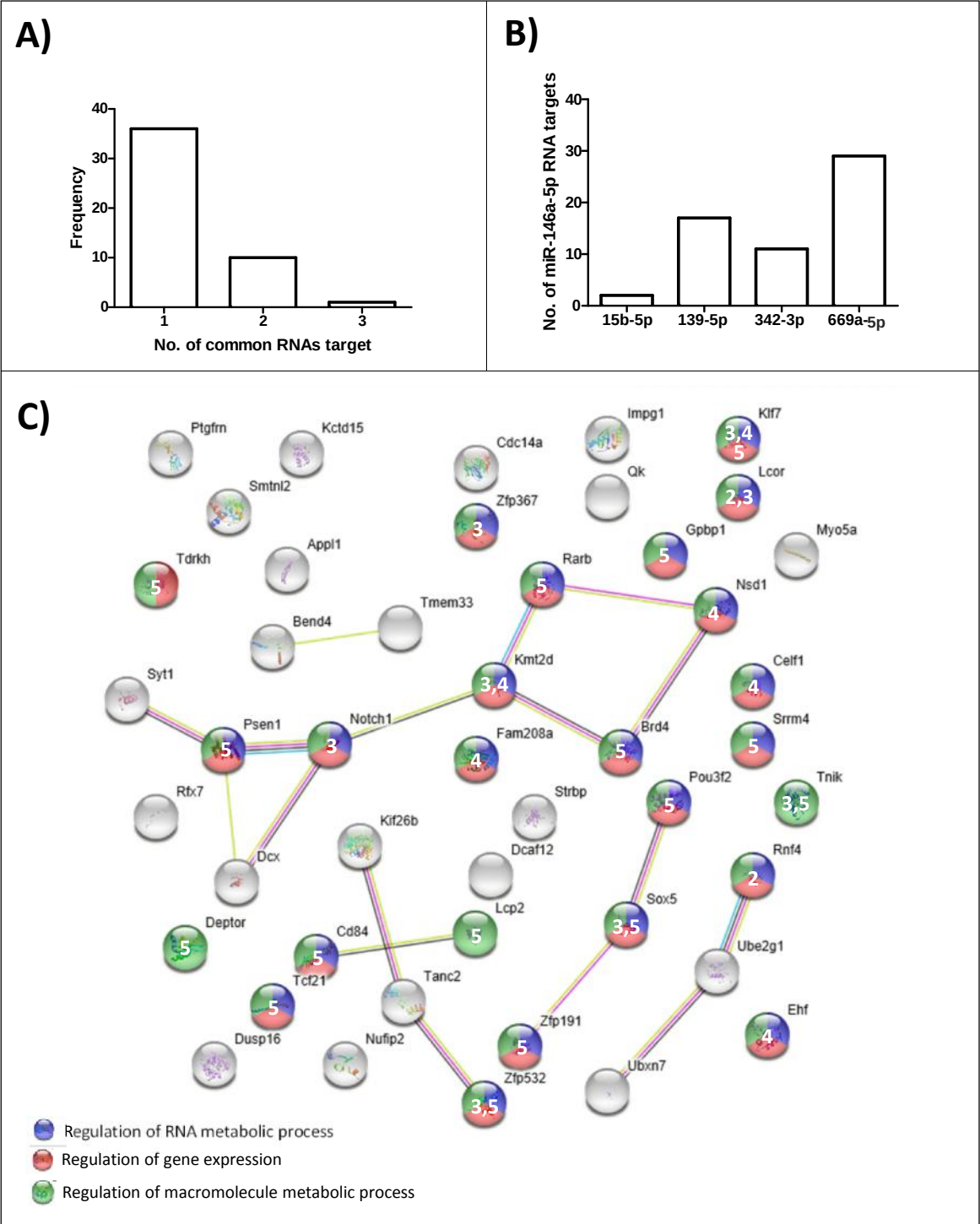
B)



c)



Supplementary Figure 3



Supplementary Figure 1: Basic quality controls of NGS miRNASeq data. A) DESeq2 normalized log transformed counts per sample are shown as box-blots. Thick bars correspond to the median, boxes to the interquartile range and whiskers to 2.5 x IQR. No significant difference in read depths were observed (ANOVA $p > 0.05$). B) hierarchical cluster analysis of all log transformed normalized counts was performed on the Eukclidean distance to identify technical outliers. Sample S55 was excluded.

Supplementary Figure 2: STRING diagram of target protein-protein interaction of the mmu-miR-146a-5p-cluster. A) LCPUFA treatment in the murine asthma model influences the regulation of proteins in A) cytokine-mediated signaling pathways and TGF-beta signaling pathway, B) in fatty acid and steroid metabolic processes and C) in extracellular matrix interaction. Significant pathway enrichments are indicated by colored bullets. Numbers in the bullets indicate the responsible miRNA (¹miR-146a-5p, ²miR-15b-5p, ³miR-139-5p, ⁴miR-142-3p, ⁵miR-669a-5p).

Supplementary Figure 3: Analysis of common targets of the miR-146a-5p-cluster. A) Frequency of miR-146a-5p targets that were also regulated by one, two or three of the other miRNAs (miR-15b-5p, miR-139-5p, miR-142-3p, miR-669a-5p). B) number of miR-146a-5p RNA targets that were also regulated by miR-15b-5p, miR-139-5p, miR-142-3p, or miR-669a-5p, respectively. C) STRING diagram of target protein-protein interaction of miR-146a-5p targets that were also regulated by at least one of the other miRNAs (miR-15b-5p, miR-139-5p, miR-142-3p, miR-669a-5p). Significant pathway enrichments for the “regulation of RNA metabolic process” (blue bullets), “regulation of gene expression” (red bullets), and “Regulation of macromolecule metabolic process” (green bullets) are shown. Numbers in the bullets indicate the responsible miRNA (²miR-15b-5p, ³miR-139-5p, ⁴miR-142-3p, ⁵miR-669a-5p).

Supplementary Table 1A

Upregulated miRNA in asthma

miRNA	log2FC	p_{adj}
mmu-miR-155-5p	1,419	6,043E-17
mmu-miR-21a-5p	0,931	1,074E-15
mmu-miR-7a-5p	1,100	4,270E-15
mmu-miR-146b-5p	0,795	2,016E-10
mmu-miR-142a-5p	1,016	7,166E-10
mmu-miR-135b-5p	2,506	6,352E-09
mmu-miR-142a-3p	0,912	1,389E-06
mmu-miR-340-5p	0,564	1,261E-05
mmu-miR-21a-3p	1,957	4,466E-05
mmu-miR-92a-3p	0,687	6,885E-04
mmu-miR-362-3p	0,785	1,708E-03
mmu-miR-652-3p	0,383	4,254E-03
mmu-miR-93-5p	0,331	6,020E-03
mmu-miR-18a-5p	0,846	7,438E-03
mmu-miR-342-3p	0,401	9,609E-03
mmu-miR-15b-5p	0,308	1,032E-02
mmu-miR-146a-5p	0,320	1,104E-02
mmu-miR-130b-5p	0,937	1,140E-02
mmu-miR-451a	0,397	1,257E-02
mmu-miR-425-5p	0,339	1,667E-02
mmu-miR-669a-5p	1,433	2,027E-02
mmu-miR-147-5p	1,921	2,407E-02
mmu-let-7i-5p	0,236	2,582E-02
mmu-miR-669l-5p	1,982	2,894E-02
mmu-miR-223-3p	0,458	2,904E-02
mmu-miR-106a-5p	1,991	2,905E-02
mmu-miR-147-3p	1,855	3,228E-02
mmu-miR-669p-5p	1,008	3,669E-02
mmu-miR-130b-3p	0,960	3,699E-02
mmu-miR-139-5p	0,471	4,385E-02

Supplementary Table 1A: NGS analysis revealed thirty upregulated miRNAs in asthmatic mice sensitized with HDM compared to control mice. Results are presented as log2FC with their respective p_{adj} -values from statistical tests of Ctrl vs. HDM (n= 3-5).

Supplementary Table 1B

Downregulated miRNA in asthma

miRNA	log2FC	<i>p</i> _{adj}
mmu-miR-125a-5p	-0,669	6,633E-06
mmu-let-7c-5p	-0,501	1,261E-05
mmu-miR-26a-5p	-0,379	1,261E-05
mmu-miR-375-3p	-0,755	1,628E-05
mmu-let-7b-5p	-0,556	1,628E-05
mmu-miR-125b-5p	-0,624	2,130E-05
mmu-miR-10a-5p	-0,601	3,503E-05
mmu-miR-1a-3p	-0,833	8,406E-05
mmu-miR-365-3p	-0,622	8,406E-05
mmu-miR-100-5p	-0,437	2,694E-04
mmu-miR-145a-3p	-0,416	2,892E-04
mmu-miR-92b-3p	-0,459	3,160E-04
mmu-let-7a-5p	-0,369	6,885E-04
mmu-miR-151-5p	-0,405	1,179E-03
mmu-let-7e-5p	-0,394	1,402E-03
mmu-miR-30c-2-3p	-0,552	3,367E-03
mmu-miR-26b-5p	-0,286	3,418E-03
mmu-miR-322-3p	-0,396	4,696E-03
mmu-miR-145a-5p	-0,368	4,732E-03
mmu-miR-1b-5p	-0,738	5,054E-03
mmu-miR-182-5p	-0,499	6,588E-03
mmu-miR-183-5p	-0,500	8,765E-03
mmu-miR-351-5p	-0,411	9,199E-03
mmu-miR-1298-5p	-0,585	1,032E-02
mmu-let-7f-5p	-0,261	1,032E-02
mmu-miR-34b-3p	-0,435	1,257E-02
mmu-miR-30d-5p	-0,223	1,538E-02
mmu-let-7d-5p	-0,295	2,027E-02
mmu-miR-30a-3p	-0,525	2,408E-02
mmu-miR-193a-5p	-0,738	3,458E-02
mmu-miR-99b-5p	-0,310	4,058E-02
mmu-miR-676-3p	-0,605	4,106E-02

Supplementary Table 1B: NGS analysis revealed thirty-two downregulated miRNAs in asthmatic mice sensitized with HDM compared to control mice. Results are presented as log2FC with their respective *p*_{adj}-values from statistical tests of Ctrl vs. HDM (n= 3-5).