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Functional importance of intra- and extracellular microRNAs and their isoforms in blood and milk

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Abstract

The discovery of miRNAs, a class of small non-coding RNA, has greatly deepened our understanding of post-transcriptional regulation of gene expression. By targeting mRNA and preventing their translation, miRNAs play a crucial role in many physiological processes. Dysregulation of miRNAs can have severe pathological effects and many disease states such as cancer are characterized by such aberrant miRNA expression profiles. Recent advances in sequencing technology unearthed a previously hidden sequence heterogeneity resulting in various isoforms of miRNA, termed isomiRs. isomiRs have subtly altered targeting behavior and display even higher specificity for their tissues of origin and the biological circumstances they were created in. Further interest, especially as biomarkers, was generated when their association to extracellular vesicles (EVs) was proven. EVs are important mediators of intercellular communication and possess the nearly unique quality of transferring RNAs, including miRNAs, between cells across tissue and even species boundaries.

So far, most miRNA expression analyses do not take advantage of the immense potential isomiRs offer for Next-Generation-Sequencing (NGS) studies of small RNAs in general and biomarker discovery in particular. To enable more investigators to perform isomiR-specific analyses, a software tool for the sensitive detection of miRNA isoforms was developed. Furthermore, a database for the identification of stably expressed reference miRNAs in EVs was established that exploits the wealth of publicly available miRNA NGS data sets and will strengthen reproducibility in EV research. Insights gained from these new bioinformatic approaches were applied to miRNAs derived from bovine milk and blood. miRNAs protected by EVs were shown to be highly stable in biofluids, and EVs in milk were able to resist gastro-intestinal conditions and were incorporated by intestinal as well as endothelial cell *in vitro*, making them prime candidates for uptake of dietary EVs. First, their potential as effectors of the “farm milk effect”, the phenomenon describing the significantly lower prevalence of asthma and allergies in children regularly consuming raw milk, was assessed. Treatment of milk by prolonged and high heat caused a drastic decline in miRNA stability and mRNA targets of affected miRNAs were involved in various

immune-related pathways relevant for asthma development or severity. To evaluate the potential of postprandial uptake of dietary EVs more directly, colostrum-derived EVs and their cargo were then characterized in their intended recipients, newborn calves. Different populations of EVs were discovered in blood and colostrum, that displayed distinct molecular composition. Blood samples taken after feeding showed significant increase in EVs bearing morphological and protein characteristics of colostral EVs. In-depth isomiR-specific analysis however revealed an endogenous origin of EV-derived miRNA and suggested different uptake mechanisms for protein and miRNA or the possibility of repackaging in intestinal cells in calves.

Zusammenfassung

Die Entdeckung von miRNA, einer Klasse von kleinen, nicht-kodierender RNA, hat unser Verständnis der post-transkriptionellen Regulation von Gen-Expression tiefgreifend verändert. miRNAs spielen eine entscheidende Rolle in fast allen physiologischen Prozessen indem sie mRNA binden und ihre Translation verhindern. Dysregulation von miRNAs kann schwerwiegende pathologische Folgen haben und viele Krankheiten wie zum Beispiel Krebs sind gekennzeichnet durch abnorme miRNA Expressionsprofile. Fortschritte in der Sequenzier-Technologie der letzten Jahre haben eine zuvor unbekannte Sequenz-Heterogenität zum Vorschein gebracht, die in Isoformen von miRNAs, genannt isomiRs, resultiert. isomiRs haben einen subtilen Einfluss auf das Bindeverhalten an mRNAs und ihre Expressionsmuster zeigen eine noch höhere Spezifizität für die Gewebe und die biologischen Umstände in denen sie gebildet wurden. Besondere Aufmerksamkeit, speziell als Biomarker, erhielten sie durch ihre Assoziation zu extrazellulären Vesikeln (EVs). EVs sind wichtige Vermittler der interzellulären Kommunikation und verfügen über die fast einzigartige Fähigkeit RNAs, inklusive miRNAs, zwischen Zellen auch über Gewebe oder Speziesgrenzen hinweg, zu transferieren.

Die meisten Expressions-Analysen in miRNAs nutzen bis heute die immensen Vorteile nicht aus, die isomiRs für Next-Generation-Sequencing (NGS) Studien im Allgemeinen und zur Entdeckung von Biomarkern im Speziellen bieten. Um mehr Forschern die Möglichkeit zu bieten isomiR-spezifische Analysen durchzuführen, wurde ein Software Programm für die sensitive Detektion von miRNA Isoformen entwickelt. Zusätzlich wurde eine Datenbank für die Identifizierung von stabil exprimierten Referenz-miRNAs eingerichtet, die sich die Vielzahl der frei verfügbaren miRNA NGS Datensätze zu Nutzen macht und einen positiven Einfluss auf die Reproduzierbarkeit in der EV Forschung haben wird. Erkenntnisse, die durch diese neuen bioinformatischen Auswertungen gewonnen wurden, wurden auf miRNAs aus bovinem Blut und Milch angewandt. Geschützt durch EVs sind miRNAs stabil in Körperflüssigkeiten und EVs aus Milch waren in der Lage der gastrointestinalen Umgebung zu widerstehen und wurden in intestinalen und endothelialen Zellen *in vitro* aufgenommen. Dies macht sie zu idealen Kandidaten zur Untersuchung der Aufnahme von EVs aus der Nahrung.

Zuerst wurde ihr Potential als Verursacher des „Bauernhof-Milch Effekts“ untersucht, das deutlich geringere Prävalenzen von Asthma und Allergien in Kindern mit regelmäßigem Rohmilch-Konsum beschreibt. Anhaltende Behandlungen mit hohen Temperaturen verursachten einen starken Rückgang der miRNA Stabilität und für mRNA Ziele betroffener miRNAs konnte eine Beteiligung an mehreren immun-relevanten Vorgängen mit Relevanz für die Entwicklung von Asthma und seinem Schweregrad gezeigt werden. Um die Möglichkeit einer postprandialen Aufnahme von EVs aus der Nahrung direkter zu untersuchen, wurden EVs aus dem Kolostrum und ihr molekulare Ladung eingehend in neugeborenen Kälbern charakterisiert. Unterschiedliche Populationen von EVs aus Blut und Kolostrum konnten identifiziert werden. Blutproben, die nach der ersten Fütterung genommen wurden, zeigten einen signifikanten Anstieg an EVs, die morphologische und Protein-Charakteristika von kolostralen EVs aufwiesen. Ausführliche, isomiR-spezifische Analysen deckten jedoch einen endogenen Ursprung der miRNA aus EVs und deuten auf abweichende Aufnahme-Mechanismen für Protein und miRNA or die Möglichkeit eines Umpackens in intestinalen Zellen in Kälbern hin.

List of abbreviations

ADAR	adenosine deaminase acting on RNA
AGO	Argonaute protein
ARF6	ADP-ribosylation factor 6
ATP	adenosine triphosphate
BTN1A1	butyrophilin
CDAR	cytidine deaminase acting on RNA
CNX	calnexin
DCP2	mRNA-decapping enzyme 2
DGCR8	DiGeorge syndrome critical region 8
DND1	deadend 1
eIF	eukaryotic initiation factor
ESCRT	endosomal sorting complex required for transport
EV	extracellular vesicle
EXP5	exportin 5
HSC70	heat shock cognate 70
HSP70	heat shock protein 70
HSP90	heat shock protein 90
IFNG	interferon γ
IFNGR	interferon γ receptor
IL6	interleukin 6
ILV	intra-luminal vesicle
isomiRROR	isomiR – references, overviews and readcounts
Linc-MD1	long intergenic non-protein coding RNA, muscle differentiation 1
lncRNA	long non-coding RNA
MFGE8	lactadherin, milk fat globule-EGF factor 8
MHC	major histocompatibility complex
miREV	miRNA References of Extracellular Vesicles
miRNA	microRNA
MISEV	minimal information needed for studies of extracellular vesicles
mRNA	messenger RNA

MVB	multi-vesicular body
NGS	next-generation sequencing
nt	nucleotides
NTA	nanoparticle tracking analysis
ORF	open reading frame
PABPC	poly(A)-binding protein complex
PACT	protein activator of the interferon-induced protein kinase
PARN	poly(A)-specific ribonuclease
PCA	principal component analysis
PD1	programmed cell death protein 1
PLD2	phospholipase D2
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
PTEN1	phosphatase and tensin 1
PTGS2	prostaglandin-endoperoxide synthase 2
PUFA	polyunsaturated fatty acids
RAN-GTP	RAs-related nuclear protein Guanosine-5’-triphosphate
RBP	RNA-binding protein
RISC	RNA-induced silencing complex
RT-qPCR	real-time quantitative polymerase chain reaction
SAM	sequence alignment map
SDG	sucrose density gradient
SMase	neutral sphingomyelinase
SNARE	soluble N-ethylmaleimide-sensitive fusion attachment protein receptors
snoRNA	small nucleolar RNA
STAT3	signal transducer and activator of transcription 3
TARBP	transactivation-responsive RNA-binding protein
TEM	transmission electron microscopy
tRNA	transfer RNA
TUT	terminal uridylyl transferases
Xrn1	5’-3’ exoribonuclease 1

1. Introduction

1.1 microRNAs and their isoforms – major players of gene regulation

microRNAs (miRNAs), a class of small non-coding RNAs of approximately 18-25 nucleotides (nt), have garnered great interest in the scientific community since their discovery in 1993 [1]. Originally described as an idiosyncrasy during the larval development of the nematode *Caenorhabditis elegans*, miRNAs and their involvement in post-transcriptional regulation of gene expression were since found to be conserved in almost all species including mammals, which lead to their recognition as a formal class of RNA in the early 2000s [2]. Although its main modes of biogenesis and subsequently most likely genomic locations were identified early [3,4], progress in novel miRNA discovery shows no signs of abating even to this day. The most comprehensive miRNA registry, miRBase [5,6], now lists 48,860 mature miRNA sequences distributed over 271 organisms, with the human and bovine genome comprising 2,654 and 1,025 sequences by themselves, respectively. Furthermore, recent advances in high-throughput sequencing unveiled a sequence and length heterogeneity of mature miRNAs far exceeding initial expectations [7]. Origin of these isoforms of miRNA, termed isomiRs, lies mainly within divergent processing of key enzymes (Drosha and Dicer) during miRNA biogenesis [8].

1.1.1 Biogenesis of microRNAs and the deviations therein that lead to isoforms

Although miRNA sequences were found to be highly conserved between different species, especially in mammals [9], locations of miRNA genes exist in a multitude of genomic contexts. Roughly 80% of all mammalian miRNA loci are situated in intronic regions, displaying a more or less equal distribution between coding and non-coding transcripts [10]. Furthermore, approximately 10% of all miRNA sequences can be found in exonic regions of non-coding transcripts with classification also depending on alternative splicing patterns in some cases. Transcription patterns of miRNAs and their corresponding host genes suggest parallel transcription of both, albeit biogenesis of exonic and intronic miRNA is most likely following different mechanisms [4]. miRNA genes were shown to have multiple transcription start sites and even individual promoters were found for certain intronic miRNAs [11]. Frequently, miRNA sequences

are clustered in close proximity to each other, composing poly-cistronic transcription units, where miRNAs are mainly co-transcribed [12]. Metazoan genomes are rife with events of gene duplications in miRNA genes, leading to not only multiple separate loci of the same miRNAs but also to the formation of miRNA families consisting of numerous, paralogous sequences [13]. miRNAs within these families are thought to act in a redundant fashion due to their mostly identical sequence but diverging expression patterns of paralogous miRNAs and new insights in miRNA binding mechanisms suggest a distinct role as well [14].

miRNA genes are dominantly transcribed by RNA polymerase II into primary miRNA (pri-miRNA) with only a minor group of miRNAs associated with Alu repeats being transcribed by RNA polymerase III [15]. pri-miRNAs are comprised of one or more local stem loops containing the mature miRNAs and flanking sequences of hundreds to thousands of nucleotides [16]. Still in the nucleus, pri-miRNA is cleaved into precursor miRNA (pre-miRNA) by the microprocessor complex consisting of RNase III Drosha and its co-factor DiGeorge syndrome critical region 8 (DGCR8) in humans [17]. Cleavage sites for Drosha are predominantly determined by the junction of single-stranded and double-stranded RNA at the base of the stem loop and generally produce a 2 nt overhang at the 3’ end [18]. Although most pri-miRNA are cleaved 11 bp away from the basal junction, it was shown that the junction at the terminal loop [19] as well as sequence elements located near both junctions can have a major influence as well [20], leading to alternative Drosha processing of pre-miRNAs and ultimately isomiRs with varying 5’ and 3’ ends [21]. Trimmed of their flanking single-stranded sequences pre-miRNAs are then exported in a energy dependent manner by Exportin 5 (EXP5) with the help of RAs-related nuclear protein Guanosine-5’-triphosphate (RAN-GTP) [22] into the cytoplasm where they undergo final maturation.

In the cytoplasm, Dicer primarily recognizes the 3’ overhang of normally 2 nt, produced by the staggered cleavage of Drosha, with the help of transactivation-responsive RNA-binding protein (TARBP) or protein activator of the interferon-induced protein kinase (PACT) and excises the terminal loop of the pre-miRNA at a fixed distance (~ 22 nt) from the 3’ end [23]. Although this behavior is likened to a ‘molecular ruler’, it is not absolute. Thermodynamically unstable pairings at the end of the stem loop can cause

Dicer to cleave in relation to the 5’ end and not the 3’end [24], while the loop sequence [25,26] and even single nucleotide bulges in the stem [27] can affect specificity of the cleavage site and consequently cause the creation of isomiRs. Similarly, TARBP and PACT not only facilitate higher processing efficiency of Dicer, they can also modulate lengths of mature miRNAs by altering the cleavage site of Dicer [28]. The resulting miRNA duplex is then loaded into an Argonaute protein (AGO1-4) where it is unwound and one of the strands, termed guide strand, is selected to form the RNA-induced-silencing-complex (RISC), the effector complex of miRNA induced RNA interference. Loading of AGO proteins with dsRNA is mediated by a chaperone complex, consisting of heat shock cognate 70 (HSC70) and heat shock protein 90 (HSP90) under consumption of adenosine triphosphate (ATP) [29]. In contrast, unwinding of the double-stranded miRNA duplex is ATP-independent and most likely driven by conformational changes when AGO reverts after opening by the HSC70-HSP90 chaperone [30] and promoted by mismatches at central positions (nt 2-8 as well as 12-15) [31]. Removal of the unrequired strand, termed passenger strand, can also occur by cleavage when the miRNA duplex is perfectly matched at central positions and loaded onto a slicing-competent AGO, i.e. AGO2 in humans, although these events tend to be rare (Figure 1) [32].

The decision which “arm” of the miRNA duplex (Figure 2, box upper right corner) matures into the final guide strand is based mainly on the difference in thermodynamic stability between its 5’ and 3’ end. An unstable terminus at the 5’ end is usually favored for retention in AGO proteins as guide strand with an additional preference for uracil or adenosine at the starting position [33]. Originally thought to be pre-determined for every pre-miRNA, guide strand selection was shown to be dynamic with events of this so called “arm-switching” occurring between different species, sexes, tissues as well as developmental stages and pathophysiological conditions [34–36]. In part, arm-switching events are thought to be caused by altered thermodynamic stabilities of miRNA [21]duplexes due to isomiRs caused by alternative Drosha and Dicer processing.

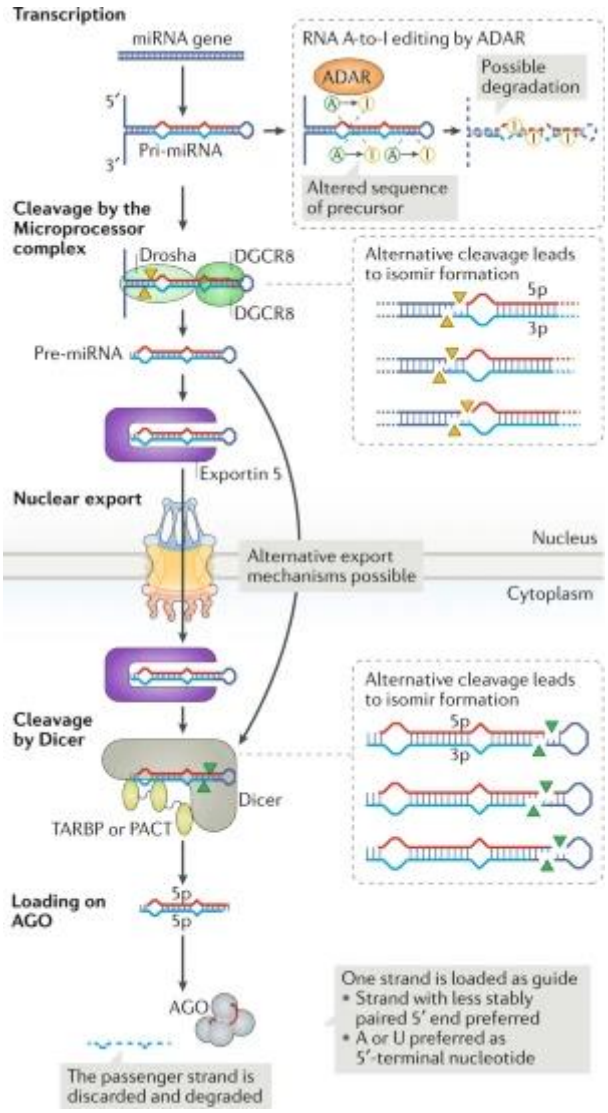

Figure 1: Canonical miRNA biogenesis pathway including deviating processing patterns that lead to isomiR formation. After transcription, primary miRNAs are trimmed to hairpin-forming precursor miRNAs and exported into the cytoplasm. Subsequently, precursor miRNAs undergo further trimming to excise their loop before loading of the guide strand into the effector protein complex. miRNA: microRNA; Pri-miRNA: primary miRNA; ADAR: adenosine deaminase acting on RNA; DGCR8: DiGeorge syndrome critical region 8; Pre-miRNA: precursor miRNA; TARBP: transactivation-responsive RNA-binding protein; PACT: protein activator of the interferon-induced protein kinase; AGO: Argonaute protein. Blue passenger strand of miRNA duplex loaded into AGO mislabeled as 5p instead of 3p. Figure reprinted from Gebert and MacRae [37]

Apart from the canonical pathway of biogenesis, Drosha- and Dicer- independent maturation of miRNAs have been discovered through the use of deficient cells. Drosha processing can be bypassed by a number of non-canonical biogenesis pathways leading to pre-miRNA like hairpin sequences that can be recognized by Dicer. pre-miRNA of certain mirtrons, miRNAs derived from intronic loci, can be directly generated

during splicing of their host messenger RNA (mRNA) [38]. After the spliceosome creates the intron lariat, a hairpin structure is formed by debranching and refolding. Excess nucleotides on either 5’ or 3’ end are then trimmed by exonucleases leading to canonical pre-miRNAs [39]. In other cases, suitable short hairpin structures are directly transcribed by polymerases which can then be recognized by Dicer [40]. Depending on their sequences and hairpin structures, substitute transporters from the nucleus are employed (e.g. Exportin 1) and in case of Pol II transcription, only miRNAs from the 3’ end are selected as guide strands, most likely due to the 5’ cap blocking AGO loading [41]. Completing the Drosha-independent miRNA repertoire is the ability of Dicer to use fragments of other small non-coding RNAs such as transfer RNAs (tRNAs) or small nucleolar RNAs (snoRNAs) and even viral RNAs as miRNA substrates [42,43]. Requirement for efficient Dicer processing is in all cases a short hairpin sequence with an overhang of at least two nucleotides at the 3’ end, making extension of shorter pre-miRNAs necessary. For instance, pre-miRNAs of the highly abundant let-7 family only possess a one nucleotide overhang necessitating mono-uridylation by terminal uridylyl transferases (e.g. TUT7) [44].

Although the majority of isomiRs are created by shifts in the cleavage sites of Drosha and Dicer during miRNA maturation, trimming or adding of nucleotides on miRNA 3’ ends by exonucleases or nucleotidyl transferases are common occurrences [45]. Allowing the formation of sequences diverging from from pri- and pre-miRNA sequence templates, this further increases isomiR heterogeneity [7]. On the other site, 5’ isomiRs tend to be rare as 5’ ends are protected by their tight binding in the AGO pocket and because alterations of the seed sequence have major consequences on miRNA target behavior in general [7]. In contrast to 5’ and 3’ end divergences by nucleotide trimming or addition, polymorphic isomiRs are the product of RNA editing prevalently facilitated by deamination. By removing an amino group, adenosine deaminase acting on RNA (ADAR) can convert adenosine to inosine, which in turn shows binding capacity for all four basic nucleotides with a slight preference for cytosine followed by adenine [46]. Less frequently, cytidine deaminase acting on RNA (CDAR) transforms cytidine to uracil, leading to further changes in targeted complementary sequences (Figure 2) [47].

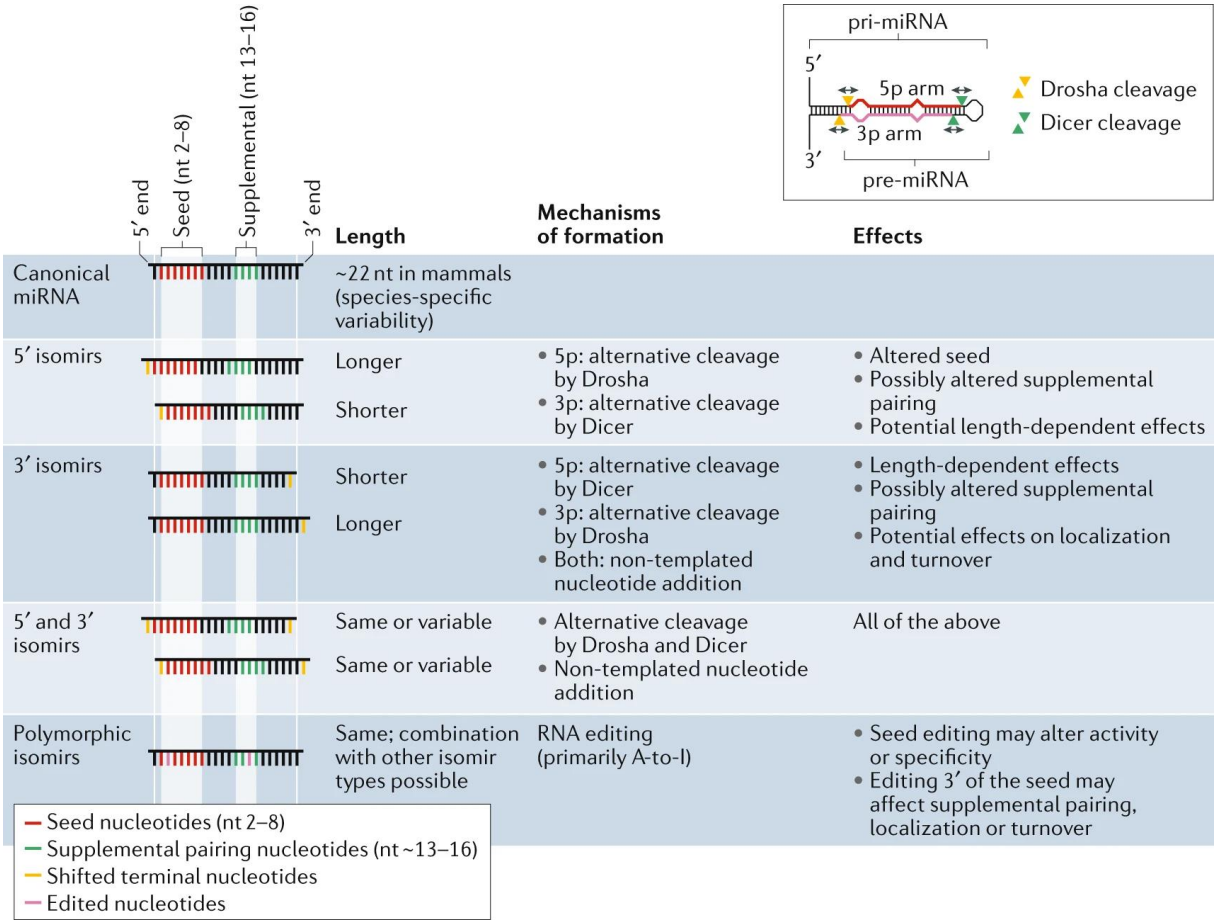

Figure 2: Classes of miRNA isoforms and their mechanisms of formation. isomiRs are characterized by their deviations from their canonical template sequence on either 5p or 3p end or by existence of nucleotide editing. Figure reprinted from Gebert and MacRae [37].

1.1.2 Post-transcriptional regulation of gene expression by microRNAs

Since their discovery, miRNAs have been found to regulate nearly every physiological pathway and an overwhelming number of pathological processes can be associated with their dysregulation including various cancers, heart diseases and neurological disorders [48]. Through prediction of target sites in the genome, it is thought that over 50% of the human transcriptome is directly under the influence of miRNAs [49]. miRNAs convey their effect mostly by binding to complementary target sites in the 3'-untranslated region (3' UTR) of mRNAs and causing translational repression by subjecting their target mRNAs to degradation [49]. Without the interference of RISCs, translation is typically initiated by circularization of mRNAs. Poly-A-tails at the 3' end of mRNAs are recognized by the aptly named poly(A)-binding protein complex

(PABPC) while the m^7G cap at the 5' end of mRNAs binds the multi-protein complex eukaryotic initiation factor 4F (eIF4F). eIF4G, a sub unit of eIF4F, can then interact with PABPC to close the loop and promote successful recruitment of the 43S pre-initiation complex, ultimately leading to full formation of ribosomes [50]. Mature miRNAs bound in AGO proteins can disrupt this process with the help of GW182 proteins, named after their abundant glycine-tryptophane repeats. Recognition of target mRNAs by miRNA is facilitated by partial sequence complementary in general and driven by Watson-Crick pairing of the seed sequence (nucleotide 2-7/8 from 5' end) [49]. By binding to the 3' UTR, AGO directs GW182 to the PABPC, causing release of eIF4G as well as deadenylation via the CAF1–CCR4–NOT deadenylase complex [51]. Silenced mRNAs are then primed for degradation through 5'-3' exoribonuclease 1 (Xrn1) by decapping of the m^7G cap by mRNA-decapping enzyme 2 (DCP2) and its co-factors [52]. Although studies have found that over 75% of changes in protein synthesis caused by miRNAs can be explained by mRNA degradation [53], silencing of mRNAs can also be attributed to prevention of initialization, prevention of progression of translation at ribosomes or, in case of perfect sequence pairing, direct endo-nucleolytic cleavage [54]. Even though direct cleavage of mRNAs is the preferred modus operandi in plants, it is a rare event in animal cells with only one of the AGO proteins in humans retaining endonuclease capacity (AGO2). In stark contrast to their translation inhibition competence, miRNAs have also been shown to promote protein synthesis under specific conditions [55]. These events however are confined to limited cases and further studies are needed to capture the extent in which miRNAs are positively regulating their targets (Figure 3).

Despite all the progress made in elucidating miRNA function, predicting targeting sites in of miRNAs remains a major challenge. While initial studies suggested that binding of a 6mer (nucleotide 2-7) from the 5' end of miRNAs to 3' UTR of mRNAs functions as the sole driver of miRNA function in mammals, recent advancements in prediction algorithms have shed new light on miRNA targeting behavior. Efficacy of miRNA repression is raised significantly when targeting sites in mRNAs include a 7^{th} Watson-Crick pairing at position 8 or, independent of miRNA sequence, an adenosine opposite position 1 of the miRNA [56]. Further cementing the importance of these extended targeting sites, is the observation that their effect is cumulative, meaning that highest

repression of mRNAs is achieved by targeting 8mer sites starting with adenosine and displaying perfect complementarity for nucleotides 2-8 of the miRNA. The primacy of the seed region in determining mRNA targets can be explained in part by the conformational topography of AGO proteins that pre-organize the 5' end in a A-form helix fashion that promotes Watson-Crick pairing [57]. This would present nucleotide 2-8 in a readily accessible fashion to the mRNA while simultaneously preventing nucleotide 1 from binding, giving a reasoning as to why complementarity at this position is inconsequential. In addition, pairing of the seed sequence can be supplemented and

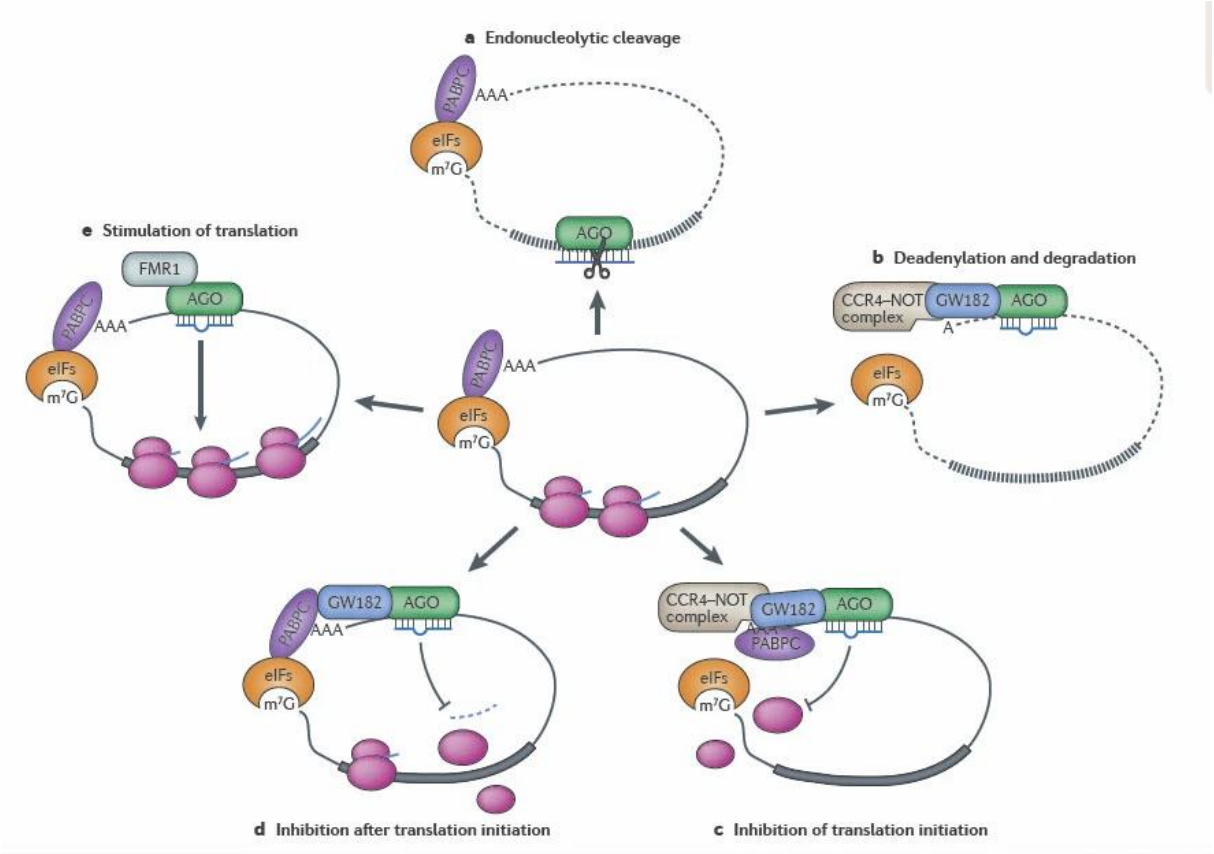

Figure 3: Regulation of mRNA translation by miRNAs. Ribosome recruitment is promoted by circularization of mRNAs through interaction of PABPC and eIFs among each other as well as their respective mRNA termini. Full sequence complementarity of miRNAs can lead to direct cleavage of target mRNAs by endonucleolytic competent AGO proteins (a). Most frequently, target mRNAs are identified by partial complementarity of miRNAs in their seed sequence. mRNAs are commonly subjected to degradation after disassociation of PABPC through GW182 and subsequent deadenylation by the CAF1-CCR4-NOT complex (b), although events of initiation blocking (c) or ribosomal drop-off (d) have been described as well. Under specific conditions, translation of target mRNAs is promoted instead of inhibited with the help of FMR1. EIFs: eukaryotic initiation factors; PABPC: poly(A)-binding protein complex; AGO: Argonaute protein; CCR4-NOT complex: Carbon Catabolite Repression-Negative On TATA-less; FMR1: fragile X mental retardation protein 1. Figure reprinted from Pasquinelli [58].

even compensated in part by extensive interaction at the 3' end of miRNAs although the frequency of these occurrences appears to be low. Supplementary pairing of nucleotides centered around position 13-17 is thought to modestly enhance target recognition [56] but extending complementarity to 9 or more adjacent nucleotides was shown to be able to compensate singular mismatches or bulges in the seed sequence [59].

Efficiency of miRNA repression is not only dependent on targeting site sequence but also on their location and abundance. Complementary binding sites can not only be found in 3' UTRs but occur within open reading frames (ORF) and 5' UTRs as well, albeit much less frequent and less effective in transcriptional regulation [56]. Anchored only by partial binding of ~7 nt, RISCs targeting these sites can easily be displaced by the translational machinery [57]. Corroborating this notion, is the observation that effective target sites start accumulating not at the stop codon but around 15 nt into the 3' UTR, coinciding with the length of RNA that enters the ribosome before the stop codon reaches its active center [56]. Evolutionary conserved target sites within 3' UTRs are not only eschewing this so-called ribosomal shadow but tend to accumulate in regions where accessibility for the miRNA silencing complex is not hindered by secondary structures. This includes in general regions not located in the center of the UTR and sequences with high AU content [56]. Expanding this intricate network that determines efficiency of post-transcriptional regulation is the fact that mRNA in general have a multitude of different target sites for many separate miRNA families on one hand and miRNAs are able to target a multitude of different mRNAs on the other hand. A study in 2009 found that on average a mammalian miRNA family has 300 conserved target sites upon which they can exert their regulatory effect [59]. Impact of multiple target sites in one mRNA is generally multiplicative, greatly increasing pressure that can be applied to single transcripts [60]. Target sites in close proximity (~40nt) of each other, strengthen this cooperative effect even further, making it possible for even small changes in miRNA expression to have a profound impact [61].

Adding another layer to this already impressive network of regulation, are opposing factors that inhibit silencing by RISCs. RNA-binding proteins (RBP) like HuR that binds to AU-rich sequences in 3' UTR can compete with miRNAs over targeting sites.

Normally located in the nucleus, HuR is exported to the cytoplasm under stress conditions and can reverse the fate of mRNAs bound for degradation [62]. Similarly, deadend 1 (DND1), a RBP expressed in primordial germ cells, identifies sequences in the vicinity of target sites and can alleviate translational inhibition of certain mRNAs by impeding accessibility for AGO [63]. Cells can also relieve pressure from repressive miRNAs by expressing transcripts with matching target sites in an attempt to titrate miRNAs from specific mRNAs. This target mimicry was realized in cell culture experiments by introducing “miRNA sponges”, RNAs highly enriched in target sites for specific miRNA families, long before researchers became aware of the similar potential of endogenous transcripts [64]. Endogenous examples include pseudogenes like phosphatase and tensin 1 (PTEN1) that acts as mRNA decoy for tumor suppressor gene PTEN [65] as well as members of the heterogeneous group of long non-coding RNAs (lncRNA) such as long intergenic non-protein coding RNA, muscle differentiation 1 (linc-MD1), which guides timing in muscle differentiation [66].

1.1.3 Influence of isomiRs on microRNA turnover and targeting behavior

Originally thought to be rare, inconsequential transcripts or sequencing artifacts, the functional importance of isomiRs was demonstrated by a number of studies in recent years. isomiRs with deviating sequences on 5’ or 3’ ends, 5’-isomiRs and 3’-isomiRs respectively, are loaded on AGO proteins correctly and form fully functional RISCs [67]. Indeed, significant changes in isomiR expression profiles could be detected between different developmental stages [68], populations [69], genders [70] and even cell lineages [71]. Furthermore, specificity of isomiR distribution patterns was shown for a variety of pathological conditions and diseases including cancer [72,73], degenerative diseases [74,75] and other proliferative [76] or viral diseases [77]. It was shown that isomiRs and canonical miRNAs cooperatively control common regulatory networks by either increasing specificity of targeting for the same mRNA or by expanding the number of affected mRNAs within the same biological pathway [78]. Even though 5’-isomiRs have altered seed sequences for mRNA targeting, they still display an overlap with their canonical miRNAs in their target repertoires and are thought to have evolutionary importance [79]. Consequentially, isomiRs were proposed to be major factors in fine-tuning miRNA interference by increasing the signal to noise ratio of

mRNA targeting [78] which makes them ideal candidates for biomarker detection (Figure 4).

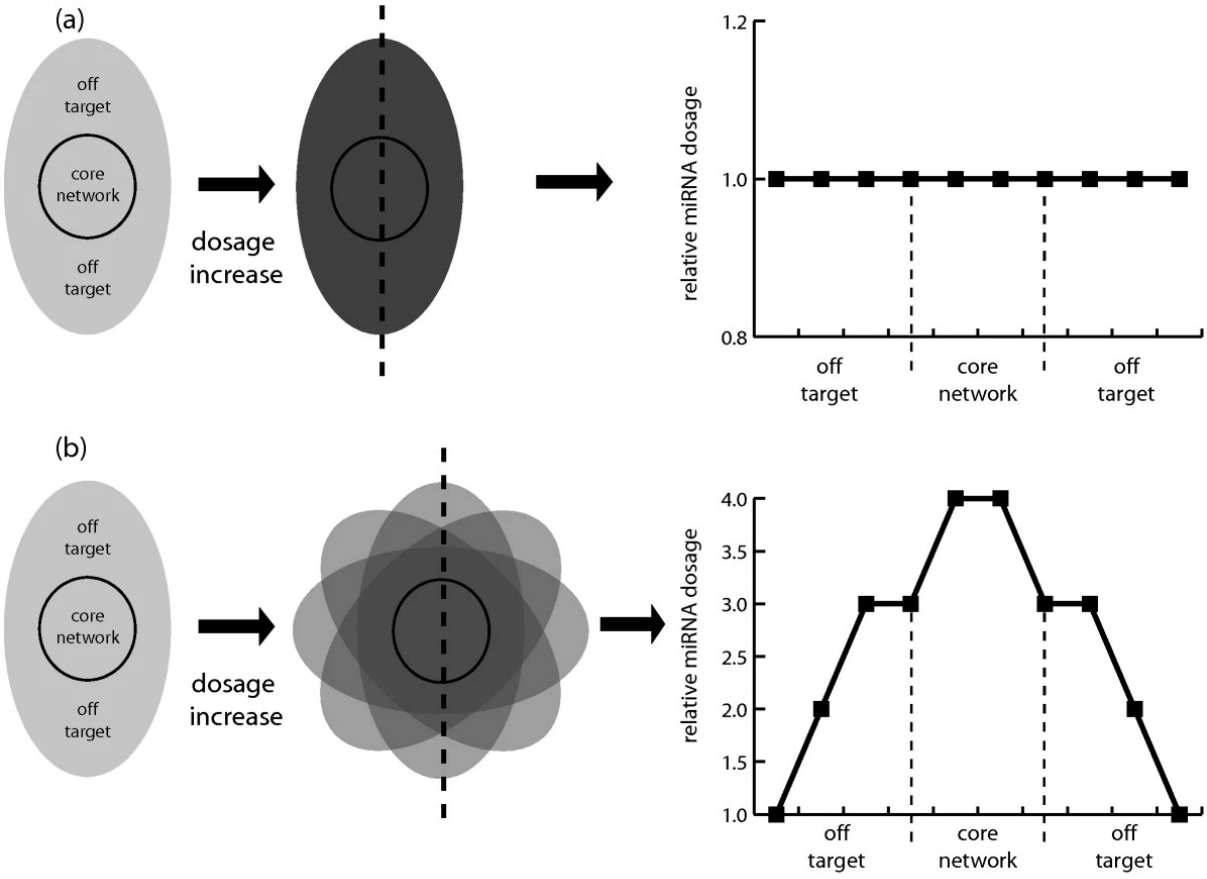

Figure 4: isomiRs can alleviate translational repression on off targets while maintaining pressure on regulation of core networks. Through slights shifts in targeted mRNAs, combined regulation of isomiRs and canonical miRNAs are thought to avoid uniform increase in translational inhibition that would be otherwise inevitable without sequence deviations (a). Overlaps in targetomes allows fine-tuning of expression regulation for specific targets without overburdening repression on the remainder of mRNAs (b). Figure reprinted from Cloonan et al. [78].

Precisioning of mRNA targeting by isoforms of miRNA is enabled by a number of factors. As already mentioned, trimming or addition on 5' end of miRNAs will shift seed sequences and alter mRNA targets. Furthermore, 5' nucleotides play an important role during AGO loading and 5' isomiRs can cause arm switching and therefore decrease abundance of certain miRNAs in RISCs [21]. 3' and polymorphic isomiRs on the other hand can be directly factored in supplementary or compensatory binding, generating subtle changes in targeting and repression efficiencies [56,59]. Translational

repression can also be hindered by transitions in cellular localization. For instance, nuclear import of mature miRNAs is triggered by a short hexamer sequence motif and its introduction into isomiRs by RNA editing is easily conceivable, which in turn would remove miRNAs physically from their traditional targets and at the same time present them to new mRNAs [80]. In addition to spatial separation, temporal expression patterns can achieve similar repression modulation by in- or decreasing miRNA turn over times and decay rates. It was shown that replacement of 5' terminal adenosines with uridin, enhances miRNA stability and abundance [81]. Due to their significantly higher occurrence, sequence variations at the 3' end are thought to play an even bigger role in miRNA stability. 3' isomiRs with adenylation or uridylation by terminal nucleotidyl transferases are among the most common sequence modifications in mammals [82]. While generally adenylation is linked to higher miRNA stability, uridylation seems to amplify miRNA decay [83]. This is consistent with studies in which deadenylation by 3'-5' exoribonucleases such as Nibbler or poly(A)-specific ribonuclease (PARN) destabilizes miRNAs and is accompanied by decreased cellular expression levels [84]. Although sequence length variations can be associated with miRNA stability, it is also thought to be an intermediate step to ensure optimal miRNA size for AGO loading with multiple additions and trimming events needed [85,86].

1.2 Extracellular vesicles – potent vehicles of intercellular communication

Similar to miRNAs, research in recent year has shed a new light on extracellular vesicles (EVs) and completely transformed our understanding of their heterogeneous populations, biogenesis and cargo composition. EVs are categorized as small membrane-contained vesicles that are released from nearly all cell types *in vitro* and *in vivo*. Release of EVs is a remarkably conserved mechanism and examples of EVs can not only be found in higher eukaryotes but also in single cell organisms like prokaryotes [87]. Earliest studies of EVs include the observation of coagulation promoting effects of platelet-derived particles in 1946 [88] as well as vesicles involved in bone calcification in 1969 [89]. The discovery of a new and more complex release mechanism for EVs in the form of fusion of multi-vesicular bodies (MVBs), containing intraluminal vesicles termed exosomes, with the cell membrane in 1983 further expanded the repertoire of EV populations [90]. At first thought to be a sole method of

disposal of unwanted cell parts, Raposo et al. revealed in 1996 the role of exosomes in immune response mediation and cell-to-cell communication in general [91]. Nevertheless, it took until 2007 for EV-related research to gain substantial momentum again, with the detection of functional RNAs in EVs, including miRNAs, which hinted at new possibilities in intercellular communication as well as their potential as biomarker sources or therapeutic targets [92]. A major hurdle in the ongoing quest to elucidate the definitive mode of action of EVs and their cargo, remains the isolation of specific subpopulations of EVs due to their overlapping physico-chemical features and surface markers [93].

1.2.1 Classes of extracellular vesicles

Although EVs encompass a broad variety of different vesicle subtypes, in general they can be categorized in one of three major groups: exosomes, microvesicles and apoptotic bodies. Transitions between these groups in size as well as other morphologies are fluent, however significant differences can be found in their biogenesis, membrane composition including surface proteins, molecular cargo and ultimately their biological function (Table 1).

Type and Size	Biogenesis	Markers	Contents
Exosomes (40-130 nm)	Endolysosomal pathway. Release by exocytosis of multivesicular bodies	Tetraspanins (CD63, CD9, CD81), Alix, TSG101, Hsp60, Hsp70, Hsp90	miRNA and mRNA; lipids (cholesterol, ceramide, sphingomyelin), cytokine receptors, MHC molecules
Microvesicles (100-1000 nm)	Cell surface. Outward budding of plasma membrane.	Integrins, selectins, metalloproteinases, Phosphatidyl-serine	mRNA, non-coding RNAs, membrane receptors, cytoplasmic proteins (cytokines)
Apoptotic bodies (50-5000 nm)	Cell surface. Release from cellular blebs during apoptosis	Phosphatidyl-serine	Nuclear fractions, cell organelles, DNA, rRNA, mRNA

Table 1: Characteristics of the three main classes of extracellular vesicles in terms of biogenesis, molecular markers and general content. Hsp: heat shock proteins; MHC: major histocompatibility complex; TSG101: tumor susceptibility gene 101. Reprinted from Perez-Hernandez et al. [94]

Differing from exosomes and microvesicles, apoptotic bodies, also called apoptotic vesicles or apoptotic blebs, are not so much secreted as released from the plasma membrane in an event called cellular blebbing during programmed cell death [95]. Cells destined for apoptosis will undergo a series of profound changes, starting with a general shrinking of the plasma membrane, accompanied by complete fragmentation of cellular organelles and compartments, including the nucleus. As the disintegration of the cytoskeleton progresses, cytoplasm flow results in the formations of irregular protrusions of the now unsupported plasma membrane and eventually their fission as apoptotic bodies. Due to the rather unregulated nature of blebbing, size and shape of apoptotic bodies are highly variable, ranging from 50 nm to 5 µm and can therefore be much larger than exosomes or microvesicles [94]. Similarly, uptake of cellular content in apoptotic bodies is an undirected process and culminates in a random assortment of nuclear fraction, broken down organelles as well as degraded DNA and RNA. Accordingly, defining characteristics of apoptotic bodies are sparse but luckily not nonexistent. Since the ultimate fate of all apoptotic bodies is elimination by macrophages, pro-phagocytic signals in the form of phosphatidylserine are heavily enriched in the outer membrane leaflets and can therefore be used as a marker of differentiation [95].

While microvesicles, also named ectosomes, microparticles or shedding vesicles, originate from the plasma membrane like apoptotic bodies, their release into the extracellular space and the resulting EVs share few similarities. Shedding of microvesicles occurs in viable cells and can be induced by external stimuli such as Ca^{2+} [96], phorbol esters [97] or hypoxia in tumour cells [98]. Outward budding of nascent microvesicles is restricted to specific sites on the membrane, characterized among others by oligomerization of cell surface receptors [99], but specific mechanisms seem to be dependent on the cell of origin. Some cells seemingly involve the same proteins to generate membrane protrusions and pinching of vesicles as exosome formation, explained in more detail below, namely parts of the endosomal sorting complex required for transport (ESCRT) machinery with the exception of ESCRT-0 [100] whereas others possess an unique mode of shedding (Figure 5). For instance, platelet-derived microvesicles, one of the earliest types of microvesicles described, are formed by cleavage of cytoskeleton proteins by protease calpain and

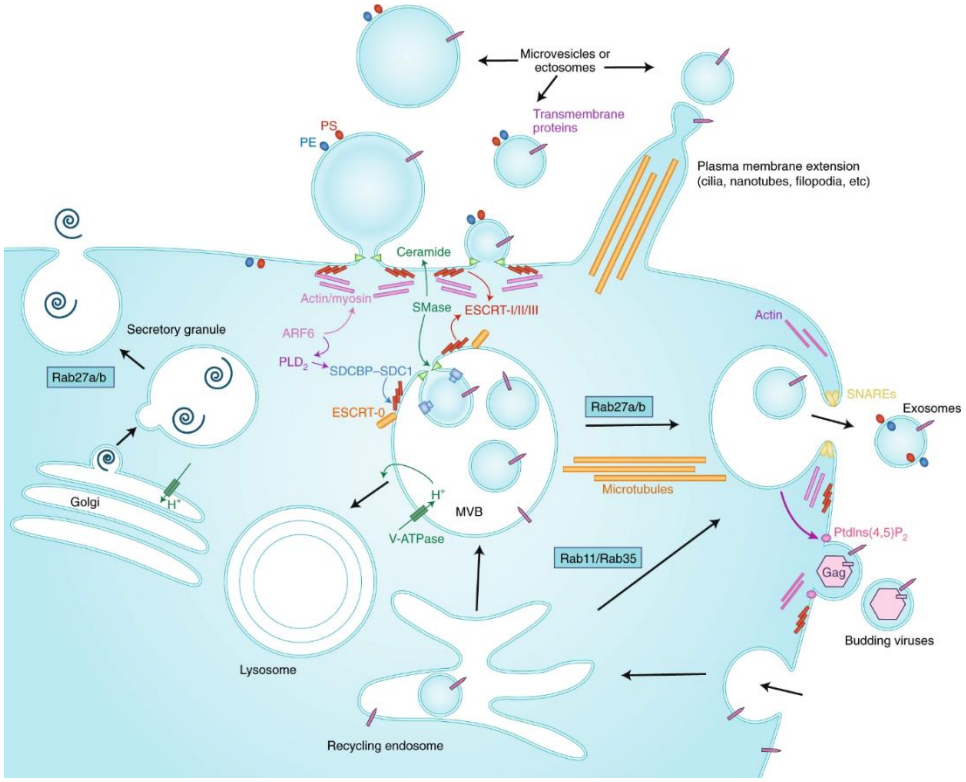

Figure 5: Proteins involved in the biogenesis of exosomes and microvesicles. Early endosomes are generated by endocytosis from the plasma membrane. Intraluminal vesicles (ILV) are formed through a second inward budding, leading to maturation of early endosomes to MVBs. Release of exosomes is facilitated by fusion of MVBs with plasma membrane. In contrast, microvesicles bud directly from the plasma menbrane. Rab: ras-related in brain GTPase; ARF6: ADP-ribosylation factor 6; PLD2: phospholipase D 2; SDCBP-SDC1: syntenin-1-syndecan-1; SMase: sphingomyelinase; V-ATPase: Vacuolar-type ATPase; ESCRT: endosomal sorting complex required for transport; MVB: multivesicular bodies; PE: phosphatidylethanolamine; PS: phosphatidylserine; SNARE: soluble N-ethylmaleimide-sensitive fusion attachment protein receptor; Ptdins(4,5)P2: phosphatidylinositol-4,5-bisphosphate. Reprinted from Mathieu et al. [110].

subsequent loss of membrane asymmetry [101]. Irrespective of their mode of biogenesis, microvesicles display a round morphology with a diameter between 100nm and 1µm, placing them in between apoptotic bodies and exosomes in terms of size [97]. Membrane composition closely resembles their cell of origin and unsurprisingly, so far, only a few components have been found ubiquitously expressed in microvesicles, such as integrins β1 and phosphatidylserine [102]. In contrast to apoptotic bodies, recruitment of nucleic acid and protein cargo is a directed process in microvesicles and defines their biological functions [103]. While microvesicles primarily convey their modulatory function by transmembrane signaling through activation of surface receptors [104], horizontal transfer of functional RNAs, including miRNAs, and proteins by uptake or direct fusion of microvesicles with recipient cells was recently

shown as well [105,106]. Biological process which involve intercellular communication by microvesicles include coagulation [107], inflammation [108] as well as tumor progression [109].

Although the role of microvesicles in cell-to-cell communications should not be underestimated, exosomes have been in the spotlight of EV researchers for years, due to their unique biogenesis, molecular composition and their tremendous potential as prognostic, diagnostic and therapeutic targets. Unlike microvesicles and apoptotic bodies, exosomes derive from a highly dynamic, endocytotic trafficking pathway involving endosomes [111]. Early endosomes originate from inward budding of the plasmamembrane by endocytosis of clathrin coated vesicles [112]. Over intermediate steps such as recycling endosomes, compartments involved in recycling plasmamembrane cargo and transportation to lysosomes [113], early endosomes can mature to late endosomes, termed multivesicular bodies (MVB) by formation of multiple intraluminal vesicles (ILVs) [114]. Unlike many other endocytotic vesicles, ILVs bud away from the cytosol and into the lumen of the MVB, thereby restoring original membrane orientation and correctly displaying surface proteins again. ILV formation and sorting of cargo is mainly driven by the ESCRT machinery and their associated proteins with each complex responsible for a different task in vesicle development. ESCRT-0 prepares endosomal membranes by sequestering ubiquitinated transmembrane proteins and recruits ESCRT-I and II which promote distortion of said membrane into buds. Fission of ILVs is subsequently realized by ESCRT-III [115]. Maturation into MVBs is not solely reliant on ESCRT however as inactivation studies have proven [116]. CD63, a transmembrane protein of the tetraspanin class, can induce ILV formation even in the absence of ESCRT [117] and so can neutral sphyngomyelinase (SMase) and phospholipase D2 (PLD2) with the help of ADP-ribosylation factor 6 (ARF6) by hydrolysis of their respective substrates into ceramide and phospatidic acid [118,119]. These concerted mechanisms not only allow for efficient generation of ILVs, they also provide a means for directed selection of RNA and protein cargo on an even higher level than microvesicles (Figure 6). Although molecular composition of exosomes is heavily influenced by their cell of origin, some proteins seem to be more ubiquitously enriched. These include ESCRT complex associated proteins like ALIX, TSG101 or RAB GTPases as well as adhesion

molecules such as tetraspanins CD63 and CD81 [121]. Since the discovery that exosomes contain mRNAs and miRNAs that can be transferred to other cells and even between species to exert their function [92], nucleic acids and especially RNA have been the focus of multitude of studies. Selection of RNA content, even more so than

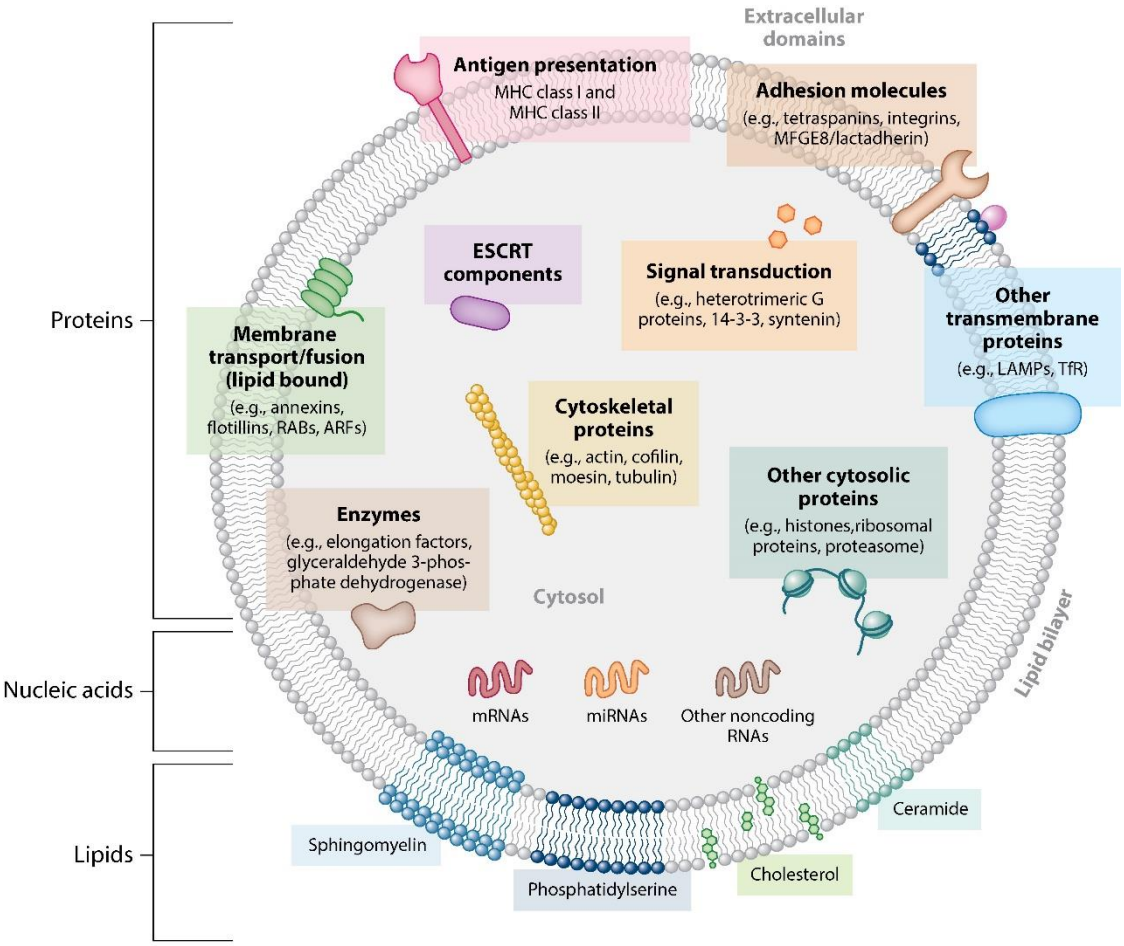

Figure 6: Molecular composition of extracellular vesicles. Figure reprinted from Colombo et al. [120].

proteins, is not a random event but produces exosomes specifically enriched in mRNA and miRNAs that differ significantly from their parent cells [122]. Underlying principles of this sorting are not yet fully understood, however recent studies reported putative export sequences for mRNA as well as miRNA [123–125]. Subsequently, transport of MVBs to the cell membrane is generally facilitated by the RAB family of GTPases, which are established players in the transfer of intracellular compartments [126]. Having arrived at the cellular border, fusion of MVBs is enabled by soluble N-ethylmaleimide-sensitive fusion attachment protein receptors (SNAREs) upon which exosome biogenesis is completed by the release of ILVs as exosomes into the

extracellular space as highly effective regulators in intercellular communication (Figure 5) [127].

1.2.2 Targeted transfer of exosomal RNA

The influence of exosomes in intercellular signaling was impressively proven for a variety of different biological contexts. To exert their function, exosomes need to bring their cargo into direct contact with recipient cells in one of two ways: either by binding of ligands presented on the surface to induce signaling cascades or by internalization of the exosome to deliver the proteins and nucleic acids contained within (Figure 7).

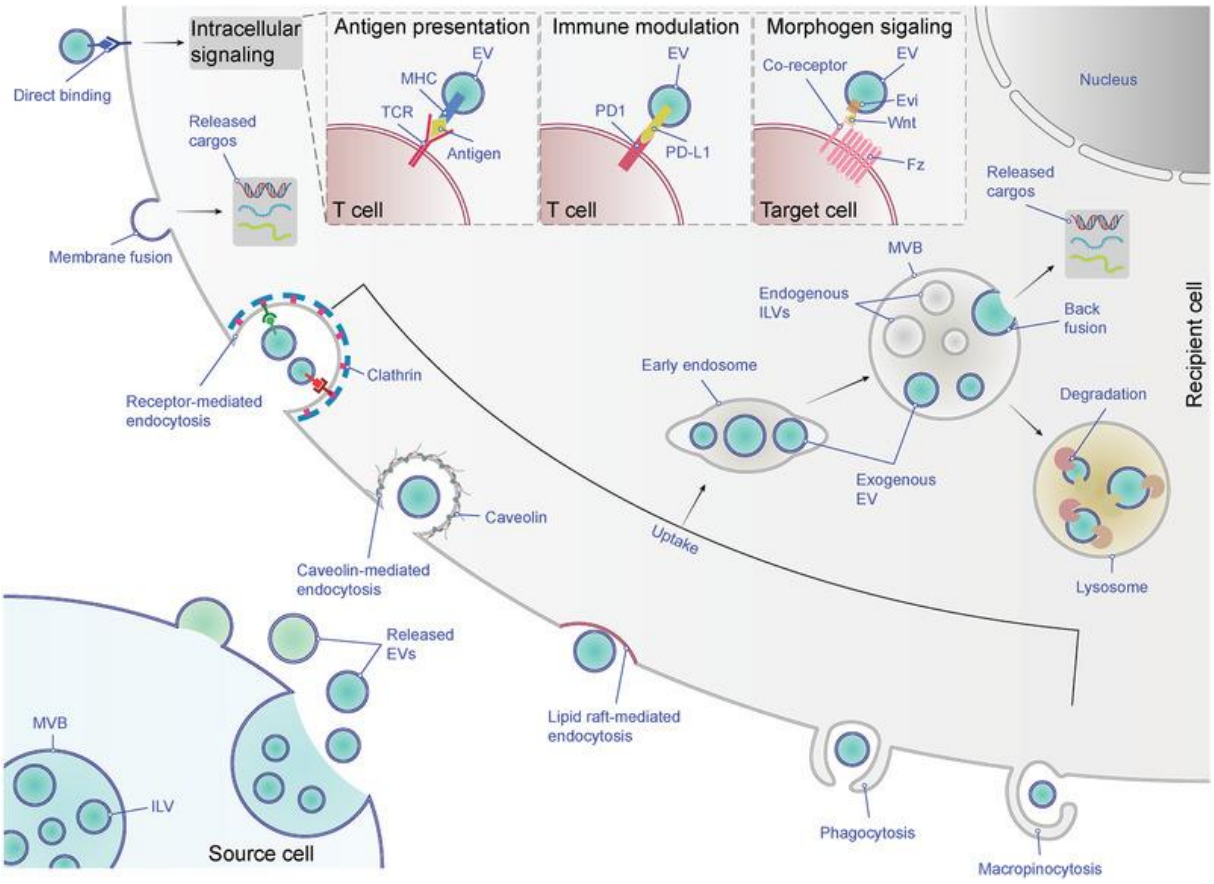

Figure 7: Interaction mechanisms between exosomes and recipient cells. Figure reprinted from Teng and Fussenegger [128].

Ligand-receptor interactions between membrane-bound proteins have been well studied in the past and play a major role in the immune-modulating capacities of exosomes. Examples include activation of T-cells through presentation of antigens on

major histocompatibility complexes (MHC) on vesicles derived from dendritic cells or B lymphocytes [91,129] or immune evasion of tumor cells by promoting apoptosis of T-cells via programmed cell death protein 1 (PD1) and its ligand from cancer derived EVs [130]. Although receptor activation can constitute extensive regulations in recipient cells, it is thought to be a less frequent event and uptake of EVs to transfer bioactive molecules, has garnered the most interest in the research community. Exosomes can gain entry into target cells either by direct fusion with the plasma membrane or, as most studies suggest, endocytosis. Mechanics of direct fusion are still not fully deciphered but seem to involve the formation of a hemi-fusion stalk followed by intermixing of the hydrophobic cores and merging of the membranes [131]. Endocytosis, as the main mode of exosome internalization, can be mediated by multiple factors. Most commonly, EVs are bound to specific receptors associated with clathrin-coated pits, that deform the membrane inward until a new vesicle containing the exosome pinches off [132]. Other forms of endocytosis include caveolae formed mainly by caveolin [133], lipid rafts enriched in flotillin [134] and ruffled membrane protrusion to capture large (phagocytosis) or small vesicles (macropinocytosis) in the extracellular space [135]. No matter which form of endocytosis was employed, exosomes are taken up in intracellular vesicles and introduced into the endosomal trafficking pathway. To avoid degradation in lysosomes, exosomes will then escape from endosomes by a process called backfusion, thereby releasing their RNA cargo into the recipient cell to fulfill their functions [136]. RNA delivery, including miRNA, by exosomes represents one of the most outstanding ways of intercellular communication, allowing distribution of signals over biofluids such as blood with a remarkable tropism for specific cells, mediated by the surface proteins necessary for endocytosis [137]. The ability of exosomes to regulate expression of target genes via miRNA interference, even in tissues distant from their origin, was impressively shown in a number of studies [92,138,139].

1.3 Regulatory properties of milk – benefits beyond nutritional value

Milk production and consumption is the defining characteristic of all mammals and plays an irreplaceable role in their development. Its nutritional composition perfectly matches the demand of newborns, but the importance of milk to promote optimal health

outcomes during early life goes way beyond the mere supply of resources to grow. Equally important for a healthy development, if not even more so, is the ability of milk and especially colostrum, the first milk produced directly after the delivery, to prime the naive, neonatal immune system and to induce tolerances to antigens [140]. Milk and in particular colostrum contains large quantities of immune-modulating molecules such as antimicrobial lactoferrin [141], factors of passive immunity such as immunoglobulins [142] as well as beneficial influencers of the developing gut microbiota to protect against infections such as milk oligosaccharides [143]. Furthermore, milk was shown to contain high amounts of vesicles and functional miRNAs, that might be able to cross into circulation from the gastrointestinal tract and were implicated in the protective effects of raw milk consumption on health, e.g. asthma and atopic sensibilization.

1.3.1 Systemic uptake of milk-derived miRNAs – a controversy

The discovery of miRNA transfer by EVs between cells even from different species [92,144] let researchers on a quest to find new sources of exogenous genetic material. An apparent choice were dietary miRNAs that could gain entry into intestinal cells and subsequently be distributed systemically. Early findings of plant miRNAs from rice and honeysuckle could be quickly dismissed due to contamination or poor study design [145,146], but analyses of milk-derived miRNAs and EVs proved to have more substance. miRNAs in milk were shown to be largely incorporated into EVs and analyses of target mRNAs proposed a high immune-regulatory impact [147,148]. Furthermore, encapsulation in EVs provided high resistance to degradation even under simulated gastrointestinal conditions [149], prolonged storage [150] or after procession to milk powder [151]. Completing this promising picture were *in vitro* experiments showing uptake of milk-derived EVs in human intestinal cells [152], endothelial cell [153] and even circulating immune cells [154]. Confirmation by *in vivo* experiments, however, proved to be difficult. Encouraging studies displaying uptake of orally administered milk EVs into blood circulation in mice linked with a decrease of related miRNAs in a control group fed with EV-depleted milk, could not be reproduced by independent research groups [155–157]. In knock-out mouse models lacking key miRNAs enriched in milk, only trace amounts of miRNAs were found after feeding newborn pups with wildtype milk [158] while studies with milk EVs marked with

fluorophores showed postprandial accumulation of vesicles in peripheral tissues [159]. In contrast to these conflicting findings, first trials with milk-derived EVs as drug-delivery vehicles showed promising results by inhibiting growth of tumor cells without inducing widespread adverse immune responses by the host [160,161]. Within the class of milk-derived EVs, vesicles from colostrum have garnered special interest. Protein and RNA composition of colostral EV is significantly diverging from later lactation stages and is thought to contain active regulators of intestinal cell proliferation and health [162]. In addition, endocytotic uptake in calves of other colostrum components in the form of immunoglobulins is a well known phenomenon in ruminants [163]. The knowledge to which extent miRNAs from milk in particular and dietary EVs in general are absorbed and systemically distributed, will have a major impact on our perception of nutrition itself and bears tremendous potential for future diagnostic and therapeutic applications.

1.3.2 The farm milk effect on childhood asthma

During the first six months of life, national health organizations and pediatricians recommend breast feeding as the optimal source of infant nutrition [164]. Beneficial effects of milk consumption, however, do not stop there. Prolonged breast feeding was associated among others with reductions in overweight as well as increased protection against breast cancer for mothers [165]. Furthermore, consumption of raw bovine milk during the first years of life and growing up in traditional farm environments could be impressively linked to significantly lowered incidences of asthma and atopic sensibilization, major health problems in industrialized countries for which no effective preventive measures are known [166]. Termed the “farm milk effect”, this observation is even more remarkable as it can be found in populations all over the world [167–169] and its effects seem to persist into adulthood [170]. Further interest was sparked when it could be shown that protective effects of raw milk consumption could be transferred to children with no association to farming environments as well [171]. Consumption of raw milk however, bears the substantial risk of infections by milk-borne pathogens such as *Escherichia coli*, *Campylobacter* or *Salmonella* species that were successfully eliminated by the introduction of pasteurization and other industrial processing techniques [172]. To which extent the protective effect of raw milk is lost due to these

mainly heat-based and homogenization treatments, has been the topic of a number of large scale, European studies such as PASTURE or GABRIELA [173,174]. Although milk composition is quite heterogeneous and influenced by a variety of external factors like cow species, feed, milking hygiene or lactation stage [175], several candidates were proposed as effector molecules for the “farm milk effect”. These include oligosaccharides, whey proteins polyunsaturated fatty acids and milk-derived miRNAs [171,175,176].

1.4 Aim of this dissertation

The goal of this study was to decipher the importance of bovine miRNAs and their isoforms by comprehensively analyzing their distribution patterns in milk and blood via high-throughput small RNA next-generation sequencing (NGS). Initially, an adaptable, bioinformatic analysis workflow for the isomiR-specific detection of miRNA transcripts was to be implemented to enable sensitive and robust quantification of all canonical miRNAs and their isoforms. Furthermore, a database for the collection of miRNA NGS results from EVs was to be established to advance understanding of the impact of different EV isolation strategies on expression profiles and facilitate detection of stably expressed reference miRNAs. With the help of these tools, changes in stability and abundance of miRNAs in differently processed milk were to be evaluated to elucidate their implication for the protective “farm milk effect” in children. Lastly, the potential of colostral-derived EVs and their cargo to be transferred to the blood circulation of neonatal calves was to be examined in order to gain insights into the postprandial uptake of dietary miRNA and EVs.

2. Methods

2.1 Development of a universal, bioinformatic workflow for the detection of canonical miRNAs and their isoforms from small RNA next-generation sequencing data

Although the relevance of isomiRs on the targeting behavior and regulation efficiency was proven in a number of studies, software for the robust identification of miRNA isoforms is still scarce. Available tools often fail to grasp the full repertoire of isomiRs (5' end, 3' end, polymorphic isomiRs and combinations thereof) and are incorporated into third party software suites that either have prohibitive licensing costs or restrict analysis to foreign servers, which raises issues with data safety. Other, free-to-use solutions are restricted to certain genomic aligners and preproccessing steps, that might hinder comparability to or necessitate time-consuming repetition of already existing analyses. Furthermore, sorting of output from existing tools requires extensive, bioinformatic comprehension and is frequently beyond reach of investigators involved in miRNA studies. In addition, most programs are limited to a few model organisms such as human or mice and rely on full genomic builds including thorough annotation of all miRNAs.

To counter shortcomings of existing isomiR analysis tools, a new bioinformatic workflow called isomiRROR (isomiR – References, Overviews and Readcounts) was established. isomiRROR generates isomiR-specific mapping indices containing the vast majority of encountered sequence variations (ranging from +3 to -6 nt on both ends). Necessary input is a sole fasta file of putative mature miRNA sequence, apart from NGS data of samples to be analyzed naturally, which is either obtained from a existing database such as miRBase [5,6] or from individually curated targets, and is not reliant on any genomic information. Putative pre-miRNA sequences can be supplied optionally to complement information whether added nucleotides on 5' or 3' end result most likely from diverging Drosha or Dicer processing or stem from nucleotidyl transferases. Resulting isomiR sequences are split by length to avoid multimapping during alignment and subsequently merged again. Furthermore, it includes algorithms to extract information on polymorphic isomiRs from SAM (Sequence Alignment Map) files, the most commonly used output format of NGS

mapping tools and automatically generates readcounts for all supplied samples. Information on sequence differences compared to canonical miRNAs is stored directly in the identifier, which allows for easy access and analysis of over-representations even by prevalent spreadsheet software. Full information on isomiRROR, including the source code, is freely available at https://gitlab.lrz.de/Physio/isomiRROR.

2.2 Development of an online database for the detection of stably expressed miRNA references derived from extracellular vesicles enriched samples

Similar to isomiR detection software, a clear deficit in databases concerning in-depth analyses of NGS data from EV experiments exists. Even though extensive collections of data for meta-analyses of EV composition were created such as Vesiclepedia [177] or the miRNA-centric EVmiRNA [178], the emphasis was put solely on the cells or tissues of EV origin. By neglecting additional factors such as EV isolation strategies, a bias with critical consequences for RNA and protein compositions in EVs [179], comparability between experiments is severely hampered. This includes studies on miRNA biomarker signatures from EVs, a research area that continues to struggle for reproducibility. Although today most miRNA biomarker profiles are discovered by small RNA NGS, technical validation by reverse transcription followed by real-time quantitative polymerase chain reaction (RT-qPCR) is fundamental to verify findings. So far, no available database for EVs allows for the detection of stably expressed RNAs, miRNAs or otherwise, that are essential for normalization between samples in RT-qPCR.

miREV (miRNA References of Extracellular Vesicles) was created with the purpose of achieving comparability between different miRNA transcriptomic studies by a standardized analysis workflow and to allow researchers the identification of stably expressed reference miRNAs for specific experimental setups, thereby avoiding time- and resource-consuming NGS screening themselves. An overview of the analysis pipeline is given in figure 8 and detailed in Hildebrandt and Kirchner et al (Appendix I) [180]. In short, raw sequencing data from publicly available or in-house data sets was processed by isomiRROR to generate readcounts of miRNAs (sums of reads mapping on canonical and isomiR transcripts). miRNA count tables were rearranged to sample

sets, accounting for all combinations of experimental variables such as EV isolation method, blood compartment (serum or plasma) and pathological context. To avoid misinterpretation of unmeaningful data, sample sets with less than ten samples were removed as well as miRNAs that could not be detected in at least 95% of the sample set. Read counts from sample sets were then normalized according to six different, commonly used normalization algorithms and finally normalized miRNAs were ranked according to their expression stability by three different algorithms for reference detection. miREV is fully implemented as a web-based data server under http://141.40.217.80:3838/miREV/, allowing free and easy access. Source code is available from https://github.com/Hildebrandt-Alex/miREV.

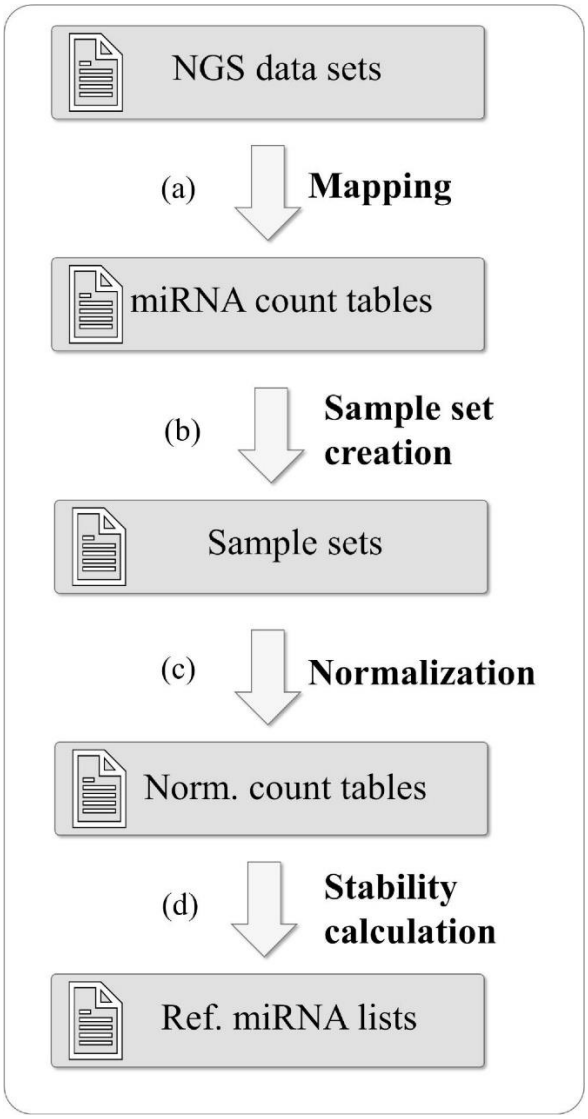

Figure 8 : Workflow of NGS data processing for miREV. Reprinted from Hildebrandt and Kirchner et al (Appendix I) [180].

2.3 Evaluating miRNA stability and abundance in processed milk

The effector molecules responsible for the asthma- and allergy protective function of raw farm milk remain elusive so far, as do mechanics that might explain the loss of this protection in processed milk. To assess whether milk miRNAs, known regulators of immune function, might play a significant role in the "farm milk effect", raw milk from three different farms was collected and processed identical to dairy industry standards (Table 2 and 3). Total RNA, including miRNA, from all milk fractions was subsequently extracted in triplicates with the miRNeasy Mini Kit (Qiagen, Germany), using an increased ratio of chloroform to account for the high fat and protein content of milk. Next, small RNA NGS was performed for all RNA isolations using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs, USA) on an Illumina HiSeq 2500 (Illumina, USA). Potentially confounding bovine sequences from other non-coding small RNA classes were filtered out before sequencing reads were mapped to human mature miRNA sequences. Differentially expressed miRNAs were detected with the help of NOISeq package in R [181] and validated by RT-qPCR using the Exiqon miRCURY LNA Universal RT microRNA PCR system (Exiqon, Denmark) on a Rotorgene cycler (Qiagen, Germany). Influences of significantly regulated human miRNA orthologes were evaluated by analysis of immune relevant mRNA targets obtained from mirTarbase (v4.0) [182]. Full details on milk processings and statistical analyses can be found in Kirchner et al. (Appendix II) [183].

	Farm		
	T	**F**	**S**
Farming type	Conventional	Conventional	Organic
Cattle breed	Fleckvieh	Brown Swiss	Fleckvieh
Feeding	Corn silage, grass silage, concentrate	Corn silage, concentrate	Grass, hay, wheat
No. of cows	13	60	30
Time point of milking for pooled samples	Morning and evening	Morning and evening	Morning and evening
Microbial contamination (CFU/mL), mean ± SD	18,500 ± 7,800	7,300 ± 3,200	4,600 ± 3,800

Table 2: Characteristics of milk processing. Reprinted from Kirchner et al. (Appendix II) [183].

	Milk fraction	Processing conditions	Day of processing
UHT	Ultra-high heat treated	Preheating at 93°C for 23 s, direct steam injection at 142°C for 5 s	1
ESL	Extended shelf life	Preheating at 95°C for 20 s, direct steam injection at 127°C for 5 s	1
BOI	Boiled milk	Preheating at >80°C for >300 s, boiling at 100°C for 30 s	2
HOM	Homogenized milk	Preheating to 55°C, 2-stage homogenization at 250/50 bar	2
SKI	Skim milk	Separation at 50°C	2
FAT	Fat fraction	Separation at 50°C	2
PAS	Pasteurized milk	72°C for 20 s	3
RAW	Native raw milk	—	3

Table 3: Characteristics of milk processing. Reprinted from Kirchner et al. (Appendix II) [183].

2.4 Characterization of milk and blood compartments including extracellular vesicles in adult cows and their neonatal offspring

Evaluation of any potential transfer of EVs relies on consequent characterization of isolates to allow for differentiation between tissues as well as organisms of origin and the recipient. In the context of milk-derived EVs, this necessitates not only discrimination between vesicles from cows and their offspring but also between EVs and the cellular compartments as well as the unfractionated biofluid they were found in. To assess the possibility of a postprandial uptake of colostral EVs in newborn calves, blood and colostrum samples were drawn on the day of parturition directly before the first ad libitum feeding of the calves as well as in regular intervals afterwards. (Figure 9 and 10). After separation of cells by centrifugation, EVs were isolated from plasma or skimmed colostrum by differential ultracentrifugation coupled with flotation into a sucrose density gradient (SDG) to remove co-isolated contaminants such as lipoproteins or protein aggregates, that can contain miRNA as well [184]. Blood and colostrum EVs from cows and their offspring underwent extensive characterization including visual confirmation of morphology by transmission electron microscopy (TEM), assessment of concentration and size distribution by nanoparticle tracking analysis (NTA) as well as detection of marker proteins by western blot (Figure 9).

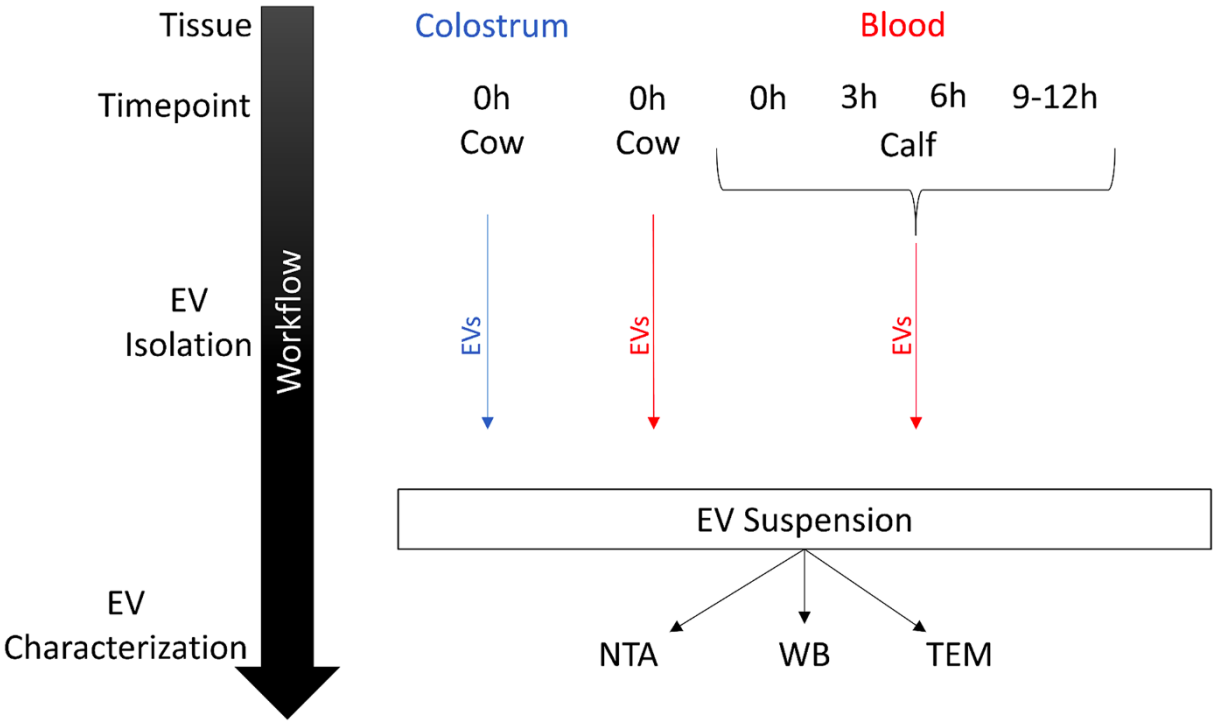

Figure 9: Schematic workflow of sample timepoints and biofluids of EV isolations. Figure reprinted from Kirchner et al. (Appendix III) [185].

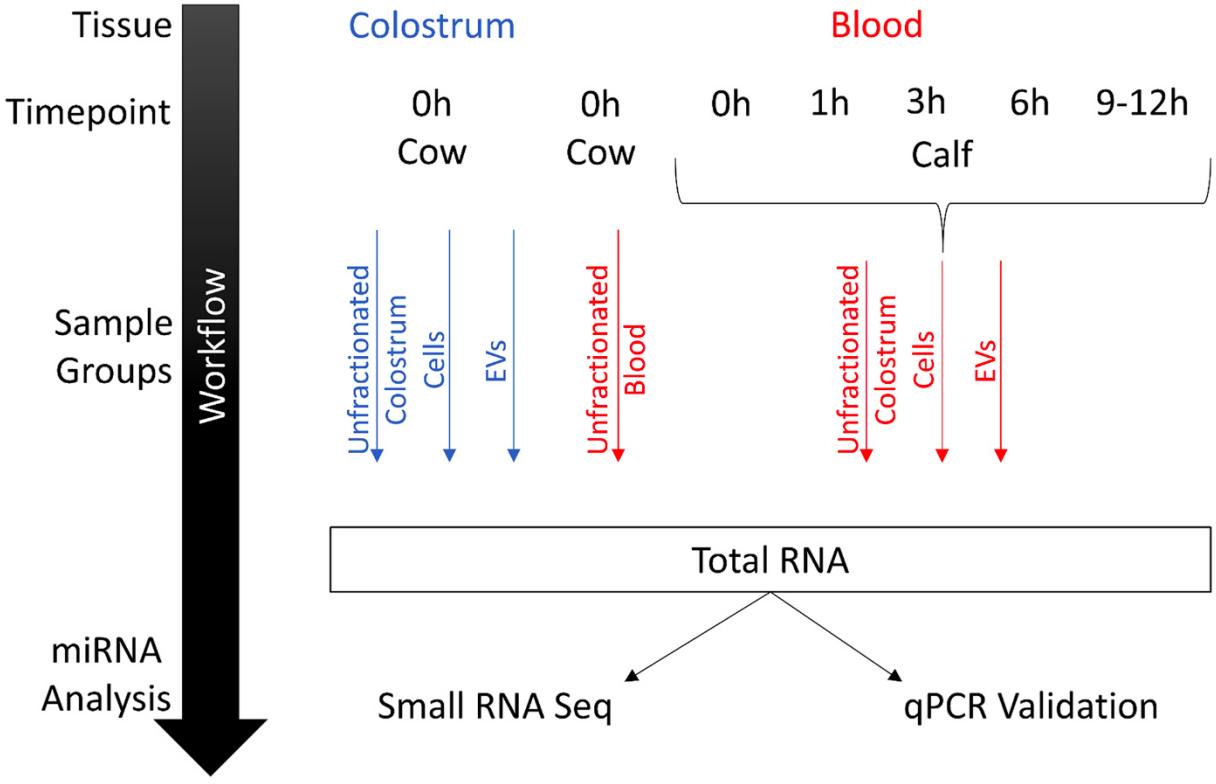

Figure 10: Schematic overview of timepoints and sample groups for miRNA transcriptome profiling. Figure reprinted from Kirchner et al. (Appendix III) [185].

To evaluate miRNA transcriptome profiles, total RNA was extracted by miRNeasy Mini Kit (Qiagen, Germany) for all EV preparations as well as unfractionated blood and colostrum and their cellular components. Small RNA NGS was performed using the (New England Biolabs, USA) on a HiSeq 2500 (Illumina, USA) and readcounts of canonical as well isomiRs were obtained using isomiRROR generated mapping references of bovine mature sequences from miRBase [5,6]. Significant changes in miRNA expression were detected with DESeq2 package in R [186] and evaluated by enrichment analysis of human homologues of mRNA targets in KEGG pathways to assess their potential implications in calf physiology by clusterProfiler [187]. Results of differential expression were validated in RT-qPCR using the Exiqon miRCURY LNA Universal RT microRNA PCR system (Exiqon, Denmark) on CFX384 Real-Time PCR Detection System (Bio-Rad, USA). Full details on EV isolation and characterization protocols as well as statistical analyses can be found in Kirchner et al. (Appendix III) [185].

3. Results and Discussion

3.1 isomiRROR – a robust and highly adaptable workflow for the simultaneous detection of canonical miRNA and isomiRs

The advent of NGS as the platform of choice for biomarker discovery studies and the resulting increase in resolution down to single nucleotides, have given unprecedented insights into the sequence heterogeneity of miRNAs. The original thought that miRNA biogenesis gives rise to a single mature transcript, had to be discarded in light of findings that proved abundances of isomiRs vary with their physiological and pathophysiological context [68,69,72,75]. Development of isomiRROR arose out of the necessity for a software tool capable of identifying the whole spectrum of sequence variations found in miRNA isoforms. With the discrepancy of the extent to which miRNAs are annotated in mind, for example 2,654 human mature miRNAs compared to 1,025 bovine in miRBase [5,6], isomiRROR was designed to work independently of existing genomes. By using a simple list of mature miRNA sequences as the sole source of input, isomiRROR is highly suitable for analysis of well-studied organisms such as humans and mice as well as less researched species including farm animals, plants and prokaryotes. Furthermore, lists with miRNAs of interest can be easily expanded to include orthologous miRNAs from closely related species and even novel miRNAs predictions [188], making it possible to take advantage of the latest research and allowing for the verification of new hypotheses. isomiRROR also enables full flexibility in terms of alignment algorithms. Although it is mapping supplied reads from small RNA NGS with bowtie by default [189], generated lists with canonical and isomiR sequences can be utilized with any available alignment software to allow for full comparability to already completed studies. In contrast to many existing isomiR analysis tools, information on mismatches during alignment is only used for detection of polymorphic isomiRs and not for the identification of non-templated additions by nucleotidyl transferases on 5’ and 3’ ends. Whereas most available software has a combined limit for alterations that deviate from genomic miRNA sequences and are therefore often overlooking isomiRs, isomiRROR is able to grasp the full repertoire of RNA editing events. Taken together with the implemented practice of mapping small RNA reads only to miRNA sequences of the same length to avoid multimapping, this enables identification of isomiRs with high sensitivity and specificity.

isomiRROR conveniently stores all sequence related information in the identifier of each isomiR, which will appear in read count lists instead of canonical miRNA names from conventional analysis (see Readme.md on https://gitlab.lrz.de/Physio/isomiRROR). Presented information includes number and sequence of nucleotides that were trimmed or added on 5' or 3' end and if new sequences are matching the pre-miRNA template. Furthermore, sequence positions including exchanged nucleotides of polymorphic isomiRs are given together with seed sequences as well as terminal nucleotides from 3' end. In doing so, isomiRROR gives researchers the opportunity to group isomiRs by matching targeting behavior, i.e. identical seed sequences, instead of canonical miRNAs, to better understand cumulative influences on mRNA targets [56]. Similarly, analysis of 3' end sequences, especially sequence alterations that lead to terminal adenines or uracils, might allow assumptions on miRNA turnover rates and temporal expression patterns [83].

3.2 isomiR specific detection increases mapping yield, differentially expressed transcripts and robustness of signal

miRNAs haven been in the focus for many years as potential sources for diagnostic biomarkers. Special attention was paid in recent years to circulating miRNA from liquid biopsies as a minimally invasive procedure and due to their association to EVs and their role in intercellular communication [190]. Although studies have shown their high potential for molecular diagnosis, prognosis and disease monitoring, a severe lack in reproducibility and specificity of reported markers is hampering their clinical utility so far [191]. One reason for this deficiency could lie within the often unaccounted sequence variants embodied by isomiRs. Methodological platforms used for miRNA biomarker detection (e.g. RT-qPCR, microarrays and NGS) vary in sensitivity and validity for certain variants and probe-based strategies generally fail to identify the full spectrum of isomiR sequences [192]. The resulting bias can be even more pronounced, if the low quantities and quality of liquid biopsies and minuscule amounts of miRNA molecules per individual EVs are taken into account as well [193].

In-depth analysis of miRNA expression profiles in bovine blood and milk samples (Appendix III) [185] revealed that transcripts of canonical miRNAs constitute less than

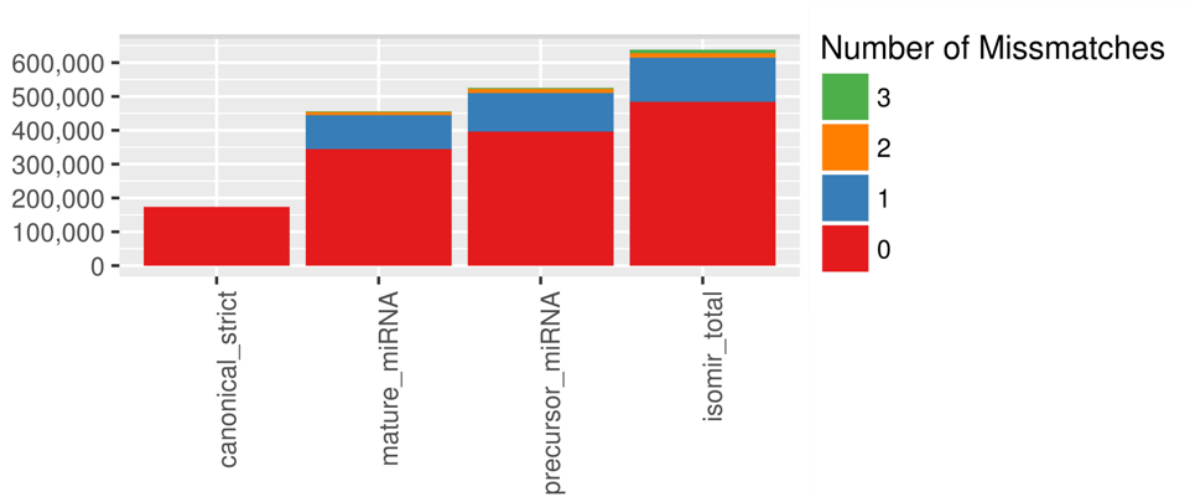

Figure 11: Reads mapping on various classes of miRNA in calf blood exosomes. Figure is representative for isomiR distribution in general. Consecutive histograms represent accumulated reads that are completely matching in length and sequence to mature miRNA sequences (canonical_strict), include trimming (mature_miRNA) or templated additions on 5' or 3' end (precursor_miRNA) as well as non-templated additions (isomiR_total). Proportion of polymorphic isomiRs is represented by the number of mismatches compared to canonical sequences. Data taken from Kirchner et al. (Appendix III) [185].

a third of all sequenced reads mapping to miRNA which is consistent with findings of other groups (Figure 11) [194]. Mapping yield varies greatly depending on the class of miRNA (mature reads or pre-miRNAs) used as alignment reference and the exactness in which query sequencing read and reference have to match. Abundances of distinct isomiRs are linked to physiological contexts and canonical miRNA sequences were often shown to not be the predominant transcript for individual miRNA families in our and other studies [195,196]. Nonetheless, variations of isomiR sequences, albeit frequent, comprise only a few nucleotides most of the times, with single trimmings or additions in 3' isomiRs being the most common (Figure 12) [194]. In contrast, 5' isomiRs, that inevitably entail changes in seed sequences responsible for targeting behavior, are rare events with potentially major implications [197].

In addition to depicting a more accurate reflection of the miRNA transcriptome and significantly increasing mapping yield, isomiR-specific detection offers further advantages compared to conventional analyses. Statistical comparisons of treatments, disease states or pathophysiological conditions are crucially relying on high ratios of signal to noise to identify significant differences [198]. isomiR-specific analyses offer the opportunity to focus on the transcripts of a miRNA family that display the highest

variance between groups by excluding isomiRs without significant changes in abundance. In rare cases bulk analysis of isomiR transcripts together with canonical miRNAs can mask the very weak expression changes researchers are trying to unveil.

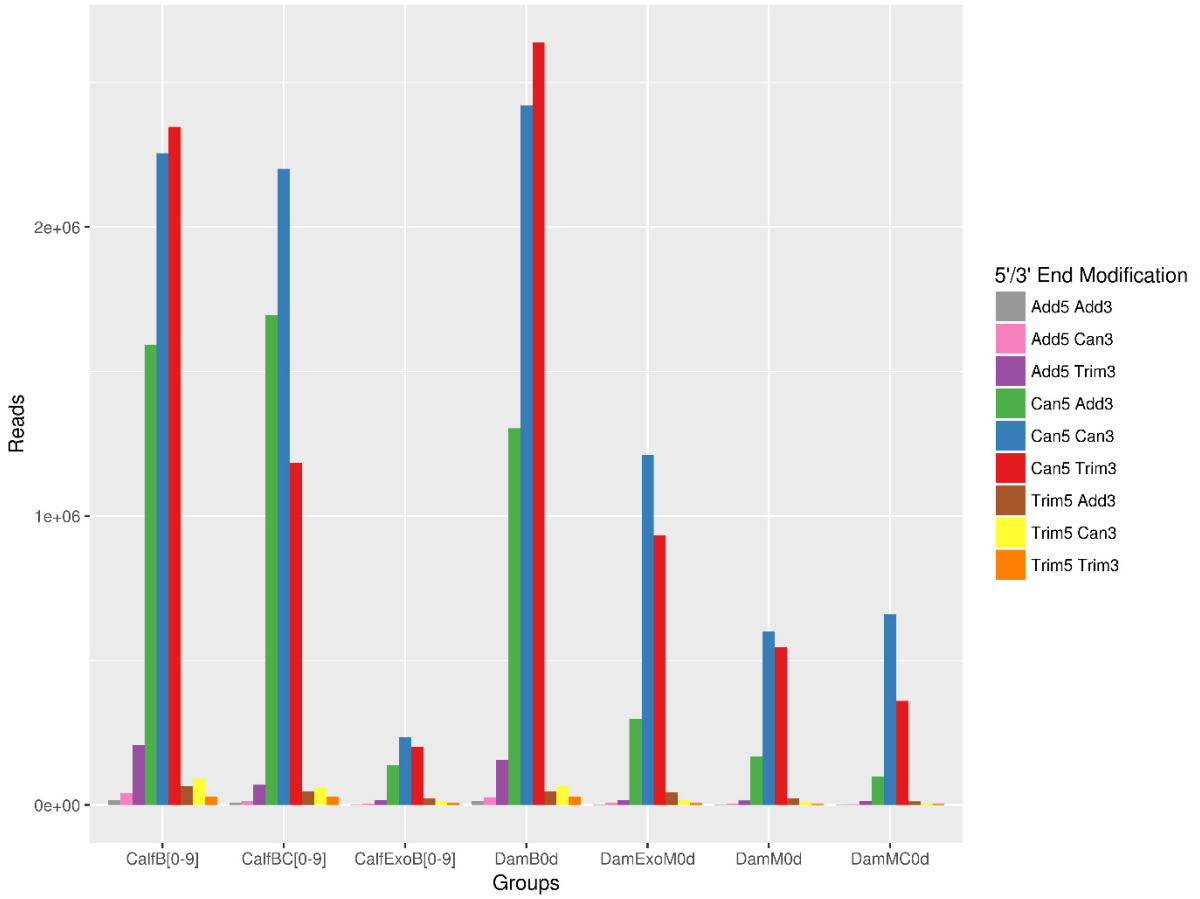

Figure 12: Abundances of miRNA sequences with modifications on 5' and 3' end in bovine blood and colostrum. Histograms represent numbers of reads with either nucleotides added (Add) or trimmed (Trim) to 5' or 3' ends as well miRNAs with no modifications (Can). Data taken from Kirchner et al. (Appendix III) [185].

If expression directions of isomiRs are opposed within one miRNA family, resulting total fold changes will be minimal and statistically non-significant. This is demonstrated in Figure 13, where canonical as well as sequences with one nucleotide trimmed from 3' end are down-regulated whereas isomiRs with two or more trimmings are significantly up-regulated.

Taking everything together, isomiR-specific analyses enable researchers to take full advantage of small RNA NGS experiments while expanding investigations on canonical miRNAs ton include isomiRs increases with mapping yields and robustness. Adaptability of isomiRROR was proven by numerous studies from various matrices

such as blood, milk, tissues, EVs or cell cultures of humans, mice or cows especially for the detection of valid biomarker signatures [199–210].

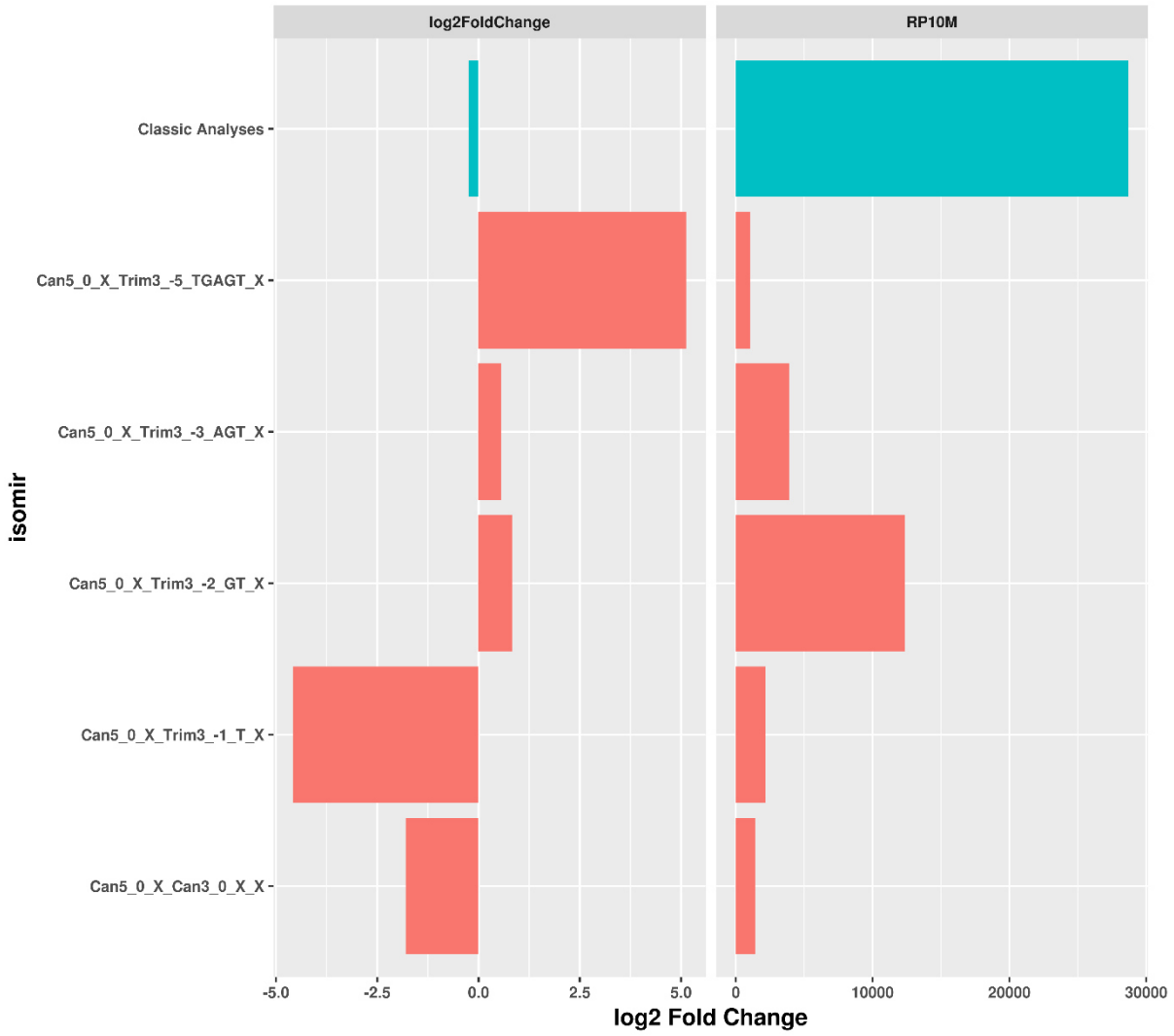

Figure 13: Exemplary overview of isomiR abundances and expression changes in bulk analysis of all miRNA transcripts (Classic Analysis) and single isomiRs. Statistically significant changes (p-value < 0.05) are marked in red, insignificant changes in blue. Data presented in Clayton et al. [196].

3.3 Time and workload reduction for the detection of stably expressed miRNAs in extracellular vesicles by miREV

Biases in miRNA and isomiR identification are not the only difficulties holding back the clinical application of EV biomarker signatures from small RNA NGS studies as we

reviewed in Buschmann et al. [198]. Experimental conditions including pre-analytical sample preparations, such as sampling itself or storage, have a major influence on research outcomes and drawn conclusions from flawed experimental design can often be misleading. A crucial but frequently underestimated source of incomparability and irreproducibility between studies is the inherent heterogeneity of EVs and the mixed subpopulations of EVs and contaminants that result from the majority of isolation protocols. Although a large number of published and commercialized isolation strategies are available to researchers [211], overlap between individual methods especially for miRNA cargo is low as we and others investigated [203,212]. Counterintuitively, purer isolations of EVs are not necessarily linked to higher discriminative power of studies or increased classification potential as biomarkers [203]. The lack of consensus on how to best extract EVs was made strikingly clear by EV-Track in 2017, a meta-study comprising more than 1,700 EV experiments that employed over 1,000 unique isolation protocols [179]. To combat this growing deficit in comparability, the research community implemented guidelines for the minimal information needed for studies of extracellular vesicles (MISEV) [213]. Even though reporting in published EV experiments has improved significantly, interpretation of the available knowledge on EV and their cargo remains difficult. Databases for protein and RNA content, including miRNAs, exist but only report their presence and not their abundance, which is crucial in biomarker studies [177]. Furthermore, no information on stably expressed transcripts can gained, which are essential in validation of NGS findings by RT-qPCR [214].

miREV was conceived as a publicly available database to investigate similarities and differences between EV studies and highlight influences of experimental conditions such as isolation methods. miREV helps researchers estimating technical as well as biological variances and provides consensus lists of stably expressed miRNA transcripts as well as raw readcounts for further analyses. The initial database consists of 428 individual small-RNA data sets from 10 different studies on blood-derived EVs that were chosen due to their publicly available NGS data and their extensive, reported EV characterization (Appendix I) [180]. The database can be explored by defining an experimental setup (blood compartment, EV isolation method and physiological state) that matches the research question at hand as close as possible. Similarities in miRNA

expression profiles can be visualized by principal component analysis (PCA) and unsupervised clustering can be verified by accentuation of experimental variables. Potential candidates for miRNA references in RT-qPCR can be obtained by defining normalization and expression stability algorithms and compared by overlap analyses. miRNA references are ranked by the consensus of different algorithms and displayed in Venn-diagrams to assess overlap (Figure 14).

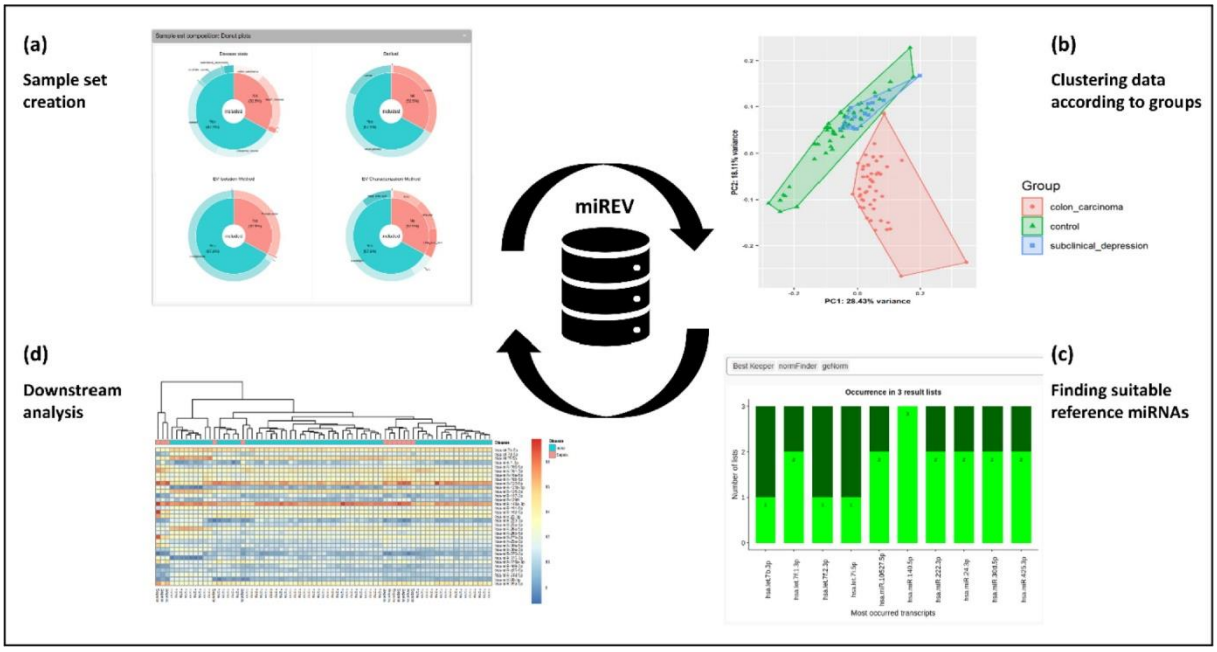

Figure 14: Overview of output plots created by an exemplary analysis in miREV. After defining experimental conditions (a), miRNA expression profiles can be examined by unsupervised clustering (b). Potential candidates for reference miRNAs are ranked by consensus and overlaps between algorithms are presented as Venn-diagrams (c). Additionally, raw read counts are available for download to conduct further downstream analyses (d). Figure reprinted from Hildebrandt and Kirchner et al. (Appendix I) [180].

miREV closes a knowledge gap in the existing landscape of databases for the molecular composition of EVs. Researchers conducting RT-qPCR experiments in EVs can save a significant amount of time and resources by avoiding lengthy and expensive NGS experiments to detect stably expressed miRNAs. The standardized NGS processing workflow via isomiRROR enables comparison and even combination of unrelated studies to increase statistical power and can give first hints on the validity of new hypotheses by evaluating the major sources of variance in PCA. In addition, the availability for download of fully processed miRNA readcounts from multiple studies, gives researchers the opportunity to reproduce findings or verify new assumptions in

large data sets, that would normally involve a substantial commitment of time and money. Furthermore, miREV provides an optimal framework for expansion and in the future, further datasets from other biofluids or cell cultures and additional experimental conditions will be included.

3.4 High-heat treatment significantly reduces miRNA abundances in differently processed bovine milk

The prevalence of asthma, allergies and other atopic sensibilizations has been constantly increasing over the last decades and poses a serious health problem in the industrialized, western countries [166]. Due to a lack of effective remedies, natural prevention as in the case of the “farm milk effect” is of particular importance. Although the beneficial effect can be in part explained by a general contact to cows or other farm animals and feed in traditional animal husbandry, studies have focused on components of raw milk since it was shown that their protective effects can be transferred to children without contact to farm environments [171]. Milk consumed by the majority of children is normally processed to inhibit pathogens and the biggest differences between raw milk and shop-bought milk is lying in the fat content and high heat treatment [172]. To assess to which extent milk-derived miRNAs play a role in the “farm milk effect”, we measured miRNA abundances in 8 differently processed milks originating from three farms (Table 2). Sequencing reads were aligned to human miRNA homologues since annotations are more extensive than in cows and, ultimately, we wanted to evaluate consequences for human consumers.

To avoid false positive alignment of small-RNA NGS, reads smaller than 16 nt in length were discarded after trimming of sequencing adapters as well as reads mapping to bovine sequences of rRNA, tRNA, snRNA or snoRNA. Relative frequencies of reads mapping to these groups or human miRNAs did not differ between farms, proving no significant influence of feeding or upbringing on mean milk miRNA expression (Figure 15A). Individual milk processing however, affected relative abundance of miRNAs greatly with the most pronounced decrease seen for milk that underwent one of the three high heat treatments (ESL, BOI, UHT) (Figure 15B). Reduction of miRNA stability could also be detected on an absolute level for individual miRNAs, as exemplified for the most abundant miRNA in the data set, miR-148a-3p (~36% of all reads) (Appendix

II, Figure 15C) [183]. Detrimental influence on miRNAs could be confirmed by unsupervised clustering in a PCA, where close to the complete variance in the data set (97.5%) could be explained by difference between high heat treatments and milks that were exposed to no or moderate temperature increases (RAW, SKI, HOM, PAS and FAT). Nearly all remaining variance was associated with fat content (Figure 15D). Validity of NGS results was confirmed by RT-qPCR with high correlations (>0.79) of fold changes between these independent methods for multiple miRNAs (see Table E4 of Kirchner et al., Appendix II) [183].

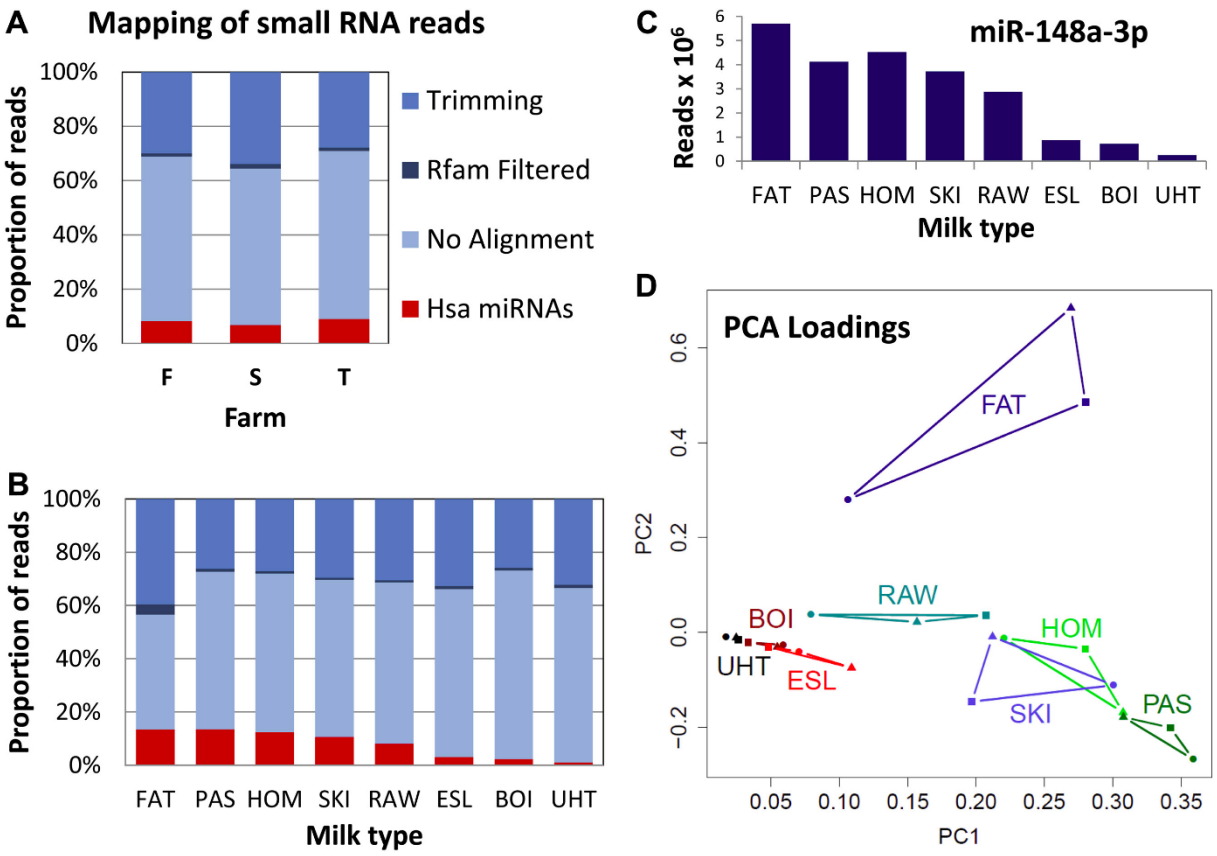

Figure 15: Overview of miRNA abundances and expression profiles in differently processed milk. No influence of milk origin could be detected in relative frequencies of mapped reads (A). In contrast, milk processing reduced relative miRNA abundance in samples undergoing high heat treatment (B). Absolute levels of the most abundant miRNA (miR-148a-3p) showed a similar pattern of high heat influence (C). Unsupervised clustering of miRNA expression profiles revealed further differences between high heat treated milk and other processings as well as divergent expression in milk fat (D). Trimming: sequencings reads < 16 nt after trimming; Rfam filtered: reads mapping to rRNA, tRNA, snRNA, snoRNA; Hsa miRNAs: reads mapping to human miRNA homologues; No Alignment: reads mapping to none of the afore mentioned RNA classes; FAT: milk fat, UHT: ultra-high theat treated milk; BOI: boiled milk; ESL: extended shelf life milk; RAW: raw milk; SKI: skim milk; HOM: homogenized milk; PAS: pasteurized milk. Figure reprinted from Kirchner et al. (Appendix II) [183].

Significant reduction in RNA content in general and miRNA in particular by high and prolonged heat treatment are in line with studies with highly processed milk such as infant formula [150,151]. Furthermore, the large contrast between raw milk and UHT is consistent with epidemiologic studies, where differences in consumption between these milk types serves as a reference category [176,215]. Interestingly, the biggest difference in miRNA expression profiles compared to high heat treated milks could be observed for pasteurized milk that underwent only moderate temperature treatments (72°C). Protective effects of pasteurized milk similar to raw milk were demonstrated before in the GABRIELA study but missed statistical significance and pasteurized milk was also shown to have a preventive effect on fever episodes in infants [171,176]. Compared to raw milk, whose consumption bears significant health risks, pathogens are completely inhibited in pasteurized milk, making it a preferable alternative for any consumer. The exact causes for the decline in miRNA stability by high heat treatments are not yet fully understood but recent findings point to the involvement of EVs. miRNAs in milk are associated to EVs to a large extent [147] which protect miRNAs from degradation and inactivation by RNAses [216]. Although EVs and their cargo were shown to be resistant to freeze/ thaw cycles and acidic conditions [149], temperatures above 90°C seem to destroy vesicle membranes and expose miRNAs. Increased miRNA content of pasteurized milk samples compared to raw milk samples might be explained by partial inactivation of RNAses at 72°C.

3.5 miRNAs affected by bovine milk processing potentially target important immune regulators in humans

In addition to the general impact of milk processing on miRNA abundances, differential expression between the three groups with the greatest difference in miRNA profiles (UHT, FAT, PAS, Figure 15D) was evaluated. miRNAs are known regulators of immune functions [148] and have been shown to exert suppressive effects in regulatory T-cells [217]. Since milk-derived EVs were shown to be resistant to gastrointestinal conditions and displayed uptake *in vitro* by intestinal and immune cells [152,154], immune-relevant mRNAs targets of differentially expressed human miRNA homologues were identified to assess a potential influence on the “farm milk effect”.

In total 52 miRNAs showed significant expression differences between UHT and PAS samples, while differential expression between FAT und PAS amounted to 25 miRNAs (see Table E5 of Kirchner et al., Appendix II) [183]. Most promising targets for miRNA regulation were determined by number of target sites of interfering miRNAs, due to the accumulative effect of simultaneous repression by multiple miRNAs (Table 3) [60]. Genes potentially repressed by three or more miRNAs exhibited high relevance in asthma and allergy related physiological processes. Signal transducer and activator of transcription 3 (STAT3) executes fundamental functions in innate immunity and has been associated with impaired lung function in asthma [218]. Furthermore, STAT3 interacts with the cytokine interleukin 6 (IL6) and regulates its pro-inflammatory effect on dendritic cells [219]. Translational repression of interleukin 13 could relieve T helper cell mediated inflammation, a major cause of asthmatic symptoms [219,220] while prostaglandin-endoperoxide synthase 2 (PTGS2) is a factor involved in the synthesis of pro-inflammatory eicosanoids [221]. Finally, interferon γ (IFNG) and its receptor (IFNGR) were implied in deterioration of asthma symptoms during acute virus infections and chronic airway obstruction [222].

Gene symbol	Gene name	Interfering miRNAs
STAT3	Signal transducer and activator of transcription 3	let-7e-5p, miR-20a-5p, miR-21-5p, miR-92a-5p, miR-93-5p, miR-125b-5p, miR-155-5p
PTGS2	Prostaglandin-endoperoxide synthase 2S	let-7b-5p, miR-16-5p, miR-26b-5p, miR-101-3p, miR-128-3p, miR-181a-5p
IL13	IL-13	let-7d-5p, let-7f-5p, let-7g-5p, let-7i-5p, miR-98-5p
IL6	IL-6	let-7a-5p, miR-26a-5p, miR-365a-3p, miR-98-5p, miR-155-5p
IFNG	IFN-γ	miR-15a-5p, miR-16-5p, miR-26b-5p, miR-29b-3p
IFNGR1	IFN-γ receptor 1	miR-98-5p, miR-155-5p, miR-181b miR-196a

Table 4: mRNA targets of differentially expressed miRNAs. Reprinted from Kirchner et al. (Appendix II) [183].

Milk-derived miRNAs are not the only class of immune relevant regulators implied in the “farm milk effect”. Accompanying our study on miRNAs were two additional investigations on whey proteins and fatty acids conducted on the same initial milk samples [223,224]. Similarly, detrimental effects of high heat treatments could be observed for whey proteins, with a reduction to about 50% of detectable total proteins.

Furthermore, a number of particularly heat sensitive proteins were identified including lactoferrin, which is a potent stimulator of the immune system and prevents overburdening reactions to microbial infections [225] and lactoperoxidase which was shown to relief airway damages and inflammation in asthmatic patients [226]. An even stronger association of milk components to “farm milk effect” was found for polyunsaturated fatty acids (PUFA) and especially ω-3 PUFAs [223]. Partial fat skimming, as is common in industrial milk processing, was characterized with a substantial decrease in ω-3 PUFAs and an increase in ω-6/ ω-3 PUFA ratio which is of particular importance since ω-3 PUFAs interfere with the production of pro-inflammatory leukotrienes from ω-6 PUFAs [227]. Due to the highly complex composition of bovine milk, an entanglement of influences from individual milk components is extremely difficult. Industrial milk processing aggregates these effects further by simultaneously altering fat content and affecting heat sensitive molecules. However, findings of beneficial association of different milk components might not be in conflict to each other as effects could be cumulative or even multiplicative through interactions. A recently started intervention study, the MARTHA trial, aims to combine the lessons we learned from milk treatments in a pragmatic and more promising approach by using a minimally processed, full-cream milk that is nonetheless microbiologically safe [228].

3.6 EV populations from blood and colostrum are highly divergent

The potential uptake of dietary miRNAs has caused great excitement in the scientific community as the implications of a general absorption would be immense [229]. The association with EVs provides dietary miRNAs with a very plausible tool for inter-animal and even inter-species transfer as studies *in vitro* have shown [92,149,152]. One of the most promising sources of such EV-associated miRNAs is milk, which was proven to be highly enriched in both, EVs and immune-relevant miRNAs, suggesting importance beyond nutritional value [147,148]. To evaluate a potential uptake of milk-derived EVs and their cargo, we assessed EVs in the circulation of newborn calves before and after their very first feeding. This approach bears many advantages, as colostrum is even more enriched in miRNAs and EVs than milk from later lactation stages [230] and calves were not in contact with any other sources of dietary EVs priorly.

Discernment of EV uptake from any source relies on extraction of uncontaminated vesicles and extensive characterization of EV populations. To minimize influence of co-isolated molecules, we isolated EVs by differential ultracentrifugation followed by separation by density on a SDG [184]. Although EV preparations of blood and colostrum from adult cows were uniform in size and in line with previous findings in bovines (Figure 16A) [231], significant differences in density, concentration and protein composition could be found between the biofluids. Even though, EV preparations from SDG fractions of 30% as well as 40-50% sucrose were in the density range expected of exosome-like vesicles (1.1270–1.2296 g/ml) [232], colostrum was significantly enriched in denser EVs by more than 10-fold while blood EVs accumulated mostly in the lower density fraction (Figure 16B).

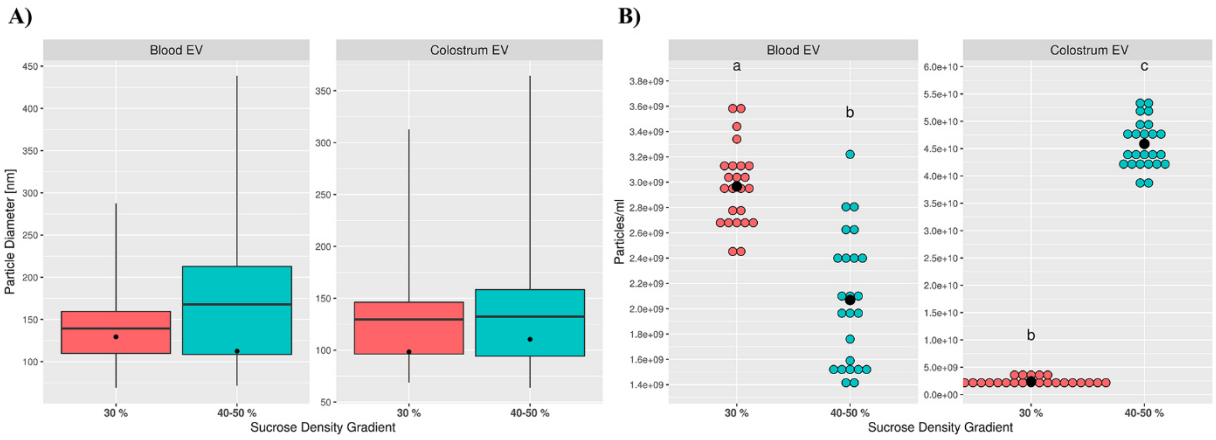

Figure 16: Particle size and abundance of extracellular vesicles in blood and colostrum from adult cows. (A) Particle sizes showed no significant differences between SDG fractions and biofluids. Whiskers indicate 1st and 99th percentiles; line: mean diameter; dot: modal diameter. (B) Particle concentration of higher density colostral EVs was significantly higher by more than 10-fold compared to EVs from the 30% SDG fraction and all blood EVs. Black dots indicate mean particle numbers and letters indicate sificantly different particle concentrations. Figure reprinted from Kirchner et al. (Appendix III) [185].

Additionally, EV isolations were characterized regarding the expression of protein markers regularly found in exosomes as well as proteins thought to be colostrum specific (Figure 17). EV preparations displayed correct expression of exosomal proteins to prove endosomal origin (tetraspanin CD63, heat shock protein 70 HSP70) and were negative for cellular fragments as determined by a marker associated with the endoplasmatic reticulum (calnexin, CNX) as proposed by the guidelines for minimal

information for studies of extracellular vesicles (MISEV) [213]. Further differences between colostral and blood-derived EVs were found in the expression of butyrophilin (BTN1A1) as well a lactadherin (also called milk fat globule-EGF factor 8, MFGE8), which could only be detected in colostrum. While BTN1A1 is so far exclusively described in studies on EVs derived from human or bovine milk [233,234], MFGE8 expression was previously described in EVs from various tissues and biofluids. Missing signal for MFGE8 in blood could stem from low starting input but was believed to be genuinely missing from SDG fraction of 40-50% sucrose.

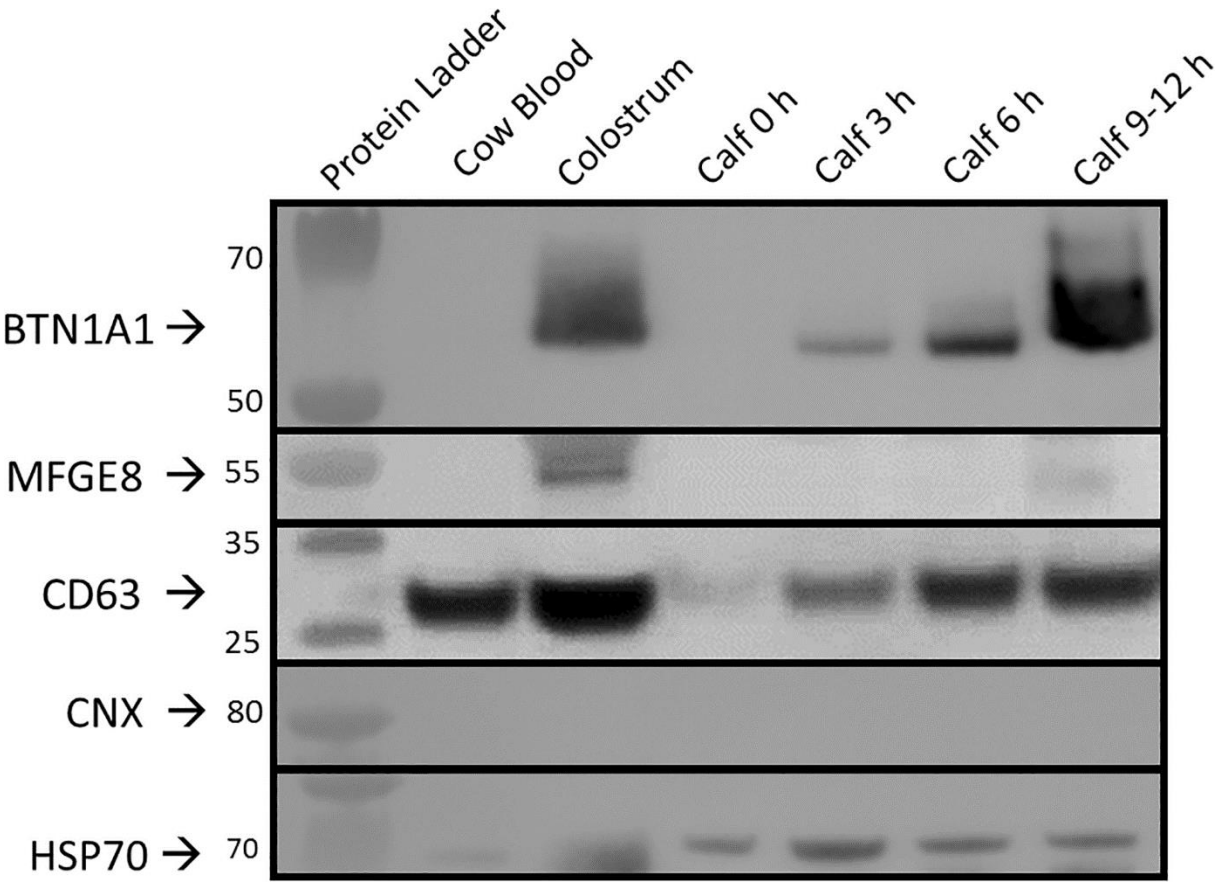

Figure 17: Protein abundances of blood and colostral EVs measured by Western blot. All EVs preparations were positive for exosomal markers CD63 and HSP70 as well as negative for CNX, a marker for cellular contamination. Expression for BTN1A1 and MFGE8 was specific for colostral EVs in adult cows but showed progressive increase in calf blood EVs postprandially. BTN1A1: butyrophilin; MFGE8: lactadherin; CNX: calnexin; HSP70: heat shock protein 70. Figure reprinted from Kirchner et al. (Appendix III) [185].

Completing the picture of distinct EV populations in blood and colostrum were results from miRNA expression analysis by small RNA NGS. miRNA profiles between blood and colostrum derived samples differed greatly with more than 150 significantly

different expressed miRNAs for all three compartments (unfractionated colostrum, cells and EVs) (see Table 1 in Kirchner et al., Appendix III) [185] and were in accordance with previous reports on bovine EVs [147,235,236]. In contrast, colostral EVs showed little to no differential expression between whole colostrum and colostral EVs, suggesting that the majority of miRNA is extracellular and protected by vesicles.

3.7 Postprandial increase of EVs bearing colostrum characteristics in calf blood

Having found molecular features distinguishing colostrum-derived EVs from vesicles in blood, we characterized EV levels in calf plasma before and after first feeding. Particle diameters were comparable to EVs from adult cows and displayed no significant differences between SDG fractions or time points, highlighting high reproducibility of vesicle preparations by ultracentrifugation and SDG (Figure 18A). Particle concentrations measured from 30% SDG, the sucrose fraction which was highly enriched in blood EVs in adult cows, were constant over all time points. Interestingly, vesicles from calf plasma with the same density of colostral EVs (40-50% sucrose) showed a significant increase in all postprandial time points with the highest concentration of particles measured directly before second feeding (Figure 18B). Comparable to colostral EVs, vesicles from calf blood were also significantly enriched in denser EVs for all time points.

Similarity of postprandial EVs in calf blood to colostrum vesicles was also corroborated by protein composition (Figure 17). Expression of positive and negative markers on all time points indicated exosomal origin of EVs similar to adult cows. Furthermore, a clear increase in expression in a time-dependent manner was found for colostrum-specific BTN1A1 and to a lesser extent MFGE8 as well as for the general exosome marker CD63, which was closely matching findings in EV concentrations by NTA. Diminished expression of MFGE8 compared to BTN1A1 discourages the notion of co-expression of these proteins on the same vesicles and suggests the existence of different exosomal sub-populations in colostrum with selective uptake mechanisms. Delayed

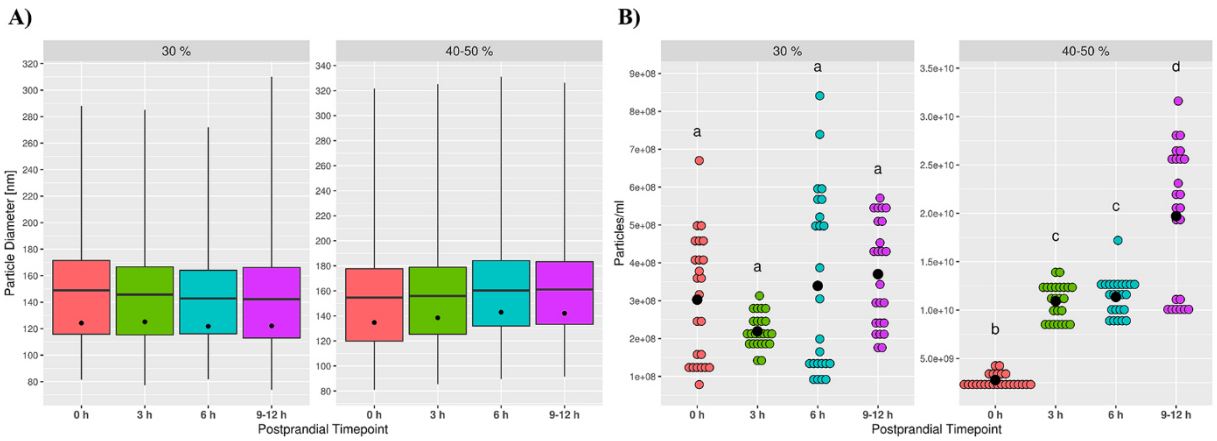

Figure 18: Particle size and abundance of extracellular vesicles in blood from newborn calves. (A) Particle sizes showed no significant differences between SDG fractions and measuring time points. Whiskers indicate 1st and 99th percentiles; line: mean diameter; dot: modal diameter. (B) Particle concentrations in 30% sucrose were constant, while particle numbers in 40-50% were significantly increased for all time points compared to pre-feeding levels. Black dots indicate mean particle numbers and letters indicate sificantly different particle concentrations. Figure reprinted from Kirchner et al. (Appendix III) [185].

appearance of MFGE8 in calf blood could then be explained by differing transfer efficiencies into the circulation or prolonged retention of EV subpopulations in intestinal epithelial cells.

On first glance, expression profiles from small RNA NGS were further supporting the hypothesis of EV cargo transfer from colostrum into the circulation of calves. miRNA expression was highly divergent between all three calf blood compartment with more than 100 differently expressed canonical miRNAs between each, revealing large differences between the intra- and extracellular space. Expression changes related to progressing time points however, could only be detected in calf EV samples (see Table 2 in Kirchner et al., Appendix III) [185]. Resemblance of calf EV expression profiles to colostral samples was further confirmed by hierarchical clustering (Figure 19). Although small RNA NGS could clearly differentiate between individual colostrum or blood compartments, the largest sub-clusters were formed by colostrum and calf blood EV samples on one side and samples derived from unfractionated blood and blood cells on the other. Uniformity over postprandial time points of non-EV samples as seen in differential expression analysis was reinforced by clusters associated to individual animals instead of elapsed time since feeding.

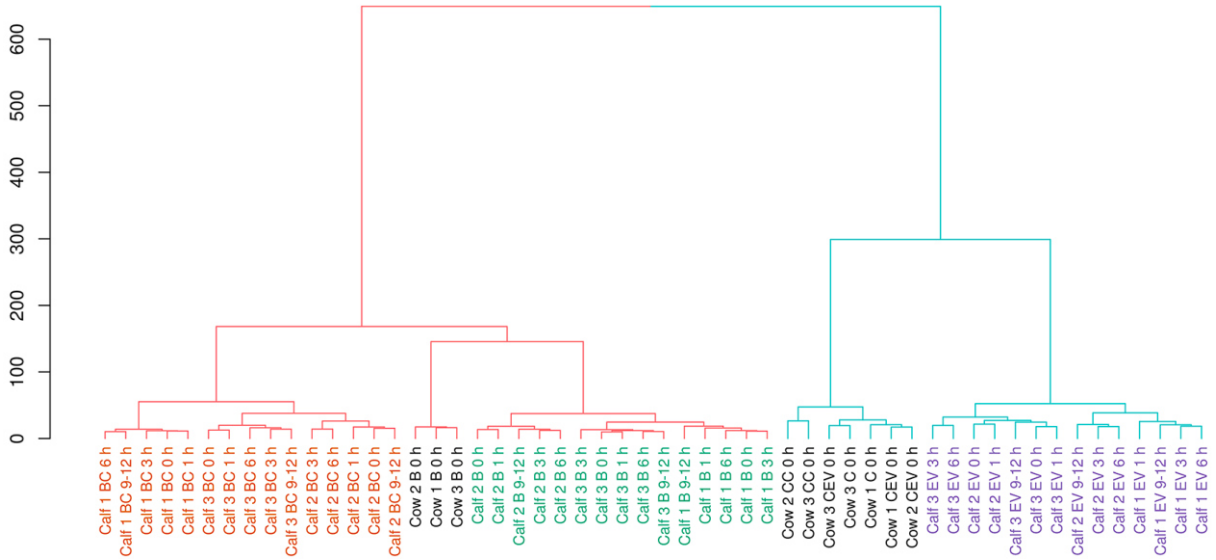

Figure 19: Hierarchical clustering analysis of expression profiles of canonical miRNAs. Clustering was driven by similarity of calf EVs to colostral samples compared to samples from unfractionated blood or blood cells. B: unfractionated blood; BC: blood cells; EV: blood extracellular vesicles; C: unfractionated colostrum, CC: colostral cells; CEV: colostral extracellular vesicles. Figure reprinted from Kirchner et al. (Appendix III) [185]

3.8 isomiR analysis of postprandial EVs shows no association to colostrum-derived miRNAs

Although canonical miRNA profiles in calf EV seemed to support an uptake of colostral EVs after first feeding in newborn calves, results might be misleading. Association between colostral and calf EVs was driven dominantly by a single miRNA family consisting of three members: miR-200a, miR-200b and miR-200c. Furthermore, differential expression analysis of canonical miRNAs between postprandial time points revealed inverse regulation for nearly half of the miRNAs. To assess whether miRNA detected in calf blood EVs could stem from dietary EVs or were actually endogenously produced, we performed in-depth analysis of isomiR profiles. isomiR specific expression analysis confirmed dominant trends seen in hierarchical clustering of canonical miRNAs by clearly separating between samples of whole blood or blood cells and colostral as well as calf EV specimen by unsupervised clustering via PCA (Figure 20). While this explained the largest part of the variance in our data set (~60%), the second largest influence could be attributed to differences between colostral samples and calf blood EVs now. Additionally, analysis of miRNA isoforms enabled separation

of calf blood EVs in a time-dependent manner with samples taken before feeding and earliest time points clustering closest to other blood compartments in calves.

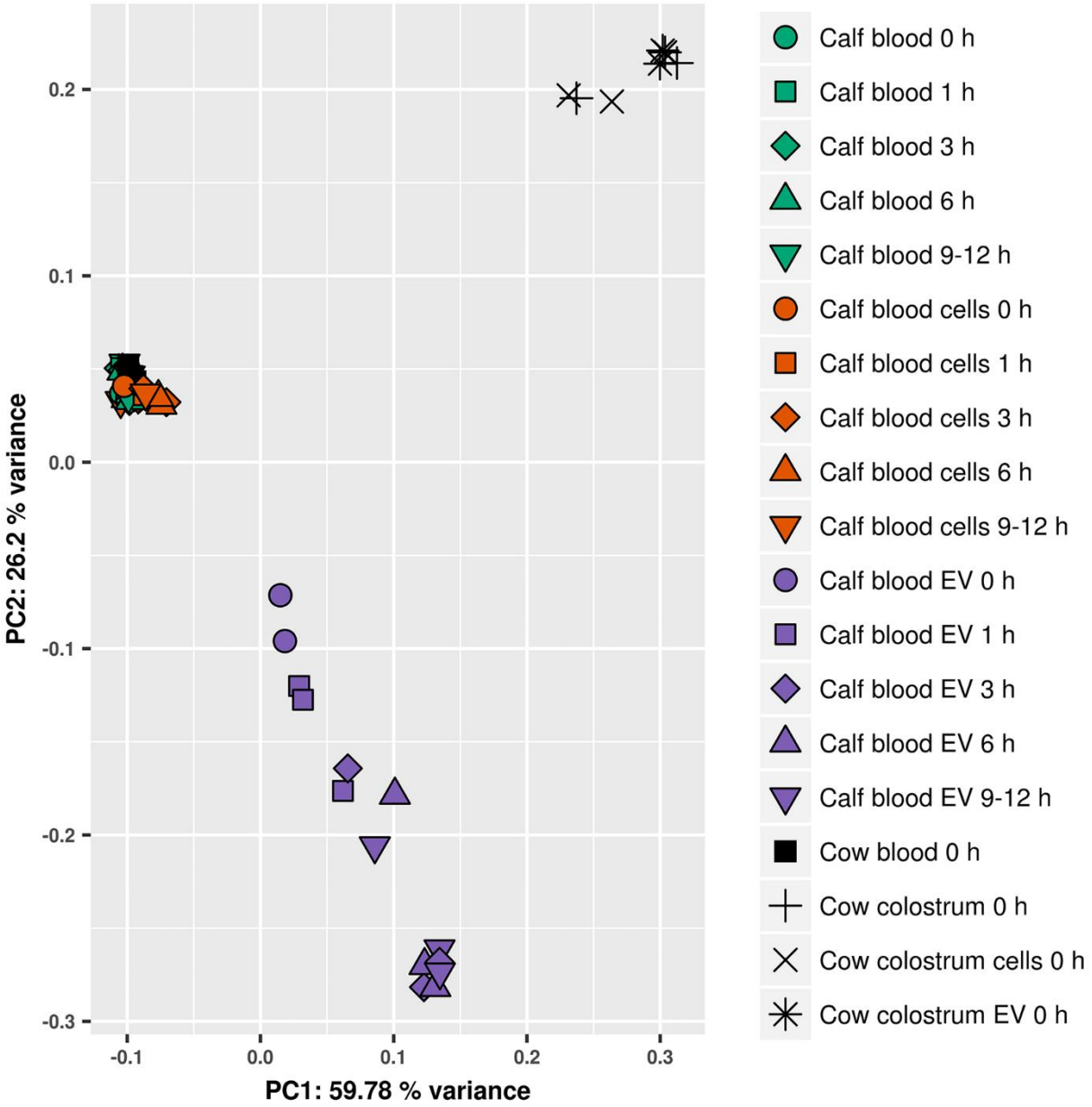

Figure 20: Principal component analysis based on expression of miRNA isoforms. Sample compartments are indicated by different colors and time points are represented by different symbol shapes. Figure reprinted from Kirchner et al. (Appendix III) [185]

Differential expression of significantly up-regulated isomiRs unfortunately only displayed limited overlap to prominent isoforms detected in colostral EVs or colostrum in general. Matching expressions were found for isomiRs belonging to the miR-200 family but the most highly abundant isomiRs in colostral EVs were missing in calf EV

profiles, suggesting endogenous origin of these miRNAs. Implications of differently regulated miRNAs in postprandial EVs was assessed by pathway analyses of experimentally verified mRNA targets. Highest enrichment of mRNA targets was shown for insulin signaling, proposing a role of calf EVs in the processing of signals from food-related energy uptake in recipient cells. This notion is backed by the well-known role of miR-200a/b/c, the only miRNAs displaying significant overlap between calf and colostral EVs, in the regulation of FOG2 and Rheb among others, that play a crucial role in mediating insulin signalling [237–239].

4. Conclusion

The discovery of miRNAs and their critical role in nearly all physiological pathways has greatly deepened our understanding of post-transcriptional regulation of gene expression and how a lack of fine-tuning or outright dysregulation can have severe pathological effects. Driven by advancements in sequencing technology in the last decade that finally saw the affordability of holistic sequencing for transcriptomes, interest in miRNAs has continually risen and their capability in diagnostic, prognostic and even therapeutic applications seems unabated. The advent of NGS technologies not only revealed their potential as biomarkers, molecular identifiers of disease or physiological states, but also unearthed the inherent heterogeneity in their sequences, leading to isoforms termed isomiRs. Adding another layer of complexity to the already intricate network of miRNA-mRNA interactions, isomiRs were shown to be involved in subtly altering targeting repertoires and turnover rates of miRNAs while displaying even higher specificity in their distribution patterns.

Special interest in the clinical use of miRNAs is generated by their ready availability from liquid biopsies which present a minimally invasive source of biomarkers and are the foundation for the promise of individualized, precision medicine. Propelled by the uncovering of their association to extracellular vesicles such as exosomes, extracellular miRNAs have become the most promising target for analysis in a variety of research questions. EVs are crucial mediators of intercellular communication and possess a number of qualities that predestine them as carriers of molecular information. miRNAs in EVs are specifically enriched for their physiological context, stable in biofluids such as blood and milk and their molecular composition reflects their secreting cells as well as enables directed transfer to recipient cells. Although our understanding of the biodistribution and uptake of EVs is still incomplete, their ability to transfer cargo across tissue and even species boundaries was demonstrated impressively *in vitro*. Unfortunately, the functional relevance of exogenous EVs *in vivo* remains elusive although first studies are showing promising results.

To facilitate correct assessment and validation of miRNAs and their isoforms from EVs and other sources, two complementary bioanalytic analysis platforms were developed and implemented into established miRNA analysis workflows. isomiRROR offers the

ability to detect miRNA isoforms in any species independent of existing genomes with high specificity, an analysis previously only available for studies in humans or mice or for scientists well versed in coding and computational analysis. miREV on the other hand, addresses the lack of a comparability in many EV-related studies by providing uniformly processed readcounts, visualizing possibly confounding differences in experimental setups and providing candidate lists for potential miRNA references for RT-qPCR validation. Furthermore, this work aimed at identifying the functional importance of miRNAs from bovine milk and blood. Implications for miRNA uptake from nutritional sources are serious and the presence of dietary EVs in milk and colostrum has fueled many hypotheses on their postprandial distribution.

One proven example of nutritional influence on the immune-system and health in general is the “farm milk effect”, that describes the significantly reduced prevalence of asthma in children living on farms and consuming raw milk and in which miRNAs were proposed as a possible effector molecule. Physical and thermal milk processing was shown to directly affect stability of miRNAs with high heat treatments drastically reducing miRNA abundances compared to raw milk or milk from moderate heat treatments such as pasteurization. Although, targets for differentially affected miRNAs included multiple mRNAs relevant for asthma development or severity, studies on other milk components have shown similar or even higher correlation of whey proteins and PUFAs with the beneficial outcome of milk consumption on the same milk samples. To evaluate the potential of postprandial uptake of dietary EVs more directly we then characterized colostrum-derived EVs and their cargo in their intended recipients, newborn calves. We discovered distinct populations of EVs in blood and colostrum that could be separated based on density, protein composition and miRNA expression profiles. Calves displayed a consistent increase of vesicles bearing morphological and protein characteristics of colostral EVs after feeding but in-depth analysis of isomiR profiles revealed little overlap between expression profiles and identified endogenous biogenesis as the likeliest origin of detected miRNAs. Differential uptake of protein and miRNA cargo in our study implied the presence of distinct exosomal subpopulations and the possibility of repackaging of protein cargo into new vesicles.

Our findings advocate the tremendous potential of miRNAs associated with EVs especially from milk as sources of biomarkers as well as biologically active compounds and highlight the yet undiscovered or unaccounted heterogeneity of vesicles and cargo composition. By providing a framework for novel identification of miRNA isoforms and the ressource-efficient detection of potential miRNA references, future studies are well equipped to build upon our results and further elucidate the function of intra- and extracellular miRNAs in biofluids.

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List of scientific communications

Published software

1) Kirchner, Benedikt
isomiRROR – isomiR references, overviews and readcounts
https://gitlab.lrz.de/Physio/isomiRROR

2) Hildebrandt, Alex; Kirchner, Benedikt
miREV – miRNA references of extracellular vesicles
http://141.40.217.80:3838/miREV/

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In: *The Journal of allergy and clinical immunology* 137 (6), 1893-1895.e13. DOI: 10.1016/j.jaci.2015.10.028.

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1) Pfaffl, Michael; Kirchner, Benedikt
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2) Kirchner, Benedikt; Paul, Vijay; Pfaffl, Michael
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1) Kirchner, Benedikt; Benes, Vladimir; Hahn, Alexander; Pfaffl, Michael
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2) Kirchner, Benedikt; Ege, Markus; Pfaffl, Michael
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Differential uptake of colostral EV protein and miRNA cargo in neonatal calves
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Universal reference transcripts for miRNA normalization – a meta-analysis on human blood EV RNA-seq data sets
Annual Meeting of the International Society of Extracellular Vesicles (ISEV), June 2019, Japan

Appendix

Appendix I:

Hildebrandt, Alex; Kirchner, Benedikt; Nolte-'t Hoen, Esther N. M.; Pfaffl, Michael W. (2021)

miREV: An Online Database and Tool to Uncover Potential Reference RNAs and Biomarkers in Small-RNA Sequencing Data Sets from Extracellular Vesicles Enriched Samples.

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Appendix II:

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microRNA in native and processed cow's milk and its implication for the farm milk effect on asthma.

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Appendix III:

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Appendix I

Substantial Contributions by Benedikt Kirchner:

- Software for miRNA alignment
- Conceptualization
- Supervision
- Debugging
- Writing – review and editing

Benedikt Kirchner 

Michael W. Pfaffl 

miREV: An Online Database and Tool to Uncover Potential Reference RNAs and Biomarkers in Small-RNA Sequencing Data Sets from Extracellular Vesicles Enriched Samples

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Abstract

Extracellular vesicles (EVs) are nano-sized, membrane-enclosed vesicles released by cells for intercellular communication. EVs are involved in pathological processes and miRNAs in EVs have gained interest as easily accessible biomolecules in liquid biopsies for diagnostic purposes. To validate potential miRNA biomarker, transcriptome analyses must be carried out to detect suitable reference miRNAs. miREV is a database with over 400 miRNA sequencing data sets and helps the researcher to find suitable reference miRNAs for their individual experimental setup. The researcher can put together a specific sample set in miREV, which is similar to his own experimental concept in order to find the most suitable references. This allows to run validation experiments without having to carry out a complex and costly transcriptome analysis priorly. Additional read count tables of each generated sample set are downloadable for further analysis. miREV is freely available at https://www.physio.wzw.tum.de/mirev/.

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Introduction

Cells release EVs from endosomal compartments or shed them from the plasma membrane. EVs include exosomes, larger microvesicles, and apoptotic vesicles released upon cell death and even more vesicle subpopulations. They can be found in various types of tissues and body fluids of most eukaryotic species.[1] Different methods are available for EV isolation, with differential ultracentrifugation (dUC) being the most widely used method. Additional isolation techniques include methods based on precipitation, filtration, buoyant density centrifugation or chromatography.[2,3] The purification of EVs from complex biofluids is a challenge. To generate reliable and comparable EV based microRNA expression data, suitable EV isolation and characterization techniques must be applied. The international standard in EV research, the MISEV guidelines, recognize that there is no consensus on an optimal or best isolation method to get pure EVs.[4] The choice of the method depends on the research question at hand and the downstream analyses. A detailed documentation of the applied methods and the characterization of EVs is important for the reproducibility of results. To be able to interpret EV-based meta-analyses correctly, all relevant aspects of a study must be summarized and made available as meta information. By considering this information, studies with different methodologies can be compared. In addition, studies with the same or similar characteristics can be combined so that the statistical power increases. Individual characteristics of EV-studies can also be weighed for different results through exploratory data analysis.

EVs play an important role in intercellular communication through the transfer of cytosolic proteins, lipids and different types of nucleic acids, i.e. miRNA, lncRNA, mRNA, and DNA.[1] In the past, numerous studies have repeatedly pointed out the high potential of EVs as a source of biomarker molecules and how EVs in liquid biopsies could be used to develop minimally invasive diagnostic protocols.[5–11] Transcriptome analysis of EVs has mostly focused on miRNAs.[12] These short non-coding RNAs play an important role in the post-transcriptional regulation of hundreds of biological processes and pathways. An atypical level of specific, single miRNAs or an altered miRNA pattern in body fluids can indicate the presence or progression of pathophysiological processes or diseases.[13–15] In this context, it should be mentioned that extracellular RNA profiles from body fluids are not exclusively due to the RNAs in EVs. Extracellular RNAs (exRNA) can also be present in protein complexes and lipoprotein particles.[16] Most EV isolation methods enrich for EV RNAs but also allow co-isolation of other exRNAs carrier.

Candidate miRNA biomarker signatures discovered in small-RNA sequencing experiments require additional validation by reverse transcription followed by real-time quantitative-PCR (RT-qPCR). The relative expression confirmation by RT-qPCR needs stably expressed miRNA reference transcripts for reliable normalization between patients or treatment groups. These reference miRNAs can be identified either by performing a transcriptome analysis through next generation sequencing (NGS), which is time-consuming and cost-intensive, or by searching for stably expressed transcripts in extensive small-RNA sequencing data sets. When using such published miRNA references, it must be ensured that these are from the same tissue type or disease as used in the new experiment. Additionally, a comparable experimental setup and workflow must be adopted. For EV-RNA analysis, important experimental parameters and confounding methodological factors are the investigated tissue type, sample handling, and the applied EV isolation method, because RNA yield and resulting miRNA profiles strongly depend on these factors.[17,18]

We here present miREV, which can be used as a database and online tool to find stable candidate reference miRNAs within a context of choice. miREV can help scientists to save valuable resources and validate their hypotheses more quickly. We here introduce miREV and show it can be used to search for stable reference miRNA candidates using the 428 small-RNA sequencing data sets of EVs currently present in the database.

Results and discussion

The transcriptional profile of a sample is strongly influenced by the pre-analytical and experimental conditions under which the sample was acquired. Preanalytical variables, EV isolation and RNA extraction methods are as important as tissue of origin and disease state of the host.[17] miREV enables scientists to investigate the extracellular miRNA transcriptome of samples and helps estimating technical as well as biological variances for selected sample sets. Figure 1 shows an example of some result plots that can be created with miREV.

(a) Sample set creation: After user of miREV has created a sample set by the selection of variables the sample composition is displayed through tables as well as through donut plots. **(b) Clustering data according to groups:** PCA plot of a sample set of 112 data sets. 47 datasets were from patients with colon carcinoma, 16 from patients with subclinical depression and 49 from healthy volunteers. Data was normalized by median ratio of expression and transformed with the variance stabilizing transformation method. The 100 microRNAs with the greatest variance were selected for the calculation of the principal components. **(c) Finding suitable reference miRNAs:** A bar plot summarizes the most occurred stably expressed transcripts of the result lists for the sample set chosen. To compare outcome of different stability measure algorithms as well as of different normalization strategies an overlap analysis is available in miREV. **(d) Downstream analysis:** Heatmap of most significant differential expressed microRNAs. This heatmap represents the clustering results based on the significant differential expressed microRNAs found with the DESeq2 package. Results where filtered with these thresholds: adjusted p-value: padj. < 0.1; log 2-fold change: log2FC > |1|. The selected sample set has 71 data sets, including 59 serum samples from healthy patients and 12 serum samples from patients diagnosed with sepsis. None = healthy patients; sepsis = sick patients.

Sample set creation

First, the user of miREV need to create an individual sample set. This can be done by selecting experimental variables. EV-isolation method, biofluid and disease type have several selections available. A sample set is created based on this selection. The composition is shown using four donut plots (Figure 1(a)). The percentage of the sample set related to the three experimental variables and the EV characterization method used for the samples is

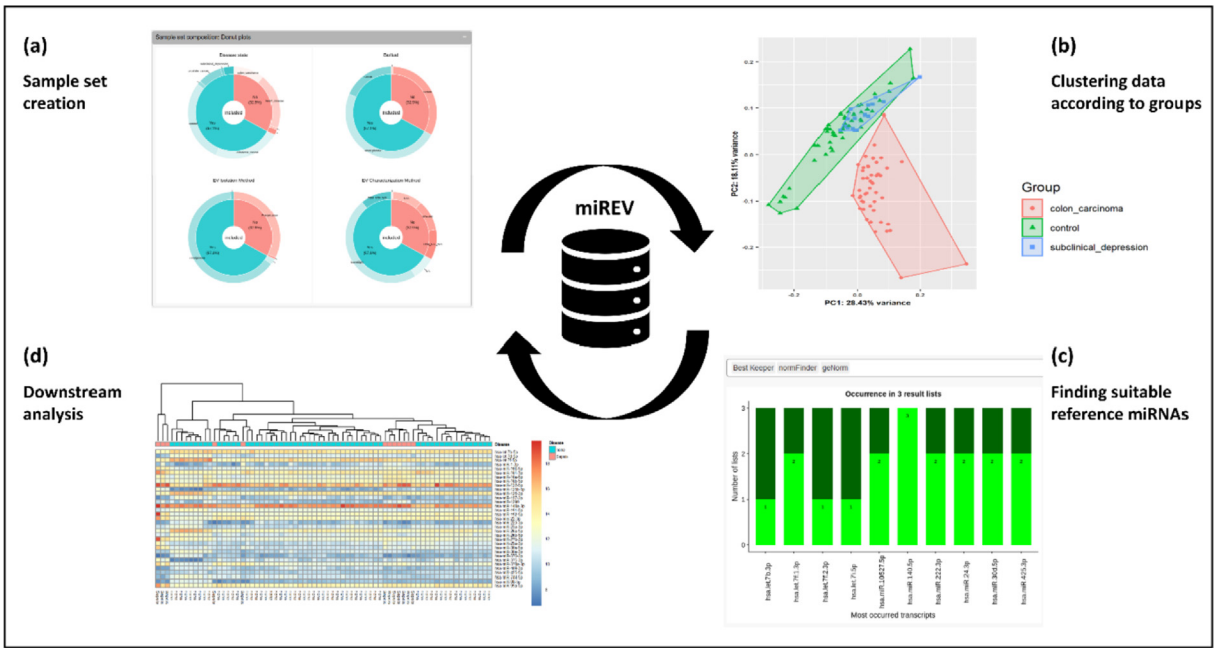

Figure 1. Exemplary result plots of miREV and a downstream analysis.

shown here. The number of samples per selection is also shown as a table in miREV for each experimental variable.

Clustering data based on metadata selection

Initial assessments can be quickly made by visualizing miRNA profiles in a principal component analysis (PCA). Figure 1(b), (Figure S3) is showing an example of the clustering of a sample set in relation to their disease status. 112 data sets are included. 47 were from patients with colon carcinoma, 16 from patients with subclinical depression and 49 from healthy volunteers. For all samples, EVs were isolated by precipitation from serum. The PCA plot shows a separation of the two diseased groups on the second component, responsible for 18.11% of total variance. In comparison, healthy controls and colon carcinoma patients are clearly separated when the first 2 components are considered, highlighting the classification potential of miREV. With a subsequent differential expression analysis (DEA), differentially expressed transcripts can be identified and further investigated. The necessary raw read counts and metadata are available to the user. To avoid misinterpretations of the PCA, one should pay attention to which experimental setup has been chosen. The sample set should differ in only one variable. For example, one cannot say anything about the origin of a variance if one has different isolation methods and different diseases in the one selected sample set. Since the PCA plot only shows one of the variables, no information about the other is visible and the variance is difficult to assign. Another possible application example would be the investigation of batch effects. These can be caused by the use of different isolation methods or biofluids for the samples within the selection. To be able to identify a batch effect with certainty, the largest possible number of data records is required. For now, the database in miREV does not provide enough statistical power to be able to carry out such investigations. However, this will be possible with the addition of new data sets.

Finding stably expressed miRNA as potential endogenous reference candidates

The quantification of miRNA or mRNA gene expression has become a standard procedure to make statements about various cellular and pathological processes. Although NGS has arrived in many laboratories, RT-qPCR is still considered the gold standard for determination of gene expression levels of a sample. The reason for this lies within the sensitivity, accuracy and speed combined with a comparably low price of this technique. Accuracy of RT-qPCR analysis, however, depends on the use of appropriate internal controls. In expression analyses, stably expressed transcripts are utilized as internal controls, according to the MIQE guidelines.[19] The normalization with these endogenous references makes it possible to compare biological samples from different contexts. Different normalization methods can be evaluated for their accuracy, precision and/or over-fitting[20] and the degree to which they influence data analysis depends, amongst others, on the data distribution.[21] miREV offers the opportunity of choosing from 6 common

normalization methods and combining them with 3 stability algorithms. This means that a total of 18 result lists are available for each selected sample set. A result list is a selection of potential reference miRNAs in descending order according to their respective stability values in the sample set. Each result list can be displayed individually or combined to review consensus results of different normalizations or stability measurements. A maximum of 30 miRNAs are listed, displaying only transcripts with the best values for stability. As an example, Figure 1 (c) and Table S4 shows the result of potentially, stably expressed miRNAs for all data sets integrated in miREV so far and lists how often individual miRNAs appear in the 18 result lists. Some of the listed miRNAs have already been described in various human studies as endogenous, stably expressed exosomal or extracellular vesicle derived RNAs. For instance *miR-103*, *miR-221* and *let-7a* have been mentioned in studies working with exosomal RNAs obtained serum with hepatocellular carcinoma, hepatitis B, and healthy patients.[22,23] Members of the *miR-30* family, such as *miR-30a* and *miR-30e*, are mentioned in studies in which the exosomal RNA was obtained from plasma.[24] Also *miR-425* is mentioned as a reference miRNA in EVs from adipose derived mesenchymal stem cells.[25] One could easily assume that the miRNAs with the highest frequency in that table can be used as universal RT-qPCR references. However, it should be noted that the stability algorithms also evaluate how often a miRNA is present within the samples of the sample set. Accordingly, a miRNA that is present in all samples of the selected sample set can be ranked as more stable than a miRNA that is only present in part of the sample set. The expression variance of the poorly ranked miRNA can, however, be smaller than that of the ubiquitous for the part of the sample set in which it is present. For example, some of the most common miRNAs in the result list (Table S4) are mentioned as differentially expressed in a wide variety of contexts. *miR-30d-5p*, for example, is discussed as an EV associated miRNA in connection with rectal cancer[26] and ovarian cancer[27] and as a regulator of conceptus-uterus interactions.[28] These examples should make clear that the result lists in miREV must always be seen in the biological context of the sample set. Top listed, potentially stably expressed miRNAs should be used as a starting point. Validation in each specific experimental setup or study is required. Information on the biological context in which the potentially stably expressed miRNAs are mentioned can give first indications about the usability.

Downstream analysis of raw read counts

miREV can be used for discovery of differentially expressed miRNAs in various diseases. This is facilitated by the fact that all data sets in miREV were processed using a standardized alignment and annotation pipeline.[29] The generated raw read counts can be downloaded for any selected sample set. The additionally available metadata makes it possible to carry out downstream analyses such as DEA or pathway analysis. Due to the uniform processing, a wide variety of sample sets can be compared with one another. There is no need for time-consuming creation of annotated read count tables. Results of an exemplary DEA are shown in Table S5 and Figure 1(d), (Figure S6). The selected sample set has 71 data sets, including 59 serum samples from healthy patients and 12 serum samples from patients diagnosed with sepsis. Since discussing all differences in miRNA abundance in this sample set would go beyond the scope of this paper, only some miRNAs that have already been associated with sepsis are highlighted. For example, *miR-223-3p* was found to be a potential biomarker for sepsis in circulating miRNAs mentioned by Zhang et al.[30] Expression levels of *miR-223* and *miR-122* are described by Wang et al. as significant higher and lower than in controls.[31] miREV does not only offer the possibility to check whether results can be reproduced in a similar context. Additional information relevant to the disease can be found. As a result of the exemplary DEA, further differentiated expressed miRNAs are listed. For example, *miR-26a-5p* is more highly expressed in the selected sample set and *miR-100-5p* is less than in the control group. The results of such a DEA can provide the user with useful additional information for their own studies. In addition, there is the possibility of comparing results and thereby strengthening a hypothesis or re-evaluating it.

Materials & methods

All data sets and calculations available in miREV were processed in advance to ensure quick response times to queries. Individual tasks of the calculation pipeline are described below and shown in Figure 2.

(a) Mapping: The mapping step processed raw NGS data sets to miRNA count tables. Data sets mapped with less than 7% to reference miRNAs were removed. **(b) Sample set creation:** Data sets were split in all possible combinations of the experimental variables. Each combination is one sample set. Resulting sample sets with less than 10 samples were removed. Additionally, miRNAs that did not pop up in at least 95% of each sample set were excluded. **(c) Normalization:** Each sample set was normalized according to six different normalization methods. **(d) Stability Calculation:** Finally, stably expressed miRNAs were determined with three different stability algorithms. For each sample set reference miRNA lists (result lists) were calculated, each representing the top 50% ranked stably expressed miRNAs.

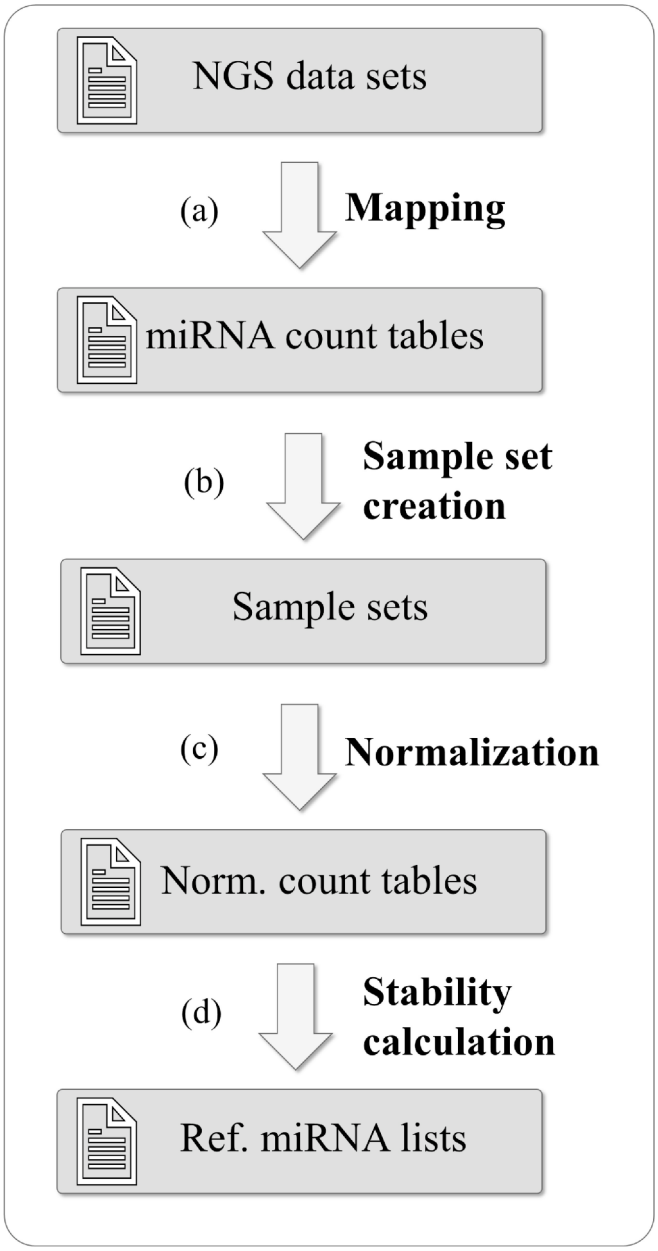

Figure 2. Calculation pipeline workflow, from raw NGS data sets to stably expressed miRNAs.

Data collection and count table generation

Currently the whole database focuses on human blood derived EVs and includes 9 different diseases and 3 different isolation methods from both serum and plasma. Appropriate and well-annotated small-RNA sequencing data sets (one data set represents all small-RNA sequencing reads of one biological sample) of circulating EVs for this meta-analysis were obtained from the gene expression omnibus database GEO.[32] Additionally, various in-house data sets as well as data sets from collaborators were added, resulting in a total of 654, which represent the starting basis of our analysis. S1 summarizes the origin of the data sets including details on EV isolation and characterization. Raw data was processed by an established alignment and annotation pipeline.[29] In brief, raw sequences were trimmed with btrim32[33] (version 0.3.0). Quality of NGS outcome was analysed for each sample with FastQC[34] (v0.11.9). The alignment of reads was performed with bowtie[35] (v.1.2.3) with a cut off for reads set to maximum one mismatch. Furthermore, parameters that limit alignment to the sense strand (--norc) and output to the single best match in terms of mismatch quality (--best) were applied. References of non-coding RNA sequences for rRNA, snRNA, snoRNA and

tRNA were downloaded from RNACentral[36] (release 12). miRNA references were obtained from miRBase[37] (release 22.1).

Sample set creation

The calculation pipeline underlying miREV utilizes read count tables to determine stably expressed transcripts of a user defined sample set. A sample set arises from the selection of experimental variables. Table S2 lists all the different experimental variables and possible selections. In total 21.483 sample sets were created to reflect all possible combinations of experimental setups.

Determination of stably expressed transcripts

Each sample set was processed by 6 popular normalization methods, all well established in next generation sequencing data analysis. Normalizing raw read counts is essential before differential expression analyses as well as for exploratory data analysis. Normalization methods are accounting for different factors such as sequencing depth, gene length and RNA composition. By scaling read counts, the expression level between samples becomes more comparable. The following methods were used: total count normalization (TC), median normalization (Med),[38] full quantile and upper quartile normalization (FQ & UQ),[39] trimmed mean of M-value (TMM)[40] and median of ratios normalization (MoR).[41] Herein applied normalization methods were shown to be the most reliable for comparison of RNA sequencing results.[42–44] All normalized sample sets were subsequently analysed by 3 different stability measure algorithms that each compute a specific stability indicator. In addition to bestKeeper,[45] which calculates the coefficient of variation (CV) across data sets, geNorm[46] and normFinder[47] were applied, which indicate expression stability via the M-value and stability measure rho, respectively. All 3 algorithms were originally designed for detecting stable mRNA or miRNA transcripts in high throughput RT-qPCR experiments, and have been cited in thousands of peer-reviewed publications.[48–50]

Filtering

The data was strictly filtered to guarantee an overall comparable and reliable sequencing data source. In a first quality filtering, data sets with less than 7% of total reads mapping to a miRNA precursor were removed to exclude sequencing results with a low miRNA level. 428 data set out of originally 654 remained. Sample sets with less than 10 samples were removed as well to get a comparable and reliable minimal size for each data set. Accordingly, 12,578 of the original 21,483 sample sets remained after this filtering step. Second, a gene filtering step was included to exclude lowly expressed genes individually for each sample set from further analysis. Genes that were not present in at least 95% of all data sets were removed, resulting in different number of miRNAs in each sample set, ranging from a few dozen to several hundreds of genes. The last filter step was applied to the result lists, which contain the proposed stably expressed miRNA transcripts. Each result list is a selection of potential reference miRNAs in descending order according to their respective stability values. miRNAs that were not amongst the top 50% of ranked transcripts were removed. This filter step ensured that only the most stably expressed transcripts are included in the result lists.

Outlook

In order for miREV to have even more selection criteria available in the experimental setup, a continuous expansion of the database is planned. Existing selection criteria are expanded and new ones, such as species, cell culture or other diseases, are added. We hereby ensure that miREV will remain freely available for at least the next five years.

CRediT authorship contribution statement

Alex Hildebrandt: Investigation, Software, Data curation, Validation, Writing - original draft, Visualization. **Michael W. Pfaffl:** Conceptualization, Writing - review & editing. **Esther N.M. Nolte-'t Hoen:** Conceptualization, Writing - review & editing. **Benedikt Kirchner:** Conceptualization, Supervision, Writing - review & editing.

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Disclosure statement

The authors reported no potential conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2021.167070.

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† These authors contributed equally to this work.

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Appendix II

Substantial Contributions by Benedikt Kirchner:

- Execution of transcriptomic experiments (NGS, RT-qPCR)
- Curation, analysis and interpretation of data
- Drafting of figures
- Writing – original draft

Benedikt Kirchner

Michael W. Pfaffl

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microRNA in native and processed cow's milk and its implication for the farm milk effect on asthma

To the Editor:

Consumption of unprocessed milk reduces the risk of asthma and atopic sensitization by 30% to 50%.[1] Already in the first year of life, consumption of unprocessed milk protects against respiratory tract infections.[2] However, consumption of raw milk is no solution because it bears a risk of potentially life-threatening infections.[2] Hence there is a need to identify those components of native cow's milk that carry this asthma- and allergy-protective, as well as anti-infectious, effect. A candidate molecule class with relation to immune functions[3] are the microRNAs (miRNAs), which have been described in both cow's milk and human breast milk.[3]

The aim of this study was to screen differently processed milk types originating from the same 3 batches of native cow's milk (see Table E1 in this article's Online Repository at www.jacionline.org) for miRNA expression and to investigate possible interference of differentially expressed miRNAs with genes and pathways implied in the pathogenesis of asthma and allergic diseases. To reflect the entire spectrum of milk treatment, we subjected the milk samples to 5 different procedures usually applied during industrial processing (see Table E2 in this article's Online Repository at www.jacionline.org): separation/centrifugation resulting in a skim milk fraction (SKI) and a fat fraction (FAT), homogenization of whole milk (HOM), pasteurization (PAS), ultraheat treatment (UHT), and heat treatment for extended shelf life (ESL) of whole milk without homogenization. We then compared these samples to whole raw (RAW) or boiled (BOI) milk samples with respect to essentially all miRNA species detectable in milk by using next-generation sequencing (NGS). We focused on the human miRNA homologues because they are more completely covered in databases than their bovine counterparts and because we were ultimately interested in their effect on the human organism.

All samples yielded a total RNA content of more than 361.8 ng, with raw milk containing the highest amount (see Table E3 in this article's Online Repository at www.jacionline.org). NGS resulted in 303,106 reads for all 24 samples, including various types of small RNA reads, such as miRNA, Rfam filtered (rRNA, transfer RNA [tRNA], small nuclear [snRNA], and small nucleolar [snoRNA]), unaligned, and trimmed sequences. The distribution of these types of small RNA did not vary between farms (Fig 1, *A*) but with milk type (Fig 1, *B*). In high heat–treated milks, RNA reads with the length of 22 nucleotides typical for miRNA were strongly reduced (see Fig E1 in this article's Online Repository at www.jacionline.org), as illustrated for miR-148a-3p, the most abundant miRNA species in milk (Fig 1, *C*).

For data reduction of the less abundant miRNA species (see Fig E2 in this article's Online Repository at www.jacionline.org), principal component analysis (PCA) was applied. The first component (PC1) explained 97.5% of total variance and differentiated well between high heat–treated milk samples (BOI, ESL, and UHT) and raw (RAW, SKI, and HOM) or pasteurized (PAS) samples, whereas the second component (PC2) explained 2.2% of the variance and separated the fat samples from all other samples (Fig 1, *D*, and see Fig E3 in this article's Online Repository at www.jacionline.org). Validation of NGS results by using RT quantitative PCR (qPCR) revealed high correlation and agreement between both methods (see Fig E4 and Table E4 in this article's Online Repository at www.jacionline.org). Differential expression of miRNAs was found for 52 miRNA species between pasteurized and UHT milk and for 25 species between pasteurized whole milk and raw milk fat (see Table E5 in this article's Online Repository at www.jacionline.org). Possible targets of the differentially expressed miRNA species were derived from the miRTarBase database, which relies on experimentally validated effects instead of *in silico* predictions. Table I shows a selection of the most relevant genes, which are targeted by more than 3 different miRNA species.

Among those is prostaglandin-endoperoxide synthase 2S *(PTGS2)*, which is involved in the synthesis of proinflammatory eicosanoids, potent mediators of allergic inflammation.[4] Eicosanoid metabolism is also influenced by intake of ω-3 polyunsaturated fatty acids, which we found to explain a substantial part of the effect of milk fat content on asthma (Brick et al, in this issue[5]).

Signal transducer and activator of transcription 3 *(STAT3)* transduces signals of receptors of innate immunity, contributes to regulation of T helper cell differentiation, and has been implied to be a factor in impaired lung function seen in asthmatic patients.[6] In addition, it mediates the inhibitory effects of the proinflammatory cytokine IL-6 on dendritic cells.[7] Like *STAT3*, *IL6* itself is regulated by the pleiotropic miRNA miR-155-5p, which also interferes with *IFNGR1* (Table I).

IL-13 induces all the cardinal features of T_H2-mediated inflammation (ie, airway hyperresponsiveness, infiltration by inflammatory cells, increased mucus production, airway remodeling, and induction of IgE synthesis).[7] Posttranscriptional regulation of *IL13* is tuned by members of the let-7 family, and intranasal administration of let-7 mimic in a murine asthma model reduced IL-13 levels in lungs and alleviated asthma features.[8]

IFNG and *IL13* have been found to be differentially expressed between acute virus-induced exacerbations of asthma and convalescence.[9] Decreased activation of *IFNG* signaling during episodes of asthma exacerbation has been implied in chronic airway obstruction.[9]

The higher miRNA levels of homogenized and skimmed raw milk compared with native raw milk might be partially explained by a shorter storage time because we had deliberately frozen native raw milk on the last day of processing. However, pasteurized milk was also frozen on the same day (see Table E2) and had comparably high miRNA levels as well. Pasteurizing might not affect miRNAs themselves but possibly inactivate

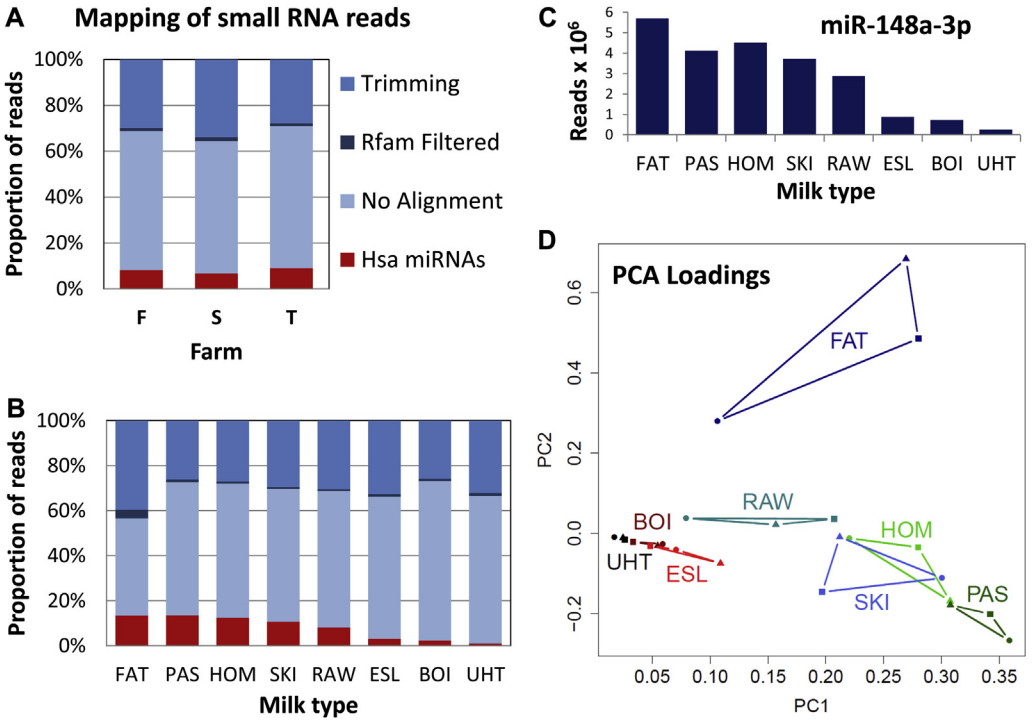

FIG 1. PCA. **A,** Mapping of small RNA reads across farms. Relative frequencies of reads are shown for the respective farms (*F*, *S*, and *T*). **B,** Mapping of small RNA reads across milk fractions. *BOI*, boiled milk; *ESL*, high-heat treatment for extended shelf life; *FAT*, fat fraction after separation; *HOM*, homogenized nonpasteurized milk; *PAS*, pasteurized nonhomogenized milk; *RAW*, native raw milk; *SKI*, skim milk after separation; *UHT*, ultra-high heat–treated milk. **C,** Distribution of reads of the most abundant miRNA species in milk (abbreviations as in Fig 1, *B*). **D,** Loading of the first 2 components of the analyzed milk samples. *Rectangles*, *circles*, and *triangles* represent samples from farms F, S, and T, respectively. Samples of the same milk type are linked by lines (abbreviations as in Fig 1, *B*).

TABLE I. Candidate genes targeted by differentially expressed miRNAs

Gene symbol	Gene name	Interfering miRNAs
STAT3	Signal transducer and activator of transcription 3	let-7e-5p miR-20a-5p* miR-21-5p miR-92a-5p miR-93-5p* miR-125b-5p* miR-155-5p*
PTGS2	Prostaglandin-endoperoxide synthase 2S	let-7b-5p miR-16-5p miR-26b-5p miR-101-3p miR-128-3p miR-181a-5p
IL13	IL-13	let-7d-5p let-7f-5p let-7g-5p let-7i-5p miR-98-5p
IL6	IL-6	let-7a-5p miR-26a-5p miR-365a-3p miR-98-5p miR-155-5p
IFNG	IFN-γ	miR-15a-5p miR-16-5p miR-26b-5p miR-29b-3p
IFNGR1	IFN-γ receptor 1	miR-98-5p miR-155-5p miR-181b miR-196a

Genes targeted by more than 3 miRNA species were differentially expressed with respect to high-heat treatment.
*These 4 miRNA species interfering with *STAT3* were also differentially expressed with respect to fat content.

RNAses. At the usual 72°C, microvesicles, such as exosomes, are still intact and protect packaged miRNAs from degradation and inactivation. This effect might be lost by high-temperature treatment and longer heat exposure, as applied in boiling and ESL/UHT milk.

Because of the strong analogy between human and bovine miRNAs, the presence of these molecules in raw milk and particularly the fat fraction might be accountable for a part of the beneficial effect of farm milk consumption on asthma and allergic diseases. A potential uptake of milk miRNAs has been shown in cell-culture experiments and after human consumption.[10] However, raw milk is not an option for future population-wide prevention strategies because of its potential health hazard. Rather, less heat-intensive procedures in industrial milk processing might help preserve bovine miRNA from degradation and maintain its favorable properties for future prevention strategies.

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Food allergies can persist after myeloablative hematopoietic stem cell transplantation in dedicator of cytokinesis 8–deficient patients

To the Editor:

Dedicator of cytokinesis 8 (DOCK8) deficiency is a highly morbid combined immunodeficiency that features recurrent sinopulmonary infections, viral skin infections, and severe food allergies.[1,2] Hematopoietic stem cell transplantation (HSCT) cures infection susceptibility in patients with DOCK8 deficiency.[3-7] Whether HSCT also cures food allergy has not been systematically examined in human subjects.[8] To gain insight into the etiopathogenesis of food allergy and its potential treatment, we studied food allergy in 12 DOCK8-deficient patients who underwent HSCT at the National Institutes of Health (NIH) Clinical Center.

First, we retrospectively evaluated 6 patients who had received either matched related or unrelated donor cells after myeloablative conditioning (patients 1-6 in Table I and see the Methods section in this article's Online Repository at www.jacionline.org).[7] Of these, patients 2 and 4 reported histories of food-induced anaphylaxis before transplantation, and patient 5 reported a new food allergy after transplantation. After transplantation, skin prick testing to 8 common food allergens and specific IgE by using ImmunoCAP (Phadia, Uppsala, Sweden) confirmed sensitization to foods precipitating the reactions. Food challenges were not performed. A fourth patient (patient 1) reported oral pruritus to lentils before transplantation and again when re-exposed more than 1 year after transplantation. Donors in these cases were confirmed to have no history of food allergy.

Our observations of persisting or new food allergies were unexpected given anecdotal reports suggesting that HSCT cured food allergies and could have reflected a selection bias.[4,5,9] Thus we prospectively studied food allergies in the next 6 DOCK8-deficient patients undergoing transplantation at the NIH (patients 7-12 in Table I). This second group included 2 patients (patients 9 and 12) who had undergone related donor haploidentical transplantations and had systemic allergic reactions to foods to which they were already allergic before transplantation. Donors were confirmed to have no history of food allergy. Patient 9 was more than 8 months after HSCT and more than 2 months off tacrolimus when she had acute oral and facial angioedema, diffuse urticaria, vomiting, and difficulty breathing within minutes of eating oatmeal fortified with egg and milk. Her symptoms resolved after receiving epinephrine, diphenhydramine, and methylprednisolone. Before transplantation, she had had anaphylactic reactions to egg and milk. However, 2 months before her last reaction, her skin prick test responses were positive to egg but not milk, suggesting egg as the culprit (see Table E1 in this article's Online Repository at www.jacionline.org). Similarly, patient 12 was 45 days out after HSCT when she had oral and periorbital angioedema and diffuse urticaria and pruritus within 10 minutes of eating a kiwi fruit, with sensitization confirmed by means of skin prick testing (Table I and see Table E1). Years before transplantation, she too had had oral pruritus and lip angioedema after eating kiwi and had subsequently avoided it entirely.

Among the patients studied prospectively, a third patient who underwent matched unrelated donor transplantation reported that a previously resolved food allergy had returned. Patient 7 was 3 months out of HSCT when he had cramping abdominal pain, vomiting, diarrhea, and headache within 15 minutes of eating scrambled eggs. His symptoms occurred on 2 more occasions following concentrated egg ingestion but never when he ate baked goods that contained eggs. He had had similar symptoms in his early school age years but had been eating eggs freely for the decade before transplantation. Skin prick testing confirmed that he had reacquired reactivity to egg after transplantation (Table I and see Table E1). The donor was confirmed to have no history of food allergy. Patient 7 also had a history of anaphylaxis to walnut as recently as 3 years before HSCT. Because of persisting positive skin prick tests, he continued to strictly avoid tree nuts after transplantation.

METHODS

Milk samples

Cow's milk samples from different breeds were collected from 3 farms located around Munich, Germany, and comprised milk from the morning and previous evening (see Table E1). The milk was stored at 1°C until further processing to minimize bacterial growth. The physical characteristics (ie, the parameters heat, pressure, and duration) of the different procedures applied to the 3 milk batches are shown in Table E2.

A total of 8 different milk fractions were obtained per farm. The high-heat treatment fractions were produced on the first day (ie, within 12 hours after milking). Separation, homogenization, and boiling were performed on the subsequent day, whereas pasteurization was completed on day 3. After processing, all milk fractions were immediately frozen at −20°C. The native raw milk samples were frozen on day 3 to avoid potential bias caused by reduced enzymatic degradation processes as a result of early freezing.

Purification of total RNA

Total RNA, including small RNA, was extracted in triplicate from all milk fraction samples by using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). Milk samples were thawed on ice, chloroform was added at a ratio of 1:5, and isolation was continued according to the manufacturer's instructions. Total RNA was eluted in 30 μL of RNAse-free water, and RNA concentrations were measured with the Qubit 2.0 Fluorometer (Life Technologies, Grand Island, NY). RNA purity was assessed by measuring absorbance levels on a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, Mass). The triplicates for each milk fraction and farm were pooled, resulting in 24 samples stored at −80°C.

Library preparation and sequencing of small RNAs

Of each milk sample, 500 ng of total RNA was converted into barcoded cDNA libraries with the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs, Ipswich, Mass). After PCR preamplification, cDNA constructs were purified with the MinElute PCR Purification Kit (Qiagen), and PCR products were separated on a 4% agarose gel for size selection. Small RNAs of approximately 135 to 160 bp (miRNA transcript plus ligated sequencing adapters) were excised and extracted with the MinElute Gel Extraction Kit (Qiagen). Successful library preparation was verified with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, Calif) by using the High Sensitivity DNA Kit (Agilent Technologies). The resulting cDNA libraries were subjected to the Illumina sequencing pipeline consisting of clonal cluster generation on a single-read flow cell (Illumina, San Diego, Calif), bridge amplification on the cBOT via TruSeq SR Cluster Kit v3-cBOT-HS (Illumina), and 50 cycles of sequencing-by-synthesis on the HiSeq2000 (Illumina). Sequenced reads were deposited in European Nucleotide Archive ENA under accession number PRJEB9597.

Alignment of sequencing reads

Raw read data were trimmed of 3′ end adapters by using Btrim,[E1] and reads without a detectable adapter sequence or with less than 16 nucleotides were excluded from the data set. Mean Phred quality scores per complete sequence and per sequencing position, as well as sequence length distribution, were assessed with the quality control software FastQC (Version 0.10.1; Babraham Bioinformatics, Cambridge, United Kingdom). Alignment was carried out by using the Bowtie short read aligner's "best alignment algorithm" of reads with a maximum of 1 mismatch within the first 15 nucleotides.[E2] Read data mapping to bovine rRNA, tRNA, snRNA, and snoRNA sequences contained in the Rfam database were removed.[E3] The remaining sequences were aligned to the most recent miRBase database (release 20) for mature human miRNAs.[E4] Read counts were generated by sorting, indexing, and calling the sum of hits per miRNA using SAMtools.[E5]

Validation by means of RT-qPCR

The presence of miRNA sequences discovered by means of small RNA sequencing (RNA-Seq) was verified by using RT-qPCR for 9 selected miRNAs. These miRNAs were chosen based on their frequency and relation to industrial milk processing. For cDNA synthesis, total RNA samples were polyadenylated and reverse transcribed with the miRCURY LNA universal cDNA Synthesis Kit II (Exiqon, Vedbæk, Denmark), and qPCR was done on a Rotor-Gene-Q (Qiagen) with ExiLENT SYBR Green master mix and the corresponding miRCURY LNA PCR primer sets (Exiqon). The potential effect of confirmed miRNAs on human health was evaluated by comparing them with experimentally verified miRNA targets of the mirTarbase v4.0 database,[E6] with a focus on 139 candidate gene for asthma, atopy, and respiratory tract infections.[E7-E10]

Statistical analysis

Raw read counts were standardized to a denominator of 10^7 reads, thereby compensating for differences in library sizes. Data reduction of expression profiles was achieved by means of PCA with R software (www.R-project.org) and the packages pcaMethods[E11] and NOISeq.[E12] Correlation between sequencing and RT-qPCR results was assessed by using the Pearson correlation coefficient *(r)*.

RESULTS

All samples yielded a total RNA content of more than 361.8 ng, with raw milk containing the highest amount of RNA (Table E3). Sequencing resulted in 303×10^6 reads for all 24 samples. High sequencing accuracy was shown by a mean Phred score of 30 or more, corresponding to a probability of correct base call of 99.9% or greater for 99% of all reads.[E13] The reads per sample ranged from 8.7×10^6 to 15.7×10^6, with an average of 12.6×10^6 reads. Because of absent cDNA library adapter sequences or shorter read lengths than 16 nucleotides, $3.9 \times 10^6 \pm 0.9 \times 10^6$ reads (mean ± SD) per sample were excluded. Additionally, $0.17 \times 10^6 \pm 0.15 \times 10^6$ reads per sample were removed (Rfam filtered) from further analysis because they mapped to bovine rRNA, tRNA, snRNA, or snoRNA sequences. Of the remaining reads, $1.01 \times 10^6 \pm 0.72 \times 10^6$ aligned against 1796 of all 2578 known mature human miRNAs. Of those miRNAs, 155 were sufficiently abundant for subsequent analyses, with 1200 reads overall corresponding to an average of 50 reads per sample.

The distribution of types of small RNA reads, such as miRNA, Rfam filtered (rRNA, tRNA, snRNA, and snoRNA), unaligned, and trimmed sequences, did not vary between farms (Fig 1, *A*). However, milk processing affected the composition of small RNA sequences (Fig 1, *B*): high heat–treated milks (BOI, ESL, and UHT) had substantially reduced miRNA read counts (5.51-fold, $P = .0012$). A similar pattern was found by comparing read lengths across milk types (Fig E1): relative mean read counts clearly peaked at a nucleotide length of 22, corresponding to the typical size of miRNAs, in all samples except the high heat–treated samples (BOI, ESL, and UHT). The proportion of aligned miRNAs among all reads with a length of 22 nucleotides was much lower in high heat–treated samples (BOI, ESL, and UHT), indicating a large proportion of degraded RNA in milk samples.

The most common miRNA sequence (36% of all reads) aligned to miR-148a (Fig E2) and was expressed in high heat–treated milk samples with substantially reduced read counts (Fig 1, *C*). All other sequences were much less abundant (<10% of all reads) and were thus analyzed by using a PCA based on the transposed

matrix of miRNA species and milk types. The first component (PC1) explained 97.5% of the total variance and differentiated well between high heat–treated milk samples (BOI, ESL, and UHT) and raw (RAW, SHI, and HOM) or pasteurized (PAS) samples, whereas the second component (PC2) explained 2.2% of the variance and separated the fat samples from all other samples (Fig 1, *D*). PC1 was mainly determined by let-7a-5p, let-7b-5p, miR-200c-3p, miR-30a-5p, and miR-22-3p; PC2 was predominantly influenced by miR-22-3p and miR-30a-5p (Fig E3). For further analyses, we used the milk samples that were separated most by the first 2 components: UHT, PAS, and FAT.

The validation of small RNA-Seq results by using RT-qPCR revealed high correlation and agreement between both methods (Table E4), as shown in Fig E4 for high, medium, and low levels of miRNA species, respectively.

Differential expression of miRNAs was found for 52 miRNA species between pasteurized and UHT milk and for 25 species between pasteurized whole milk and raw milk fat (Table E5). The strongest effects by heat treatment ($\log_2$ fold change >4) were found for miR-125b-5p, miR-101-3p, miR-27b-3p, miR-365a-3p, miR-125a-5p, miR-30c-5p, miR-128-3p, miR-151a-3p, miR-26b-5p, miR-192-5p, let-7b-5p, and let-7a-5p, whereas the strongest effects by fat content ($\log_2$ fold change >2) were found for miR-30c-5p, miR-125a-5p, miR-200c-3p, and miR-125b-5p.

Genes targeted by 4 or more miRNA species differentially expressed with respect to high heat treatment and/or fat content comprised *STAT3* (7 times) and *PTGS2* (6 times), followed by *IL6* and *IL13* (each 5 times) and *IFNG* and IFN-γ receptor 1 (*IFNG* and *IFNGR1*; each 4 times; Table I). For differences between pasteurized whole milk and the fat fraction, *STAT3* also appeared most frequently, with 4 occurrences (Table I). Differential miRNA expression between the respective milk samples was also confirmed by using RT-qPCR for all miRNA species listed in Table E4 (data not shown).

DISCUSSION

Batches of milk samples from 3 different farms were processed by using different procedures, resulting in 8 milk fractions. From all 24 samples, total RNA was extracted, selected for size, and sequenced for short reads (small RNA-Seq). After removal of noninformative reads, the remaining miRNA sequences were equally distributed across farms but occurred much less frequently in all high heat–treated milk samples. Most of the variance in the data set was explained by the discrepancy between high heat–treated and raw or pasteurized samples, particularly in the expression of let-7a-5p, let-7b-5p, and miR-200c-3p. Some variance was also attributable to the difference between whole milk and the fat fraction, particularly by miR-22-3p. High-heat treatment affected miRNAs interfering with the genes *STAT3*, *PTGS2*, *IL6*, *IL13*, *IFNG*, *IFNGR1*, and others. Also, fat separation reduced expression of miRNAs interfering with *STAT3*.

In a related manuscript (Brick et al, in this issue[E14]) we demonstrate that the protective effect of raw milk consumption on asthma can partially be attributed to a higher fat content of farm milk and particularly to higher levels of ω-3 polyunsaturated fatty acids. The effect of fat content seems to protect predominantly against mild and reversible forms of asthma. However, a similar or even stronger effect is clearly related to the contrast between raw milk and high heat–treated milk, particularly UHT milk, which is often used as a reference category in epidemiologic studies.[E15-E17] Here we also included ESL milk because it increasingly replaces traditionally pasteurized milk for the convenience of longer shelf life but has been studied rarely in epidemiologic studies.

Mild heat treatment, such as pasteurization, still maintains some but not all beneficial properties, as demonstrated in the GABRIELA study, in which pasteurized milk halved the risk of asthma similarly to raw farm milk, although it narrowly missed statistical significance.[E18] In contrast, boiled farm milk had no effect on asthma insofar as it was comparable with UHT milk. On atopic sensitization, hay fever, and eczema, only unboiled farm milk exerted a protective effect. In infancy respiratory tract infections were prevented by farm milk irrespective of boiling, whereas fever episodes were additionally prevented by pasteurized milk.[E17]

Superficially, the described pattern seems to be inconsistent. On a closer look, however, it reflects a multitude of protective effects on closely related but distinct disease entities. In addition, milk is a rather complex mixture of organic molecules, with rather specific functions reflecting the entire evolution of mammals. Consequently, unravelling the underlying variants of the "milk effect" is rather challenging. For a first approach, we examined well-known and clearly defined molecule classes, such as whey proteins,[E18] fatty acids,[E14] and miRNAs.

Thus far, miRNAs have been described in human breast milk[E19] and cow's milk.[E20] These small regulatory molecules circulate in body fluids and regulate physiologic and pathologic processes by interfering with mRNA. In milk and other body fluids they are stored and transported in microvesicles,[E20] which renders them resistant to RNAse and acidic conditions, such as those found in gastric fluid. The majority of miRNA species are related to immune functions[E19]; are differentially expressed in T_H1, T_H2, and regulatory T cells[E21]; and have even been implicated in mediating suppressive effects of regulatory T cells on T helper cells.[E22]

The primary finding of this analysis was a substantial reduction of miRNA levels in high heat–treated milk samples. Infant formula, which is strongly heated, has been reported to contain only minimal miRNA residues.[E23] Rather unexpectedly, pasteurized, homogenized, and skimmed milk contained higher levels compared with raw milk. For homogenized and skimmed milk, this might be explained in part by a shorter storage time until freezing because we had deliberately frozen raw milk on the last day of milk processing to avoid any bias caused by early freezing of raw milk. However, pasteurized milk was also frozen on the same day (Table E2), thereby suggesting an individual effect of pasteurization. A potential explanation might be found in the rather mild temperature of 72°C not affecting miRNAs themselves but possibly inactivating RNAses. Moreover, microvesicles, such as exosomes, which contain and protect miRNA from degradation and inactivation, are still intact at 72°C.[E13] In contrast, high-temperature treatment and longer heat exposure as applied in boiled and ESL/UHT milk might destroy protective microvesicles. Nevertheless, we have to acknowledge that the raw milk samples of farm S almost clustered with the high heat–treated samples in the PCA, and therefore we cannot exclude artificial degradation of miRNA in this sample caused by contamination during storage. Yet the most abundant miRNA in milk, miR-148a-3p, was found at higher RT-qPCR

levels in pasteurized compared with raw milk in the other 2 farms (Fig E4, *A*); ultimately, this argues in favor of some preservation of miRNAs by pasteurization. Obviously, miRNA levels vary with the circadian rhythm and lactation status[E23]; however, we assessed milk samples pooled over morning and evening milk of more than a dozen cows per farm, thereby leveling out such potentially confounding effects.

NGS with small RNA-Seq was chosen as an approach to screen for essentially all miRNA species present in milk. We focused on the human miRNA homologues because they are more completely covered in databases than their bovine counterparts and because we were ultimately interested in their effect on the human organism. The use of NGS as a fully quantitative method is questionable, and therefore we validated it against RT-qPCRs targeting mRNA species with low, medium, and high abundance. The strong agreement of both methods justified the approximation by read counts in the quantitative models. For data reduction, we performed a PCA and used the strongest contrasts (ie, the milk types at the extremes of the first 2 dimensions [PAS, UHT, and FAT]) for the subsequent analyses. In addition, we deliberately avoided raw milk as a comparator because it is not an option for future population-wide prevention strategies because of its potential health hazard. In light of the high number of detected miRNAs (n = 155) and our limited sample size (n = 24), we performed association analyses on the transposed data matrix, which enabled us to correct for multiple testing.

For the prediction of candidate genes targeted by the detected miRNAs, we used the mirTarbase v4.0 database,[E6] which relies on experimentally validated effects instead of *in silico* predictions. Our list of detected gene targets is obviously influenced by the choice of database and the selective focus of the scientific community on immunity genes. Nevertheless, we consider the resulting pool of miRNAs and their target genes as a helpful starting point for further confirmatory analyses in population-based samples.

The detection of *PTGS2* as a major target of several differentially expressed miRNAs points to an interference with the synthesis of proinflammatory eicosanoids, such as prostaglandin D_2, a potent mediator of allergic inflammation.[E24] The metabolism of eicosanoids is also influenced by the intake of ω-3 polyunsaturated fatty acids, which we found to explain a substantial part of the effect of milk fat content on asthma.[E14]

STAT3 transduces signals of receptors of innate immunity, contributes to regulation of T_H cell differentiation, and has been implicated in impaired lung functions in asthmatic patients.[E25] In addition, it mediates the inhibitory effects of the proinflammatory cytokine IL-6 on dendritic cells.[E8] IL-6 itself is a key cytokine of inflammation, is involved in airway obstruction in neonates,[E26] and triggers C-reactive protein release in the liver.[E27] In the PASTURE birth cohort we found high-sensitivity C-reactive protein as a marker of low-grade inflammation to be inversely related with the consumption of raw milk in infancy[E17] and with the ω-6/ω-3 polyunsaturated fatty acid ratio of cow's milk consumed at preschool age.[E14] Like *STAT3*, *IL6* itself is regulated by the pleiotropic miRNA miR-155-5p, which also interferes with *IFNGR1* (Table E5). The role of miR-155-5p in this context is possibly ambivalent because it has been described to rather enhance type 2 immunity.[E28] Furthermore, *IL6* shares effects of miR-98-5p with *IL13* and *IFNGR1*, thereby suggesting some redundancy in the effect of milk-born miRNAs.

IL13 has a similarly important role but on the effector side of allergic inflammation; it induces all the cardinal features of T_H2-mediated inflammation (ie, airway hyperresponsiveness, infiltration by inflammatory cells, increased mucus production, airway remodeling, and induction of IgE synthesis).[E8] Polymorphisms in the *IL13* gene are associated with both childhood-onset asthma and serum IgE levels.[E9] Posttranscriptional regulation of *IL13* is tuned by members of the let-7 family, and intranasal administration of let-7 mimic in a murine asthma model reduced *IL13* expression in the lungs and alleviated asthma features, such as airway hyperresponsiveness, airway inflammation, and goblet cell metaplasia.[E29]

Finally, the primary T_H1 cytokine *IFNG* and its receptor *IFNGR1* appear on the list of target genes. Both, *IFNG* and the T_H2-related cytokine *IL13* have been found to be differentially expressed between acute virus-induced exacerbations of asthma and convalescence.[E10] Decreased activation of *IFNG* signaling during episodes of asthma exacerbation has been implicated in chronic airway obstruction.[E10] In experimental rhinovirus infection in human subjects, *IFNG* production by T cells was associated with lower viral load and less severe common cold symptoms.[E30] Respiratory syncytial virus infection resulted in increased *IFNG* levels, thereby prompting activation of natural killer and T_H cells.[E31] A possible mechanism might be found in miR-15b–induced upregulation of *IFNG* production by human natural killer cells.[E32]

Altogether, the common denominator of the miRNA target genes seems to be their involvement in the inflammatory response rather than other features of asthma. This notion is in line with our observation that consumption of unprocessed milk is less likely to affect severe forms of asthma; rather, it might temper airway inflammation and alleviate features of asthma possibly even before they manifest clinically.[E14]

In summary, we found the contents and composition of miRNA in cow's milk to be substantially altered by high-heat treatment. A distinct set of miRNAs was enriched in the fat fraction. Several of the differentially expressed miRNA species are known to interfere with mRNAs coded by genes previously implicated in the development of asthma and allergies. Because of the strong analogy between human and bovine miRNAs, the presence of these molecules in native milk and particularly the fat fraction might be responsible for part of the beneficial effect of farm milk consumption on asthma and allergies. Less heat-intensive procedures in industrial milk processing might help preserve bovine miRNAs against degradation and maintain their favorable properties for future prevention strategies.

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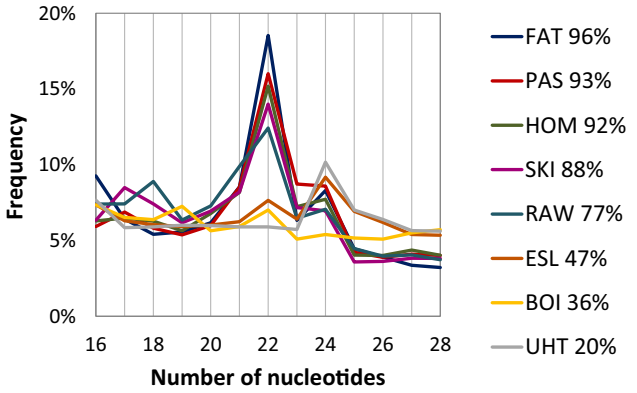

FIG E1. Distribution of RNA read length across milk types. Distributions of RNA read length are shown for the respective milk types. Percentages refer to proportion of read counts mapping to mature human miRNA sequences.

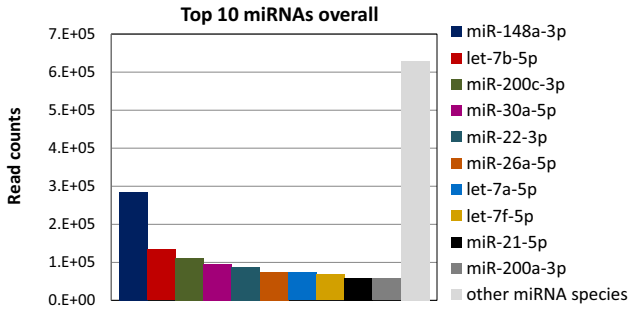

FIG E2. The most abundant miRNA species.

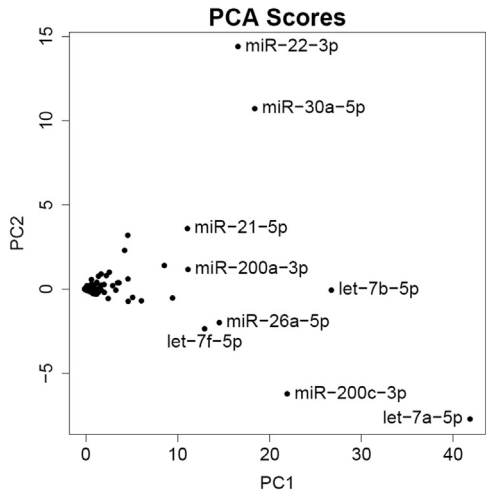

FIG E3. PCA scores. Scores per 104 reads of the first 2 components (PC1 and PC2) represent the detected miRNA species. Names are given only for species scoring high on either component.

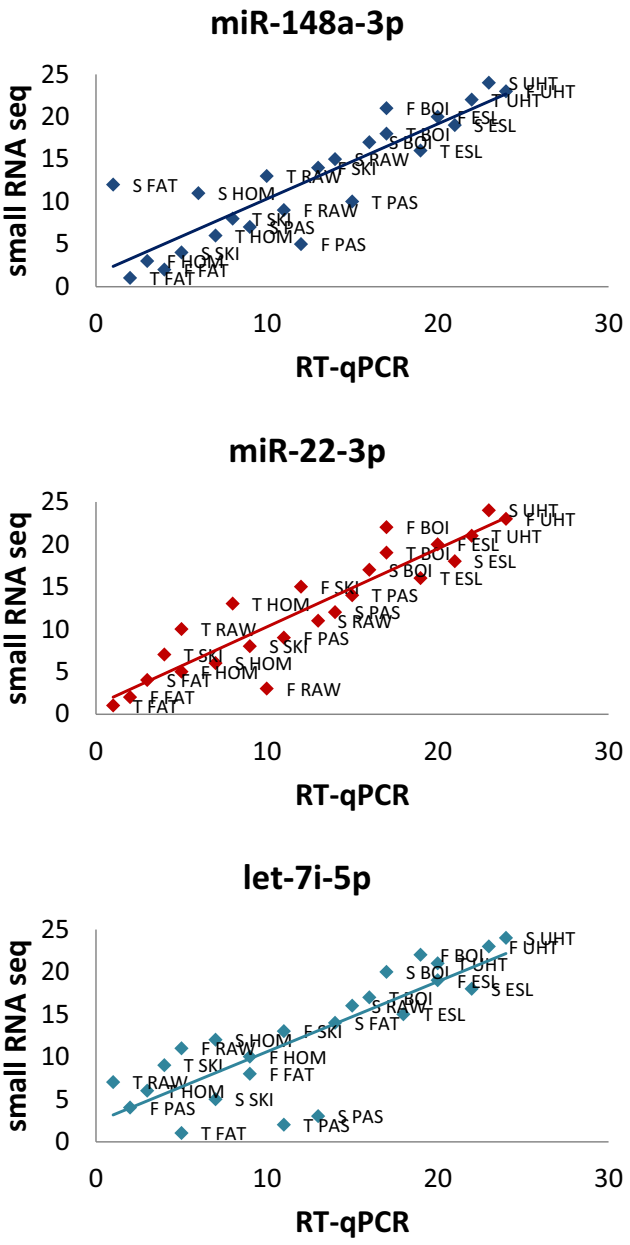

FIG E4. Rank correlation of quantification methods (small RNA-Seq vs RT-qPCR).

TABLE E1. Sources of raw milk

	Farm		
	T	**F**	**S**
Farming type	Conventional	Conventional	Organic
Cattle breed	Fleckvieh	Brown Swiss	Fleckvieh
Feeding	Corn silage, grass silage, concentrate	Corn silage, concentrate	Grass, hay, wheat
No. of cows	13	60	30
Time point of milking for pooled samples	Morning and evening	Morning and evening	Morning and evening
Microbial contamination (CFU/mL), mean ± SD	18,500 ± 7,800	7,300 ± 3,200	4,600 ± 3,800

CFU, Colony-forming unit.

TABLE E2. Treatment of milk samples

	Milk fraction	Processing conditions	Day of processing
UHT	Ultra-high heat treated	Preheating at 93°C for 23 s, direct steam injection at 142°C for 5 s	1
ESL	Extended shelf life	Preheating at 95°C for 20 s, direct steam injection at 127°C for 5 s	1
BOI	Boiled milk	Preheating at >80°C for >300 s, boiling at 100°C for 30 s	2
HOM	Homogenized milk	Preheating to 55°C, 2-stage homogenization at 250/50 bar	2
SKI	Skim milk	Separation at 50°C	2
FAT	Fat fraction	Separation at 50°C	2
PAS	Pasteurized milk	72°C for 20 s	3
RAW	Native raw milk	—	3

TABLE E3. RNA yields stratified for milk type

Milk type	RNA concentration (ng/μL)	Coefficient of variation
UHT	12.00	8%
ESL	8.89	50%
PAS	14.57	18%
HOM	17.77	20%
FAT	24.30	50%
SKI	17.10	11%
BOI	12.30	25%
RAW	46.33	11%

TABLE E4. Correlation of small RNA-Seq and RT-qPCR results

miRNA	Spearman correlation
let-7a-5p	0.88
let-7i-5p	0.83
miR-21-5p	0.79
miR-22-3p	0.92
miR-30a-5p	0.89
miR-92a-3p	0.83
miR-140-3p	0.81
miR-148a-3p	0.88
miR-200c-3p	0.84

TABLE E5. Differentially preserved miRNA levels between PAS, UHT, and FAT milk

miRNA	PAS vs UHT	PAS vs FAT	Targeted candidate genes
let-7a-5p	4.01	1.54	*IL6*, *ITGB3*, *VDR*
let-7b-5p	4.03		*IFNB1*, *PTGS2*
let-7d-5p	3.67	1.80	*IL13*
let-7e-5p	3.59		*STAT3*
let-7f-5p	3.81	1.64	*IL13*
let-7g-5p	3.88		*IL13*
let-7i-5p	3.48		*IL13*, *TLR4*
miR-15a-5p	2.67	−2.40	*IFNG*
miR-16-5p	2.84		*DAP3*, *IFNG*, *PTGS2*
miR-17-5p	3.65	1.23	*SMAD3*
miR-19b-3p	2.48		*TLR2*
miR-20a-5p	3.59	1.68	*STAT3*
miR-21-5p	3.90		*IL1B*, *STAT3*, *TGFB1*, *TLR4*
miR-24-3p	3.57		*TGFB1*
miR-25-3p	3.18	1.20	*ACP1*, *CCL26*
miR-26a-5p	3.94		*IFNB1*, *IL6*
miR-26b-5p	4.11		*AGT*, *C3*, *GSTP1*, *HLA-DQB2*, *IFNG*, *IFNGR2*, *IL3*, *MUC7*, *PTGS2*, *STAT6*, *TIMP1*
miR-27b-3p	4.48		*EDNRA*, *VDR*
miR-29b-3p	3.33		*IFNG*, *TBX21*
miR-30c-5p	4.34	2.50	*ITGB3*
miR-30d-5p	3.25		*IRF2*
miR-34a-5p	3.50		*IFNB1*
miR-92a-3p	3.57		*CTLA4*, *GATA3*, *GSTP1*, *RORA*, *STAT3*, *TLR1*
miR-93-5p	3.08	1.15	*IRF1*, *STAT3*
miR-98-5p	3.58		*C3*, *CCL5*, *IFNGR1*, *IL13*, *IL6*, *ITGB3*
miR-101-3p	4.65		*PTGS2*
miR-103a-3p	3.48		*IFNGR2*
miR-106a-5p	3.45		*IL1*
miR-106b-5p	3.64		*DAP3*, *RORA*, *TLR2*
miR-125a-5p	4.41	2.40	*CCL5*
miR-125b-5p	4.76	2.05	*STAT3*, *VDR*
miR-128-3p	4.22		*PTGS2*
miR-148a-3p	3.99		*HLA-G*
miR-148b-3p	3.83		*ADAM33*, *CCL11*, *HAVCR1*, *HLA-G*, *PTGER2*, *SLC22A5*
miR-151a-3p	4.15		*ACP1*
miR-152-3p	3.90		*HLA-G*
miR-155-5p	3.36	1.48	*CTLA4*, *EDN1*, *IFNGR1*, *IL13RA1*, *IL6*, *IL8*, *NOS3*, *SMAD3*, *STAT3*, *VCAM1*
miR-181a-5p	3.65		*PTGS2*
miR-181b-5p	3.90		*IFNGR1*
miR-191-5p	3.09	1.77	*IL1A*
miR-192-5p	4.08		*IL15*
miR-196a-5p	3.53		*IFNGR1*
miR-200a-3p	3.97		*SMAD3*, *VCAM1*
miR-200c-3p	3.74	2.29	*EDNRA*
miR-203a	3.04	1.01	*EDNRA*, *TNF*
miR-215-5p	3.83		*IL15*
miR-296-3p		−1.98	*CHRM3*
miR-320a	3.33	−1.42	*MIF*
miR-365a-3p	4.44		*IL6*
miR-423-3p	3.04	1.90	*CYFIP2*
miR-744-5p	2.45	1.71	*MIF*, *TGFB1*
miR-769-5p	3.30		*MIF*

NOISeq analyses were done with the transposed milk sample × miRNA matrix. Changes are presented as $\log_2$ fold change, and only significant associations corrected for multiple testing are shown.

Appendix III

Substantial Contributions by Benedikt Kirchner:

- Conceptualization and execution of experiments
- Curation, analysis and interpretation of data
- Drafting of figures
- Writing – original draft

Benedikt Kirchner

Michael W. Pfaffl

PLOS ONE

RESEARCH ARTICLE

Postprandial transfer of colostral extracellular vesicles and their protein and miRNA cargo in neonatal calves

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Abstract

Extracellular vesicles (EVs) such as exosomes are key regulators of intercellular communication that can be found in almost all bio fluids. Although studies in the last decade have made great headway in discerning the role of EVs in many physiological and pathophysiological processes, the bioavailability and impact of dietary EVs and their cargo still remain to be elucidated. Due to its widespread consumption and high content of EV-associated microRNAs and proteins, a major focus in this field has been set on EVs in bovine milk and colostrum. Despite promising i*n vitro* studies in recent years that show high resiliency of milk EVs to degradation and uptake of milk EV cargo in a variety of intestinal and blood cell types, *in vivo* experiments continue to be inconclusive and sometimes outright contradictive. To resolve this discrepancy, we assessed the potential postprandial transfer of colostral EVs to the circulation of newborn calves by analysing colostrum-specific protein and miRNAs, including specific isoforms (isomiRs) in cells, EV isolations and unfractionated samples from blood and colostrum. Our findings reveal distinct populations of EVs in colostrum and blood from cows that can be clearly separated by density, particle concentration and protein content (BTN1A1, MFGE8). Postprandial blood samples of calves show a time-dependent increase in EVs that share morphological and protein characteristics of colostral EVs. Analysis of miRNA expression profiles by Next-Generation Sequencing gave a different picture however. Although significant postprandial expression changes could only be detected for calf EV samples, expression profiles show very limited overlap with highly expressed miRNAs in colostral EVs or colostrum in general. Taken together our results indicate a selective uptake of membrane-associated protein cargo but not luminal miRNAs from colostral EVs into the circulation of neonatal calves.

Introduction

MicroRNAs (miRNAs) are small non-coding RNAs of approximately 21 nt that can regulate gene expression post-transcriptionally by hybridizing to complementary sequences in the 3'-untranslated region of mRNAs or in their coding region [1]. High degree of complementarity between miRNA and mRNA leads to destabilization and subsequent degradation, while a

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weaker pairing of primarily the seed region prevents translation to proteins at ribosomes [2]. Although miRNA biogenesis is a well-studied topic, resulting mature sequences display a heterogeneity far greater than originally assumed [3]. The complexity of these isoforms of miRNAs (isomiRs) stems mostly from divergent processing by DROSHA and DICER during cleaving of pri- and pre-miRNAs [4], but additional variants, diverging from pri- and pre-miRNA sequence templates, are also generated via exonucleases, nucleotidyl transferases and RNA editing [3]. isomiRs were shown to be incorporated into RNA-induced silencing complexes (RISC) [5] and achieve functional importance by cooperatively regulating common biological pathways [6]. Moreover, the potential and specificity of isomiR distribution patterns as biomarkers in development [7], cancer research [8], and even gender studies [9] were clearly demonstrated.

Although the general importance of miRNAs is undisputed, the functional relevance of dietary miRNAs still remains to be elucidated. Initial discoveries on uptake of plant miRNAs from rice [10] and honeysuckle [11] were followed by a number of studies that failed to reproduce the original findings [12–14] and were able to plausibly attribute them to contamination, poor study design or sequencing artefacts [15,16]. Even though the bioavailability of plant-derived miRNAs in food appears to be arguably refuted, it seems a special case can be made for dietary miRNAs associated with extracellular vesicles (EVs). EVs such as exosomes are key regulators of intercellular communication [17] and various studies in recent years have highlighted their importance in development [18] or promotion of disease progression [19].

One dietary source rich in EV-encapsulated miRNAs is milk [20]. Studies have shown that a large proportion of miRNAs in milk is localised in exosomes and exosome-like EVs [21]. Despite a significant reduction during processing or storage [22,23], a majority of milk miRNAs are stable and readily detected in commercial dairy products and even milk powder [24]. More importantly, miRNAs in milk EVs were proven to resist degradation under simulated gastrointestinal tract conditions [25,26], and *in vitro* experiments verified their ability to enter human intestinal cells [27], endothelial cells [28] as well as circulating immune cells [29]. Unfortunately, results of *in vivo* studies on orally administered milk miRNAs remain inconsistent. Baier *et al*. demonstrated a meaningful uptake of miRNAs after consumption of physiological amounts of bovine milk in adult humans and mice, coupled with a decrease in murine plasma levels of these miRNAs after feeding a diet depleted of bovine milk exosomes [30]. Independent replication of this study on the same samples, however, could not reproduce the results albeit this study suffered from technical problems during sample storage and EV degradation could not be excluded [31]. Well-designed experiments in knockout mice deficient in milk-enriched miRNAs failed to detect any significant postprandial miRNA increase in newborn pups after feeding wild type milk [32] and are therefore conflicting with studies that showed accumulation of orally administered fluorophore-labelled bovine milk exosomes in peripheral tissues in adult mice [33]. While efficiency of EV-associated miRNA transfer remains disputed, a systemic effect of orally administered milk EVs was proven in multiple studies including an attenuating effect on arthritis in mouse models [34]. Furthermore milk derived EVs might prove to be important players in drug delivery. Agrawal et al showed a significant tumor growth inhibition by drug loaded EVs compared to intraperitoneal injection [35] and drug delivery by milk derived EVs seems to induce less or no adverse immune and inflammatory responses [36,37].

So far, the vast majority of experiments has focused on the potential uptake of miRNAs from commercially available bovine milk in adult non-bovine species, neglecting the unique advantages of newborn calves and colostrum as a model for transfer of dietary miRNAs. It stands to reason that an uptake of milk or colostrum-derived EVs is just as likely to be observed between mother and direct offspring as a cross-species transfer to adult individuals.

Colostrum not only contains significantly higher amounts of miRNAs compared to mature milk, it was also shown that these are largely associated with EVs [20]. Moreover, transfer of colostral protein such as immunoglobulins to the circulation has already been studied extensively in ruminants. Although the intestinal uptake can be receptor-mediated, it is mostly driven by unspecific pinocytosis and occurs exclusively during the first days after parturition [38]. Furthermore, immediate postnatal colostral EVs were shown to be highly enriched for immune relevant proteins and factors involved in intestinal cell proliferation and displayed a protein composition quite dissimilar to milk EVs from later lactation stages and even colostrum EVs from day 2 or 3 [39]. While this prevents further conclusions on mature milk EVs or a potential uptake in other species, given the unique nature of colostral EVs [40], studying the effects of their very first dietary EVs on new-borne calves harbours great potential for insights in EV transfer in general.

The potential implications of a widespread absorption of dietary miRNAs are far-reaching and intimidating. Given the unique role miRNAs play in almost all physiological and pathological processes [41], it would cause no less than a paradigm shift in our perception of nutrition in general [42]. The objective of this study was to assess the potential transfer of colostral EVs and their cargo to the circulation of newborn calves by analysing colostrum-specific protein, miRNA and isomiR markers.

Material and methods

Sample collection

Blood and colostrum were sampled from randomly selected, healthy, multiparous, pregnant Brown Swiss cows (n = 9) housed at research station Veitshof (TU Munich, Weihenstephan) on the day of parturition. Blood samples from calves were taken directly before the first ad libitum feeding with colostrum (0 h) as well as 1 h, 3 h, 6 h afterwards plus directly before second feeding (9–12 h). All blood samples were drawn from *vena jugularis* in 9 ml K3 EDTA-Vacuette tubes (Greiner bio-one) with single-use needles (20G x 1", Greiner bio-one). Plasma and blood cells were separated within half an hour after sampling by centrifugation at 1850 g for 20 min at 4 ˚C. Comparably, colostrum was centrifuged at 1850 g for 30 min at 4 ˚C within one hour post sampling. After removal of the fat layer, skimmed colostrum and colostrum cells were collected. All samples including whole blood and colostrum were stored at -80 ˚C until further analysis. Colostrum was collected as total quarter milk and fed within 2 h of parturition, and calves were monitored to prevent autonomous feeding before blood sampling. Animals were housed and fed according to good animal attendance practice under permanent surveillance of a veterinarian, and all efforts were made to minimize suffering (permission number 55.2-1-54-2531-5-08). All animal trials were approved by the government of Upper Bavaria according to the German Protection of Animal Acts and no animals were sacrificed for this study and all animals were housed for further research.

Isolation of extracellular vesicles

EVs were isolated from defatted and cell-free sample fractions (~ 4 ml of plasma and 66 ml of skimmed colostrum) by differential ultracentrifugation as described previously [21,43] followed by flotation into a sucrose density gradient (SDG) [43]. In short, samples were diluted in PBS if necessitated by uneven fill levels, and pre-cleared by low-speed centrifugation (12,000 g, 1 h, k-factor: 2335.3) removing any remaining fat and cell residues. Plasma EVs were pelleted from 12,000 g supernatant (100,000 g, 2 h, k-factor: 278.3). For skimmed colostrum samples, the 12,000 g supernatant was subjected to further centrifugation steps at 35,000 g (1 h, k-factor: 797.4) and 70,000 g (3 h, k-factor: 397.9) before EVs were pelleted at 100,000 g (1 h, k-

factor: 278.3). To increase EV purity, pellets were resuspended in PBS and separated on a discontinuous top-down sucrose density gradient (30%, 40%, 50%, 60%) at 200,000g (18 h, k-factor: 110.3). Sequential fractions were diluted 1:10 in PBS and washed by ultracentrifugation (100,000 g, 1 h, k-factor: 278.3). Sucrose gradient fractions of 40% and 50%, corresponding to a density of 1.1764 g/ml and 1.2296 g/ml, respectively, were pooled to increase yield, as results showed these to be most enriched in colostrum EVs (S1 Table). Resulting EV pellets were either resuspended in PBS for further characterization, or directly lysed in QIAzol (Qiagen) for RNA extraction. All centrifugation steps were carried out at 4 ˚C using an Optima LE-80K ultracentrifuge and a SW40 or SW60 rotor (Beckman Coulter).

Total RNA extraction and characterization

Total RNA from blood and colostrum EVs (SDG fraction 40–50% only), cells from 1 ml of whole blood or 100 ml of colostrum as well as 1 ml of each non-fractionated fluid was isolated using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. A workflow for all RNA-related procedures is provided in Fig 1. To assess RNA quantity and exclude potential contaminations, samples were analysed on a NanoDrop spectrophotometer (Thermo Fisher Scientific), and total RNA profiles were assessed on the 2100 Bioanalyzer (Agilent Technologies) via RNA 6000 Nano and Small RNA Assay (Agilent Technologies).

Library preparation and Next-Generation small RNA sequencing

Comprehensive miRNA expression profiles of three randomly selected cows and their offspring were generated for all extracted compartments and time points (total n = 57) by small RNA-Seq. To focus on the evaluation of a potential transfer of EVs and their cargo from colostrum to the circulation, only the predominant colostral EV fractions corresponding to 40–50% SDG were sequenced. The library preparation was carried out as described previously [44–46] utilizing the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England BioLabs). Libraries were prepared from 100 ng total RNA except for calf plasma EV samples, where, due to their low concentration, the entire RNA yield was used. Small RNA specificity was achieved by size selection of PCR products using high resolution 4% agarose gel electrophoresis and retrieving bands corresponding to miRNA-adaptor-constructs (130–150 base pairs). Prior to 50 cycles of single-end sequencing on a HiSeq2500 (Illumina), fragment length and library purity were further confirmed by capillary electrophoresis (2100 Bioanalyzer High Sensitivity DNA Assay, Agilent Technologies). Raw sequencing reads were deposited in the European Nucleotide Archive (ENA) under the accession number PRJEB28002 (http://www.ebi.ac.uk/ena/data/view/PRJEB28002).

Data analysis

Sequencing data was processed using a self-compiled bioinformatic pipeline as described previously [47,48] with the added functionality of discovering and quantifying isomiRs. In short, 3'-end adaptor sequences were trimmed using Btrim [49], and length distribution and sequencing quality were monitored via FastQC [50]. To prevent false positive mappings to miRNAs and isomiRs, reads matching rRNA, tRNA, snRNA or snoRNA sequences obtained from RNAcentral [51] along with reads shorter than 16 nt were excluded from further analyses. Filtered reads were then aligned to a newly designed mapping reference consisting of all bovine miRNAs (miRBase v21) [52] and their respective miRNA isoforms. isomiR sequences were derived from canonical miRNA sequences by consecutive trimming of up to 6 nt or addition of 3 nt on 5'- and 3'-end, and included mismatch information obtained during mapping.

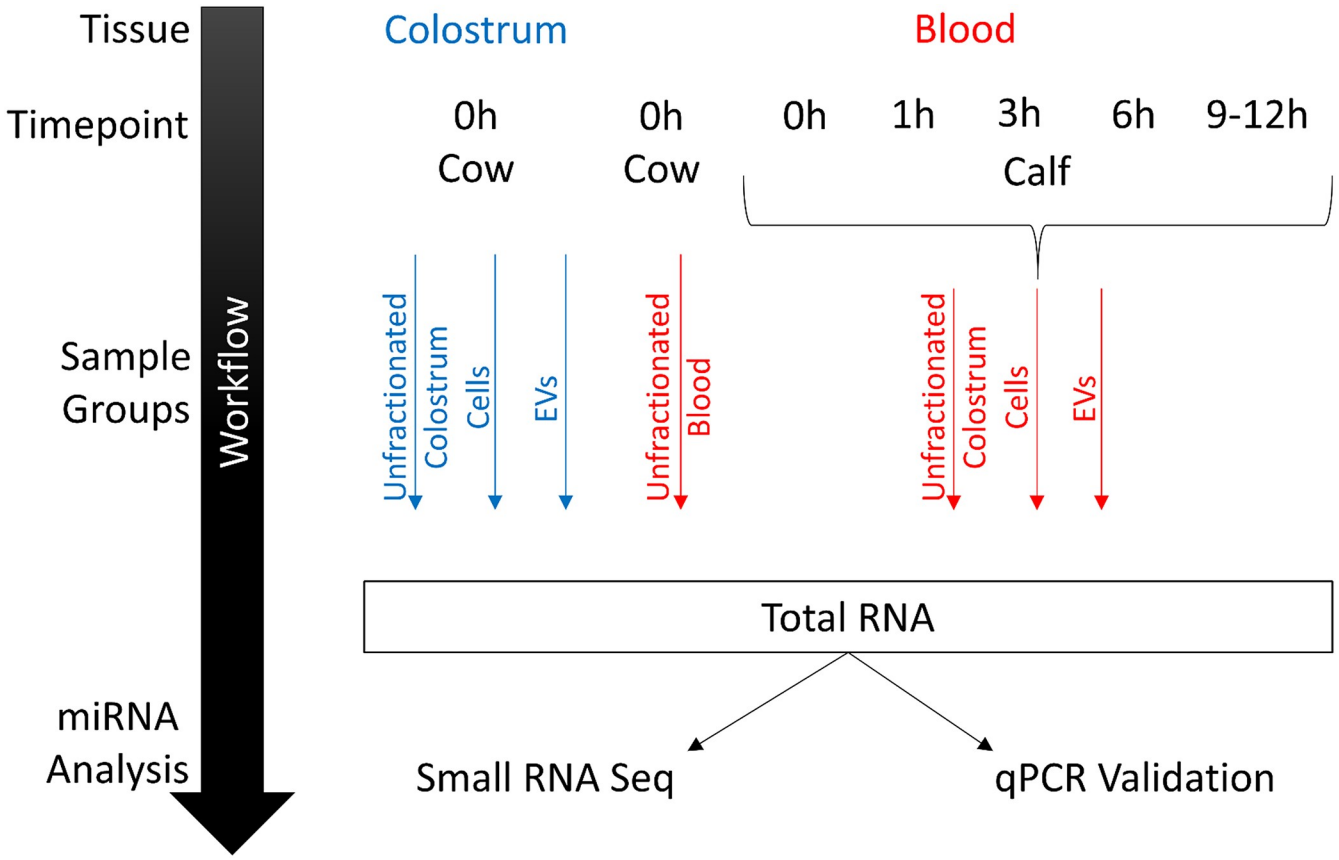

Fig 1. Schematic overview of sample groups for RNA extraction and transcriptomic analyses. Total RNA was extracted from unfractionated, cellular and EV compartments of colostrum and calf blood samples, as well as unfractionated blood from cows before first feeding and at four defined postprandial time points (n = 3 each) before being profiled by small RNA-Seq. Expression of differentially regulated and highly abundant miRNAs was subsequently assessed by RT-qPCR in an expanded animal cohort (cows n = 6, calves n = 8).

https://doi.org/10.1371/journal.pone.0229606.g001

Alignment was performed using Bowtie [53], allowing for a single mismatch over the whole sequence, and applying the 'best' algorithm. From the resulting SAM files, isomiR read count tables were generated by incorporating mismatch information that describes potential polymorphic isomiRs together with sequence additions and trimmings, and finally calling the sum of each individual sequence. Differential gene expression profiles were obtained employing the DESeq2 package (v1.18.1) [54] from bioconductor and the included normalization, testing and false discovery correction algorithms. Expression changes of isomiRs and underlying miRNAs were considered significant if adjusted p-values were ≤ 0.05 and $\log_2$ fold changes $\geq |1|$ combined with a minimal abundance of baseMean ≥ 50. Similarities of isomiR expression profiles were visualised via hierarchical clustering (euclidean distances, ward's method), principal component analysis of regularised log-transformed, normalised read counts, and Venn diagrams using R (v3.4.3) [55] and relevant packages [56–59]. Potential implications of significantly up-regulated miRNAs in calf EV fractions were evaluated by enrichment analysis of KEGG pathways [60,61] following the advice from Godard and van Eyll to minimize the false positive effect of single miRNAs with multiple targets within the same pathway [62] for experimentally verified mRNA targets of human homologues with strong evidence obtained from miRTarBase [63].

RT-qPCR

The Exiqon miRCURY LNA Universal RT microRNA PCR system (Exiqon) was used to validate miRNAs selected from NGS results in a larger cohort comprised of 6 cows and their 8 calves including two twin births (total n = 144). Reverse transcription and qPCR were performed according to the manufacturer's instructions with 10 ng of total RNA as starting input except for calf plasma EV samples, where 2 μl of undiluted sample was used due to their low yield. Assays for validation included: bta-miR-21-5p, bta-miR-26b, bta-miR-30a-5p, bta-miR-141-3p, bta-miR-144, bta-miR-146a, bta-miR-146b-5p, bta-miR-148a-3p, bta-miR-200a, bta-miR-200b, bta-miR-451 and bta-miR-2285t. Specificity of all assays and samples was ensured by using non-template and negative RT controls in representative sample pools from each fraction. All qPCR reactions were measured on a CFX384 Real-Time PCR Detection System (Bio-Rad). Statistical significance on geo-mean normalised data [64] was tested using F-test for normality and Student's t-test.

EV characterization

Isolated EV suspensions were further characterised in terms of morphology, particle size and concentration as well as protein cargo. Calf EV samples 1 h postprandial had to be omitted since plasma yield was very low and no EV isolations could be performed. A workflow for all EV characterization related analyses is provided in Fig 2.

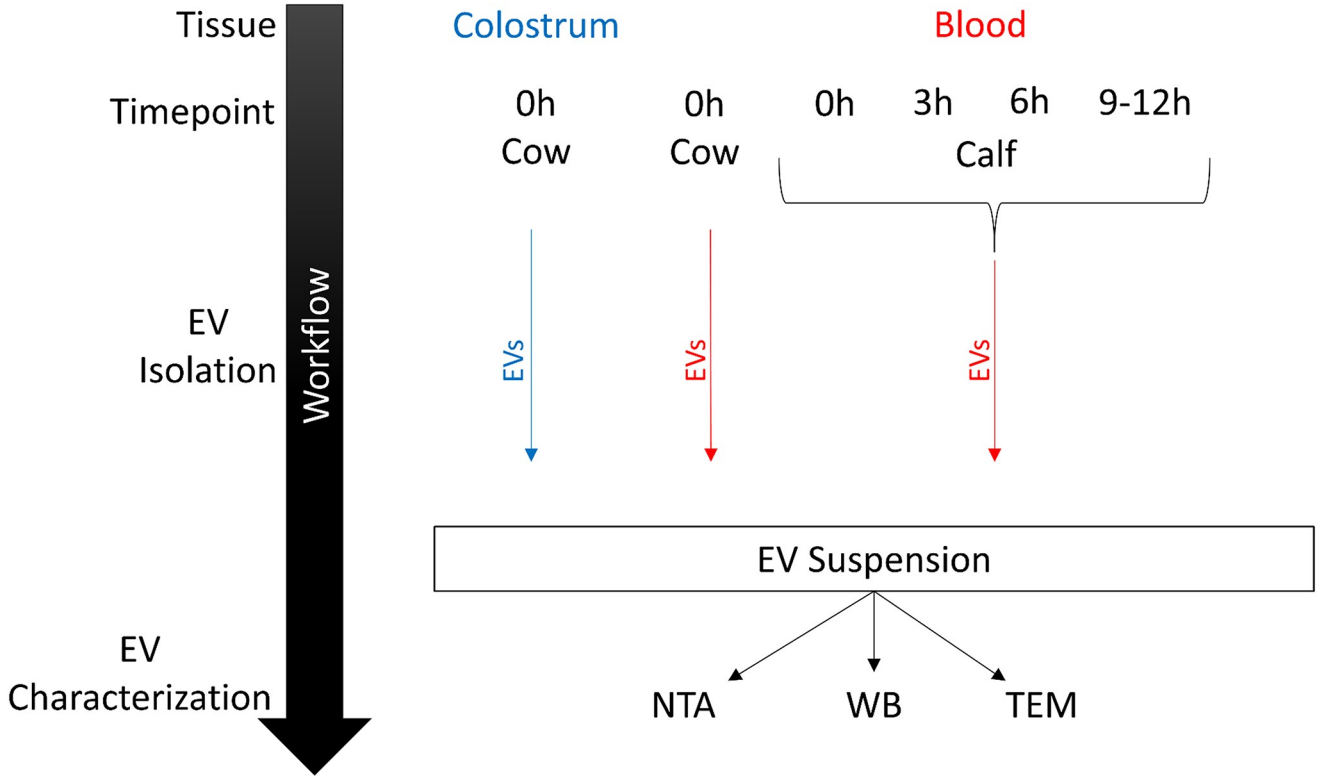

Fig 2. Schematic overview of sample groups for EV characterization. EVs were isolated by differential ultracentrifugation and sucrose density gradient in colostrum and blood samples from calves and cows before first feeding and at three defined postprandial time points (n = 3 each). EV morphology and protein cargo were characterized by Nanoparticle Tracking Analysis, Western blot and transmission electron microscopy.

https://doi.org/10.1371/journal.pone.0229606.g002

Nanoparticle tracking analysis

For assessment of particle concentration and size distribution, samples from 30% and 40–50% sucrose density gradient fractions were analysed using NTA. EVs were diluted in particle-free PBS and measured on a NanoSight NS300 (NTA 3.0 software, Malvern Instruments) device outfitted with a 405 nm laser and a high-sensitivity sCMOS camera. Samples were injected manually, and eight videos of 45 s each were captured under previously optimised conditions (25 frames/sec, camera level 14, FTLA algorithm). For analysis, a conservative detection threshold, auto settings for blur and minimum track length as well as a minimum of 4000 completed tracks per sample were used. Final concentrations were calculated in relation to 1 ml of plasma and skimmed colostrum.

Transmission electron microscopy

Diluted EVs were adsorbed onto glow-discharged, carbon-coated copper grids (Quantifoil) for 2 min before manually removing excess liquid by filter paper. Grids were negatively stained in 2% uranyl acetate for 2 min and air-dried prior to imaging. All images were taken on a JEOL JEM 100CX electron microscope at 100 kV.

Western blot

EV fractions were lysed in ice-cold detergent lysis buffer (0.1% Triton X-100 in PBS) supplemented with protease inhibitors (cOmplete Mini Protease Inhibitor Cocktail, Roche). To enhance rupture of membranes, lysates were sonicated for one minute in a water bath prior to protein quantification using BCA assay (Sigma Aldrich). For SDS-PAGE, samples were heated for 5 min at 70 ˚C in reducing Laemmli buffer transferred to 0.45 μm nitrocellulose membranes (GE Healthcare Life Sciences) using NuPAGE transfer buffer (Invitrogen) supplemented with 10% methanol. Post transfer, membranes were blocked with 1% skim milk powder in PBST for 1 h at room temperature and incubated with primary antibodies at 4 ˚C overnight. After three washes with blocking buffer, HRP-conjugated secondary antibodies were applied to membranes for 1 h at 4 ˚C. Blots were developed using Luminata Classico Western HRP substrate (Merck KGaA). Primary antibodies were purchased from Santa Cruz (goat anti-CD63, sc-31214, 1:1000; goat anti-BTN1A1, sc-324834, 1:1000), Sigma-Aldrich (rabbit anti-MFGE8, HPA002807, 1:2000), Abcam (mouse anti-HSP70, ab2787, 1:800) and Biomol (goat anti-Calnexin, WA-AF1179a, 1:2500). Secondary antibodies were from Abcam (goat anti-rabbit HRP, ab97080, 1:6700; rabbit anti-goat HRP, ab97105, 1:6700).

Results

Blood and colostrum from adult cows bear two distinct EV populations

Particles with characteristical EV morphology and size were found in blood as well as colostrum EV preparations by TEM (S1 Fig). No apparent size differences between tissues or sucrose density fractions could be detected and the majority of vesicles were less than 150 nm in diameter. Subsequent analysis by NTA confirmed these findings with mean and mode vesicle diameters ranging from 129.6 nm and 98.3 nm in 30% SDG colostrum EV samples to 167.8 nm and 112.4 nm in 40–50% SDG blood EV samples, respectively (Fig 3A). Although particles showed little size heterogeneity, striking differences were found for particle concentrations. EV preparations from colostrum showed very high mean particle numbers per ml (4.59E10 ± 4.25E9 P/ml) and consisted predominantly of particles floating in 40–50% SDG with only a very minor amount of particles originating from the 30% SDG fraction ($p \leq 0.05$, Fig 3B). Contrary to that, total particle numbers in cow blood were reduced by over 10-fold

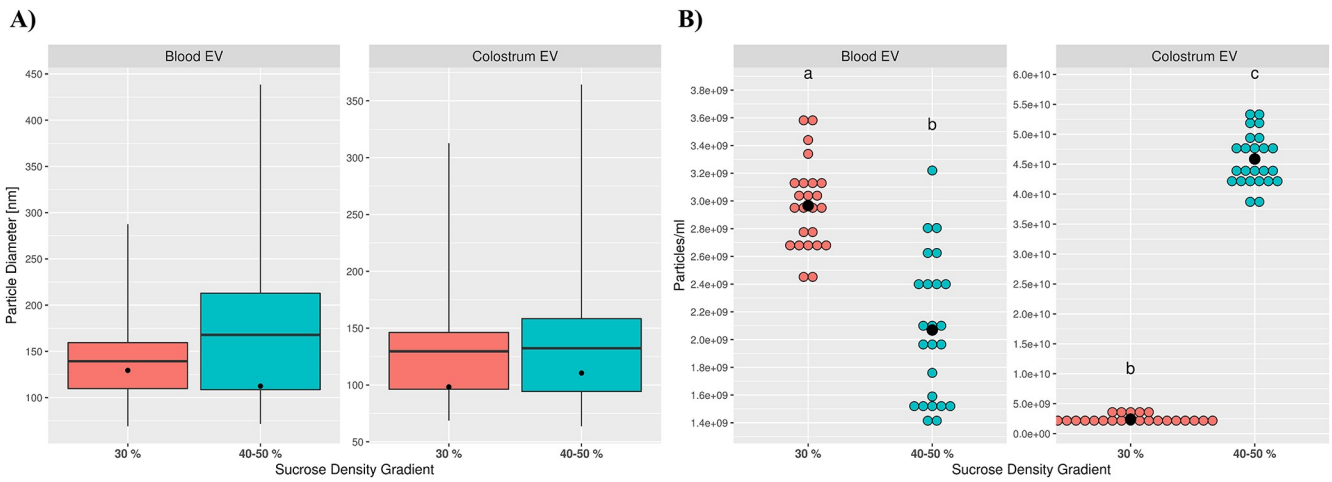

Fig 3. Analysis of particle size and concentration by NTA in adult cows. No significant differences in particle size were detected in blood- and colostrum-derived cow EV suspensions (A). Whiskers indicate 1st and 99th percentiles; line: mean diameter; dot: modal diameter. Blood- and colostrum-derived cow EV suspensions showed diverging concentrations of particles from different flotation densities of SDG (B). Mean particle numbers per ml of colostrum or plasma are depicted as black dots; binned coloured dots indicate individual measurements of three animals, each repeated eight times; differing letters above dots indicate significant differences in particle numbers ($p<0.05$).

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(2.97E9 ± 3.15E8 P/ml) and were significantly enriched in 30% SDG particles ($p \leq 0.05$). Additional information on particle size and concentration measurements from NTA can be found in S1 Table.

To further differentiate between blood and colostrum particles, EV- and milk-specific proteins as well as a negative marker were assessed by Western blot for 40–50% SDG preparations (Fig 4). Particles from both fluids were positive for CD63 and HSP70, two commonly used markers for vesicles, with EVs isolated from colostrum showing higher intensities, while no signals could be detected for calnexin, a marker for non-vesicular membrane contamination. A different pattern was observed for MFGE8 and BTN1A1, both of which were previously found to be highly associated with milk vesicles [65,66]. Colostrum EV isolates displayed very strong signals, whereas protein lysates from blood EV preparations were negative for these markers, which we therefore considered specific to milk EVs.

Calf plasma levels of EVs that share colostrum characteristics are increased after feeding

Comparable to EV isolates from adult cows, particles in EV preparations from calf blood showed similar size ranges with mean and mode diameters ranging from 142.3 nm and 122.1 nm to 160.3 nm and 142.9 nm, respectively, and no significant size differences between sampling time points or SDG fractions (Fig 5A). Particle concentrations for 30% SDG were stable over all time points with no significant changes in particle numbers (Fig 5B). Meanwhile, concentrations of vesicles isolated from 40–50% SDG were consistently and significantly ($p \leq 0.05$) increased for every time point compared to pre-feeding samples, reaching a maximum concentration of 1.97E10 ± 7.33E9 P/ml at 9–12 h postprandial ($p \leq 0.05$) and were significantly more abundant compared to 30% SDG particles. Further similarities between EVs isolated from colostrum and calf plasma after feeding were found by Western blot analysis. CD63 and the colostrum-specific BTN1A1 were detected with increasing signal intensities with progressing time points, albeit with no or only weak expression for 0 h samples.

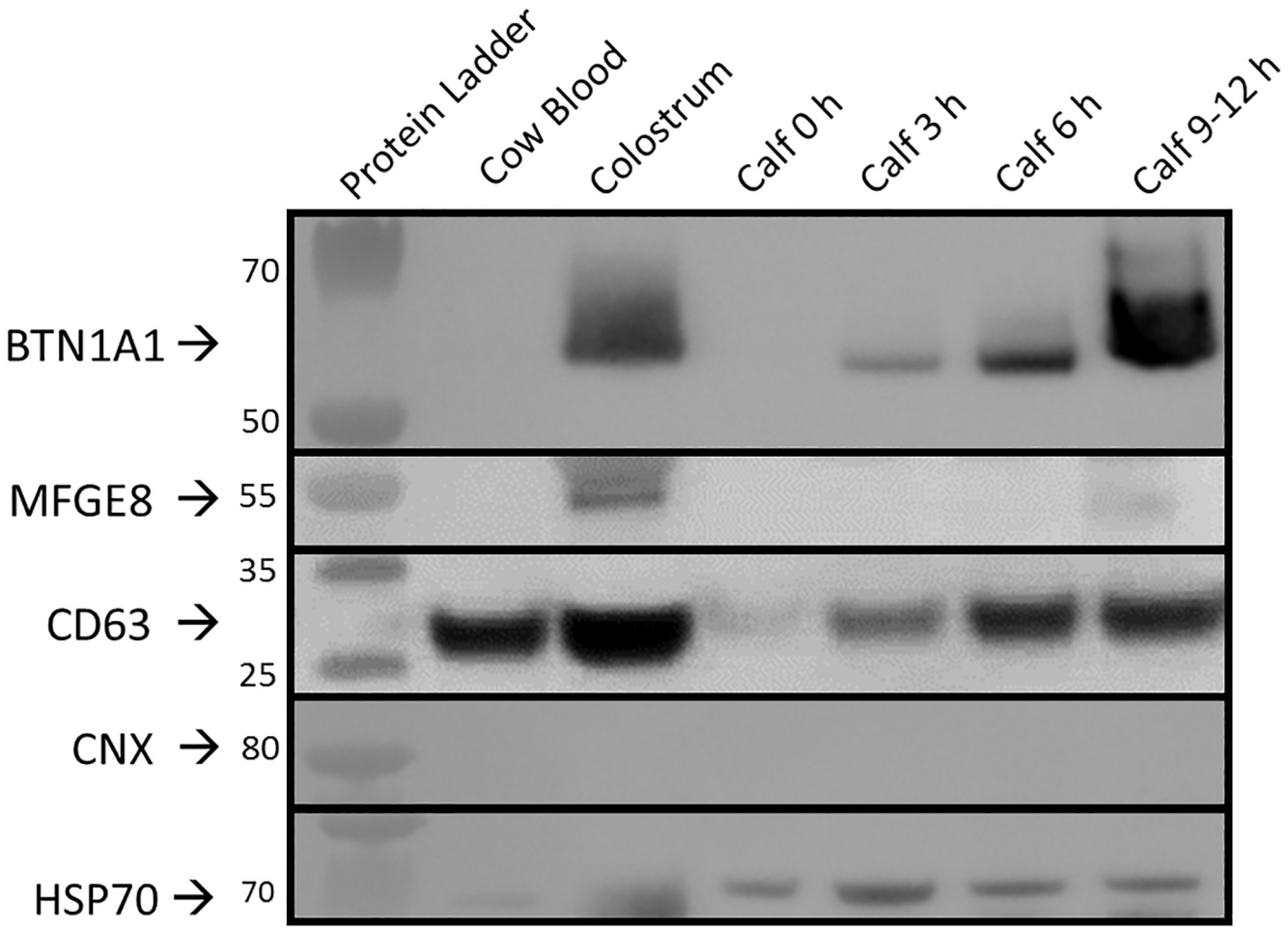

Fig 4. Protein expression analysis of EVs of colostral and blood EVs from adult cows and calves by Western blot. Milk specific markers BTN1A1 and MFGE8 were found in colostral and postprandial calf EV isolations but not in cow blood EVs or calf EVS pre-feeding. Presence of EV markers was confirmed by transmembrane protein CD63 and cytosolic protein HSP70 expression in all sample groups while CNX, a marker for contamination with cellular fragments, was negative. Results are representative for three biological replicates for both cow and calf samples as well as postprandial time points.

https://doi.org/10.1371/journal.pone.0229606.g004

Postprandial samples were positive for MFGE8 as well, but for no more than a single time point in each calf and only after 6 or 9–12 h (Fig 4).

Analysis of tissue- and compartment-specific small RNA profiles by NGS

Mean library sizes generated by small RNA sequencing differed within expected dimensions, ranging from 5.01E6 ± 1.30E6 in calf EV samples, potentially reflecting low input amounts for library preparation due to low RNA yield, to 1.34E7 ± 2.17E6 in colostrum cells. One sample each from whole colostrum, colostrum cells as wells as calf EV 0 h groups had to be excluded from further analysis, since they failed to amplify correctly during sequencing. Differences in small RNA profiles were assessed by aligning reads against miRNA and isomiR sequences as well as other major small RNA classes (rRNA, tRNA, snRNA, snoRNA) and plotting them together with unmapped and short reads (<16 nt) as percentages of total library size per group (Fig 6). Highest enrichment for isomiRs including canonical miRNAs was seen in whole blood samples from cow and calf along with calf blood cells (>80%) with little to no other small RNA species present. On the other hand, libraries from calf EVs were dominated by a large

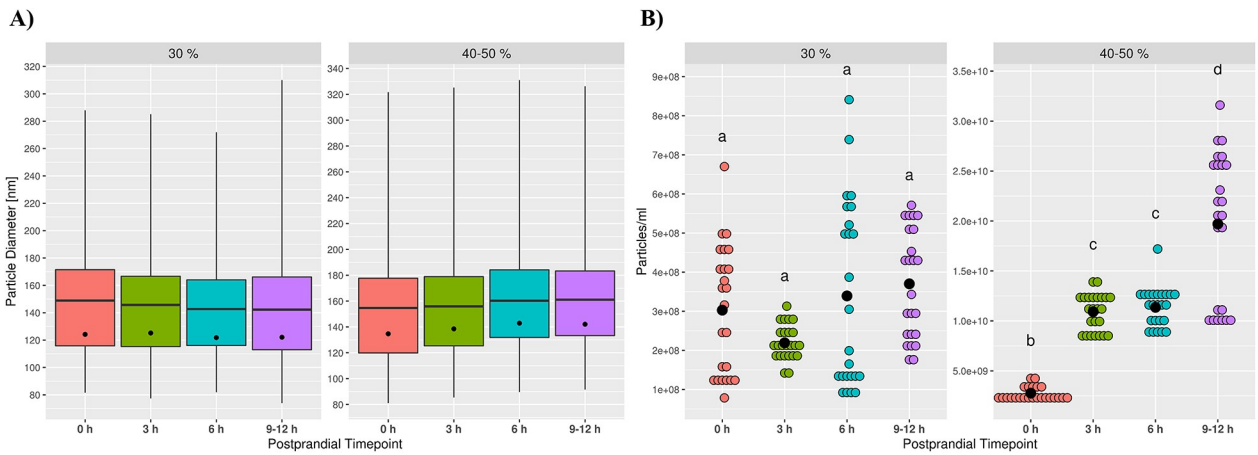

Fig 5. Analysis of particle size and concentration by NTA in calves. No significant changes in particle size were detected in calf EV suspensions by NTA analysis (A). Whiskers indicate 1st and 99th percentiles; line: mean diameter; dot: modal diameter. Particle numbers were significantly increased in progressing postprandial time points for EV isolates from higher density SDG fractions (40–50%), while concentrations from 30% SDG EV suspensions remained stable (B). Mean particle numbers per ml of plasma are given as black dots; binned coloured dots indicate individual measurements of three animals, each repeated eight times; differing letters above dots indicate significant changes in particle numbers.

https://doi.org/10.1371/journal.pone.0229606.g005

number of unmapped sequences not belonging to any of the major small RNA classes, and a comparatively low number of miRNAs. Similar to blood, colostrum-derived samples exhibited a clear distinction between extracellular, EV-associated samples and unfractionated or cellular groups, respectively. All colostrum samples displayed higher relative numbers of tRNA reads compared to blood samples, which was most pronounced in colostrum cells. Unfractionated colostrum and colostrum EVs, on the other hand, contained the highest frequencies of short reads. Additionally, a strong relative enrichment of miRNA reads was observed in colostrum EVs compared to colostral cellular samples. Further information on alignment distributions and library sizes can be found in S2 Table.

Analysis of canonical miRNAs reveals an influence of colostrum feeding on expression profiles in calf EVs only

Differential regulation of canonical miRNAs between colostrum and blood compartments along with postprandial time points within the same sampling group was assessed using DESeq2 and applying conservative filtering criteria (adjusted p-value ≤ 0.05, $\log_2$ fold changes $\geq |1|$, baseMean ≥ 50). Unfractionated colostrum differed minimally from colostrum EVs with only 9 significantly regulated miRNAs, while both groups showed a considerably different miRNA expression profile compared to colostrum cells (Table 1), reflecting RNA species distribution seen during alignment. The biggest expression changes in the data set could be found between all three colostrum-derived sample groups and unfractionated cow blood with over 150 differentially expressed miRNAs in each group (Table 1). Albeit expression profiles of calf blood-derived samples displayed high diversity between individual compartments with numbers of significantly regulated miRNAs ranging from 105 to 147 (Table 1), expression changes within different postprandial time points of blood compartments were scarce with the exception of calf EVs. Similar to calf blood cell samples, which exhibited no alterations at all over all time points, miRNA expression in unfractionated blood samples was very stable with a total of two significant regulations after 9–12 h (Table 2). On the other hand, expression changes in postprandial calf EVs samples compared to pre-feeding samples ranged from two (after 1 h) to 24 (after 9–12 h) miRNAs and continually increased in magnitude with progressing time

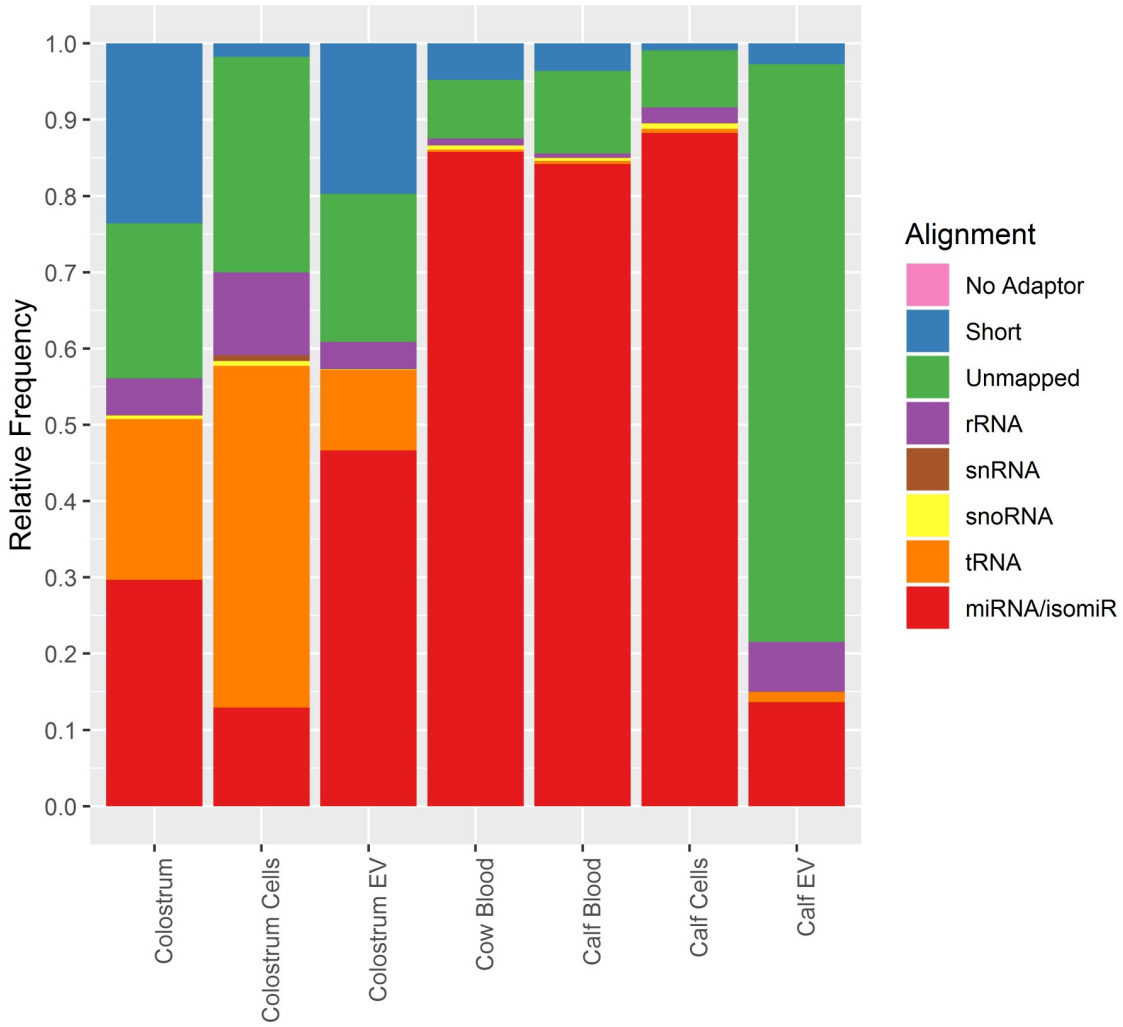

Fig 6. Distribution statistics of analysed small non-coding RNA (ncRNA) species in relation to total library sizes of raw reads. Distinctive RNA profiles of small ncRNA are recognizable for samples from different body fluids (colostrum, blood) as well as between unfractionated, cellular and EV-related samples. No Adaptor: reads without detectable adaptor sequence at 5'-end; Short: reads shorter than 16 nt; Unmapped: reads not mapping to either rRNA, tRNA, snRNA, snoRNA or miRNA/isomiRs sequences. Relative alignment frequencies are given as mean percentages of total library sizes for each sample group.

https://doi.org/10.1371/journal.pone.0229606.g006

Table 1. Numbers of canonical miRNAs differentially regulated between unfractionated, cellular and EV-related sampling groups in colostrum and blood.

	Colostrum EVs	Colostrum	Colostrum Cells	Cow Blood
Colostrum EVs				
Colostrum	9			
Colostrum Cells	92	69		
Cow Blood	167	165	158	
	Calf Blood EVs	**Calf Blood**	**Calf Blood Cells**	
Calf Blood EVs				
Calf Blood	147			
Calf Blood Cells	122	105		

https://doi.org/10.1371/journal.pone.0229606.t001

Table 2. Numbers of differentially regulated canonical miRNAs between postprandial time points and pre-feeding samples (0 h) for calf blood-derived samples.

	Postprandial Time Points			
	1 h	3 h	6 h	9–12 h
Calf Blood	0	0	0	2
Calf Blood Cells	0	0	0	0
Calf Blood EVs	6	15	24	26

https://doi.org/10.1371/journal.pone.0229606.t002

(Table 2). Although 28 out of the total 30 canonical miRNAs with significant postprandial expression changes were up regulated, 12 of those miRNAs showed significantly smaller abundances in colostral EV samples compared to calf EV 0 h samples. Furthermore, out of the top 15 most highly expressed miRNAs in colostral EVs only three showed a significant increase after feeding with all of them belonging to the canonical miR-200a/b/c family. Differentially expressed miRNAs together with corresponding $\log_2$ fold changes of key comparisons as well as raw read counts are provided in S3 and S4 Tables.

Hierarchical clustering analysis of total miRNA expression confirmed expression profile changes detected in DESeq2 results by clearly separating unfractionated from cellular and EV-associated sample groups (Fig 7). Gene expression differences within unfractionated calf blood, blood cells and, to a smaller degree, blood EVs displayed remarkable homogeneity, resulting in groups defined by individual animals rather than clusters of particular postprandial time points. Furthermore, miRNA expression in calf blood EVs resembled colostrum profiles much more closely than any other blood-derived sample from cow or calf. Continuing the pattern revealed in RNA species distribution and differential gene expression, colostrum cells could be clearly separated from unfractionated colostrum and colostrum EVs. The potential physiological impact of up-regulated miRNAs in postprandial calf EVs was evaluated by enrichment analysis of KEGG pathways as proposed by Godard and van Eyl [62]. The most highly enriched pathway across all time points was insulin signaling, followed by TGF-beta

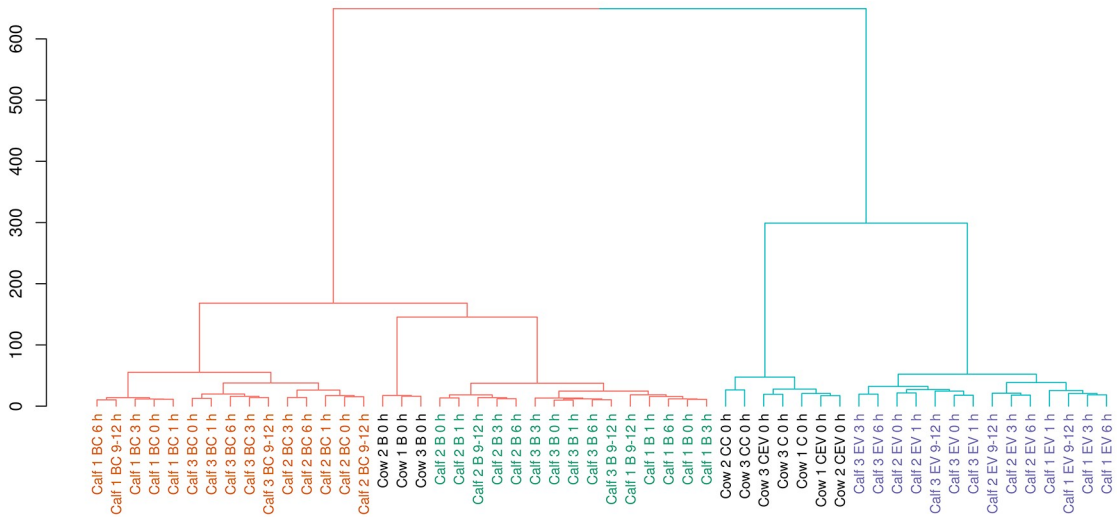

Fig 7. Hierarchical clustering analysis of canonical miRNAs across all sample groups and postprandial time points. The two dominant clusters were composed of colostral and calf EV samples (right) and unfractionated blood and blood cells (left). B = unfractionated blood; BC = blood cells; EV = blood extracellular vesicles; C = unfractionated colostrum, CC = colostral cells; CEV = colostral extracellular vesicles.

https://doi.org/10.1371/journal.pone.0229606.g007

signaling and cytokine-cytokine receptor interaction, but no apparent overall regulation pattern could be discerned. Top 20 enriched pathways along with involved miRNAs can be found in S5 Table.

isomiR expression profiles in postprandial calf EVs suggest non-colostral origin of up-regulated miRNA isoforms

To assess whether miRNA regulation changes in calf EVs could be attributed to colostral EV cargo, the distribution of miRNA isoforms was analysed. Since isomiR expression is highly specific for tissues and developmental stages [7–9], it allows for more precise clustering and higher confidence in determining the possible origin of expression changes in transfer studies. Unsupervised clustering of the 500 highest variance isomiRs via principal component analysis (PCA) highlighted uniformity of expression in colostral and non-EV blood samples and revealed an underlying expression pattern correlating nicely with advancement of postprandial time points (Fig 8). Although PCA uncovered a progressing overlap in expression between calf EV and colostrum-derived samples, evident on the first principal component (PC1), calf EV samples were even stronger characterised by an isomiR expression distinctively different from colostrum (PC2). Furthermore, the majority of postprandially up-regulated isomiRs showed limited overlap with isomiRs highly expressed in colostrum EVs (Fig 9). Out of the top 100 most abundant isomiRs in colostrum EVs, constituting 74.5% of all isomiR reads in these samples, only 30 could be rediscovered in postprandial calf EVs as significantly up regulated. Similar to canonical miRNA analysis, 15 of these isomiRs belonged to the same miRNA family of bta-miR-200a/b/c.

Discussion

The role of dietary miRNAs and their transfer to consumers has recently provoked a lot of discussion in the scientific community. Although modes of transfer, uptake and distribution to recipient cells are still not clearly deciphered, the possible ramifications on our understanding of environmental influences on gene expression are colossal due to the essential role of miRNAs in post-transcriptional gene regulation. The debate is further fueled by the association of many dietary miRNAs with EVs, providing not only a robust carrier to resist digestion but also a conceivable mean of transport to the gastrointestinal tract [25–29]. In this study, we assessed the potential transfer of colostral EVs and their protein and miRNA cargo based on biochemical, molecular analyses and high-throughput sequencing of small non-coding RNAs. As recent findings on the bioavailability of milk miRNAs are partly in direct contradiction to each other [30,31], we chose samples from blood and colostrum of multiparous cows as well as from the circulation of their direct offspring before and after their first feeding as the model with a high probability to demonstrate an uptake of dietary EVs.

The ability to isolate reasonably pure vesicles is a prerequisite for any viable EV study [67,68]. Following well-established protocols for differential ultracentrifugation in combination with flotation into a density gradient [21,43] yielded particles in the size range of typical small EVs in all biofluids. Modal diameters of particles (~98 to 112 nm) were slightly above exosome-like size (30–100 nm) [69] and in line with previous findings on EVs from bovine or human milk and blood [25,44,70] (Fig 3A). No significant differences in size could be detected between blood and colostrum samples or particles recovered from different sucrose density fractions, hinting at a high reproducibility of EV isolation methods and low inter-individual variability. Quantitative analysis of particles on the other hand, revealed prominent differences in particle composition and concentration in blood and colostrum (Fig 3B). In adult animals, EV numbers were more than 10 times higher in colostral samples compared to blood samples.

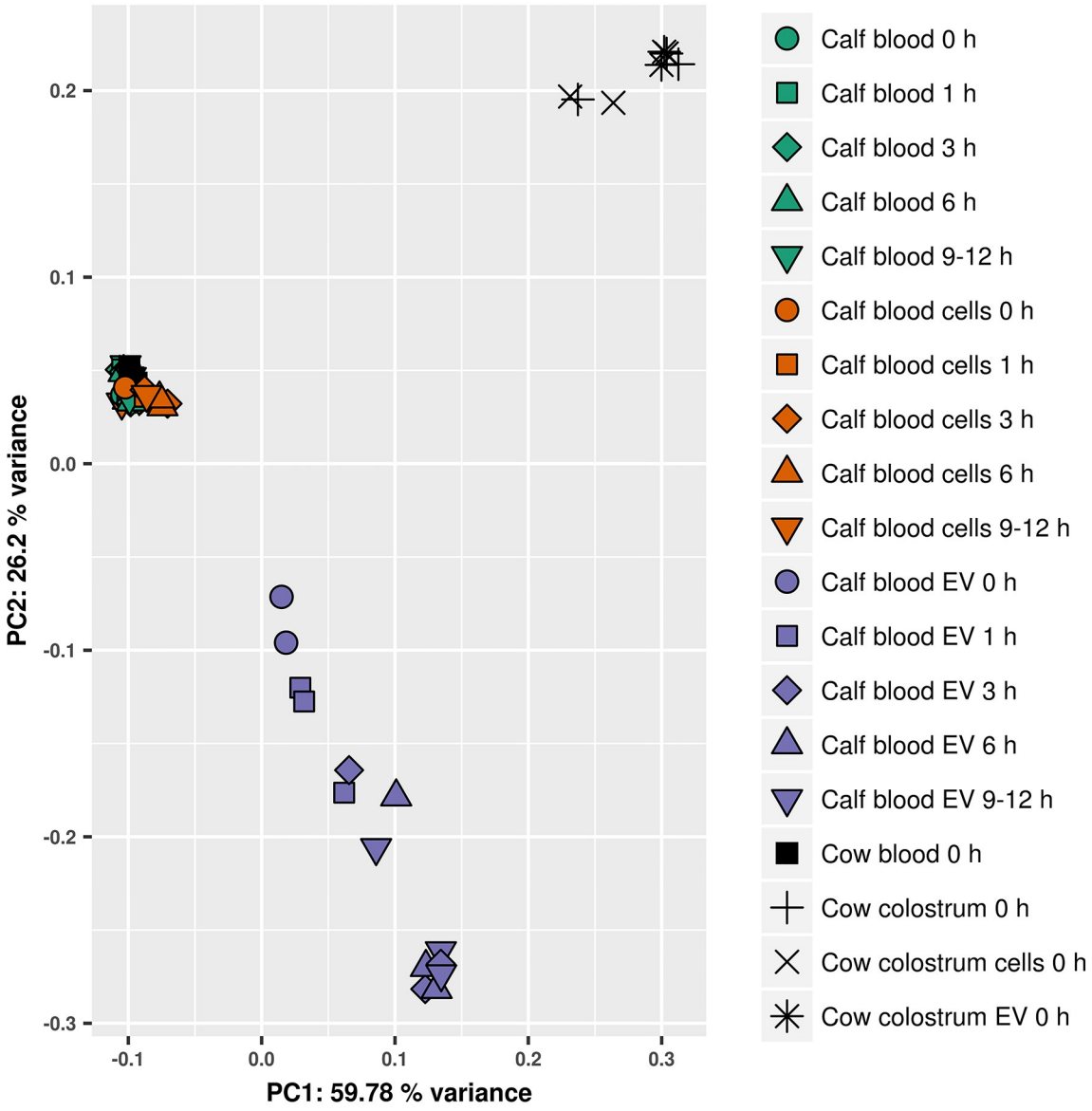

Fig 8. Principal component analysis of the top 500 most varying isomiRs across all samples. Calf sample groups are denoted by different colours while postprandial time points are indicated by symbol shapes. Cow samples are given in black with different symbol shapes depicting sample groups.

https://doi.org/10.1371/journal.pone.0229606.g008

While this stands in contrast to the findings of Koh et al, who found concentrations of milk particles to be ~3.2 fold lower than plasma particles [70], it is corroborated by previous reports that have shown that miRNAs associated with colostrum EVs are highly enriched compared to milk from later lactation stages [20,21,24]. Furthermore, the majority of colostrum EVs were significantly denser (40–50% SDG) than blood-derived EVs (30% SDG), although all isolated particles generally fell in the density range expected for exosome-like vesicles (1.1270–1.2296 g/ml) [69]. Predominant EVs of high densities in colostrum were in agreement with findings from Hata et al., showing highest content of vesicle-associated proteins and RNAs from density fractions corresponding to 1.20 g/ml compared to lower densities [21]. While density of

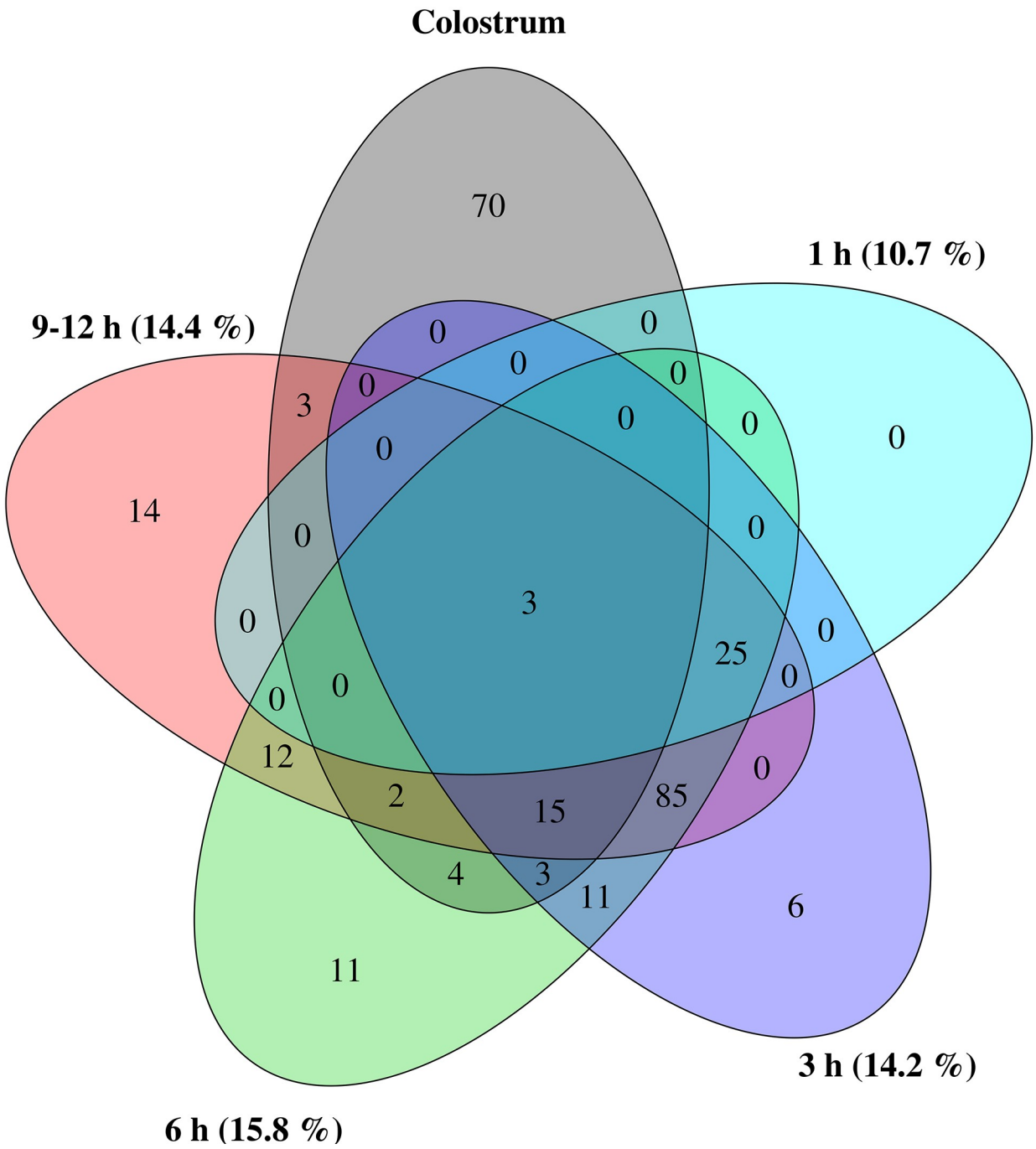

Fig 9. Venn diagramm displaying the overlap between the top 100 most abundant isomiRs in colostrum EVs and significantly upregulated isomiRs in postprandial calf EV samples of all time points. Number in brackets denote the percentage of common isomiRs per time point.

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particles from plasma was in line with numerous reports [71,72], this is to the best of our knowledge the first time that a significant shift in density between bovine EV populations of blood and milk has been reported. It should be noted, however, that concentration measurements of particles by NTA are prone to overestimation due to co-isolated contaminants such as lipoproteins and protein aggregates, especially in vesicle preparations of low purity [73–75].

Particularly preparations from milk or colostrum, with its high content of fat globules and senescent, ex-foliated epithelial cells seem prone to accumulate unwanted particles that can mimic EV properties for example by aggregation during ultracentrifugation [76]. Nevertheless, detected EV concentrations in adult blood and colostrum seem genuine as most contaminants should be discarded in SDG fractions of higher (protein aggregates) or lower (aggregated small fat globule membranes) densities [43]. Endosomal origin of membranous EV preparations as well as higher concentrations of particles in colostrum were further supported by Western blot analysis of positive (CD63, HSP70) and negative (Calnexin) vesicle markers (Fig 4). Positive expression of BTN1A1 in colostrum-derived EVs is in concordance with recordings on Vesiclepedia and ExoCarta [77,78], the biggest databases on molecular data of extracellular vesicles, which list BTN1A1 as exclusively associated with bovine and human milk EVs [65,66]. Expression of MFGE8 however, was reported to be widespread in EVs originating from a large variety of sources including B cells and platelets [79,80]. Although MFGE8 was never reported to be associated with bovine EVs apart from milk [66] and we considered absent expression in cow blood to be genuine, it could also not be excluded that a lack of signal originated from low starting input material due to the fact that only samples from 40–50% SDG fractions were analysed in Western blots.

Differentiation between blood- and colostrum-derived samples and especially EVs was further driven by abundances of small RNA species analysed by Next-Generation Sequencing. High throughput sequencing has become the tool of choice for analysing nucleic acids due to its high precision in quantifying of single RNA sequences and its accurate detection of diverse RNA compositions. Clear differences were found in relative frequencies of miRNA and tRNA as well as reads shorter than 16 nt between whole blood and colostrum-derived samples in cows (Fig 6). Higher incidences of short reads most likely stem from an increased number of degradational products tracing back to ex-foliated, senescent epithelial cells present in colostrum, while enrichment of miRNAs in colostrum vesicles compared to the cellular fraction has been reported before by Sun et al [20]. Additionally, varying expression both between blood- and colostrum-derived samples and within cellular and extracellular fractions in colostrum were confirmed by differential analysis of canonical miRNAs (Table 1). In general, miRNA expression profiles in colostrum-derived samples were in concordance with previous reports with a substantial overlap in the most abundant miRNAs [21,81,82]. miRNA expression in blood and colostrum was completely dissimilar, duplicating results obtained from milk and peripheral blood cells [83] as well as circulating miRNAs [84]. However, differences between unfractionated colostrum and colostrum EVs were minuscule compared to colostrum cells, suggesting that the majority of miRNAs in colostrum is present in the extracellular compartment and the removal of the upper fat layer and its associated miRNAs is of minor consequence. Nonetheless, a potential impact from colostral fat derived miRNA cannot be ruled out completely and should be analysed in further studies.

Taken together, quantitative and qualitative analyses of EVs in cows indicate distinct populations of vesicles in blood and colostrum, enabling the identification of a potential postprandial transfer of colostrum EVs into the blood circulation based upon EV concentrations, protein cargo and miRNA expression. Vesicles identified in calf blood after feeding indeed shared characteristics classified as specific for colostrum EVs (Fig 5). Particle concentrations for preparations increased significantly in a time-dependent manner solely for 40–50% SDG, from levels similar to cow blood to approximately half the abundance in colostrum. Contrary to adult cow blood EVs, calf particle numbers from the 30% SDG fraction even at pre-feeding time point were significantly lower by an order of magnitude compared to high-density vesicles. Similar distributions of EVs as encountered in cow blood EVs could potentially be acquired later during development, but it is also conceivable that higher amounts of 40–50%

SDG EVs at time point 0 h are the product of autonomous but very limited feeding before first blood samples could be drawn even though all calves were under the supervision of a milker. Protein expression of high-density particles was positive for both milk-specific markers, although only BTN1A1 followed the progressing pattern of increase indicated by particle concentrations. Divergent expression of MFGE8 with only low abundances detectable after 6 or 9–12 h suggests either the existence of multiple milk EV subpopulations in 40–50% SDG fractions, with greatly differing transfer efficiency through the intestinal epithelium and implying selective mechanisms for uptake, or a breakdown of milk EVs in epithelial cells followed by repackaging of protein cargo in a selective and directive manner.

Small RNA expression profiles and in-depth analyses of isomiR distributions in calf blood-derived samples however, painted a somewhat different picture of the bioavailability of dietary EVs and their cargo from colostrum. While miRNA expression contrasted significantly between all three blood-derived samples (Table 1), postprandial changes within sample groups could only be detected for calf EVs (Table 2). Absence of miRNA expression changes in unfractionated calf blood and blood cells was also apparent from hierarchical clustering analysis with sub-clusters within sample groups being defined by inter-animal differences rather than postprandial time points (Fig 7). Even though a time-dependent influence of colostrum feeding on expression of miRNA and their isoforms in calf blood EVs was evident (Table 2 and Fig 8), ostensible similarities with colostrum EVs might be misleading. In fact, the only consistent overlap for highly expressed miRNAs between colostrum EVs and calf blood EVs from postprandial time points that could be detected, was from a single miRNA family consisting of miR-200a, miR-200b and miR-200c. Furthermore, nearly half of all significant expression changes in post-feeding EV samples were inversely regulated compared to colostrum EVs. To help differentiate between endogenously produced miRNAs and miRNAs potentially taken up from dietary sources, isomiR patterns were analysed. Although a number of miR-200 isoforms could be detected with comparable differential expression, the most prominent isomiRs from colostrum EVs were missing in postprandial calf EVs and vice versa (Fig 9). A more likely explanation for the significant increase of miR-200a/b/c, instead of an uptake from dietary sources, might be its role in the processing of food-related signals. Next to its well-documented function in tumor development and progression [85], the miR-200 family regulates key players (e.g. FOG2, Rheb) in the insulin signaling pathway [86–88]. The enrichment analysis of KEGG pathways, indicating insulin signaling as the top result, further promotes the idea that up-regulated miRNAs in calf EVs after colostrum feeding are primarily involved in the regulation of food-related energy uptake in recipient cells.

In conclusion, our findings on the bioavailability of colostrum-derived EVs in the bloodstream of neonatal calves suggest an even more complex mode of uptake than previously assumed [10,30]. The unequal uptake of protein and miRNA cargo discourages the hypothesis of para- and transcellular transport of all intact dietary EVs through the intestinal epithelium into the blood. The reason that we readily found colostrum-specific protein markers but could not detect any meaningful uptake of miRNAs could well be attributable to their respective localization in or on the EV. Postprandial expression of BTN1A1 as well as MFGE8, both membrane-associated proteins, is in line with studies on orally administered labeled exosomes that utilised a lipophilic membrane dye [33]. miRNAs, on the other hand, are thought to be incorporated into the lumen of the vesicle and, like us, a number of studies have failed to detect an uptake of dietary miRNAs so far [13,31,32]. One possible conclusion to this decoupling of membrane-associated cargo from luminal miRNAs, might be an uptake of dietary EVs into intestinal epithelial cells, followed by disassembly of the EV prior to repackaging of EV protein cargo and delivery into the blood. Evidence for this was recently

given by a small study deducted by Manca et al., who showed distinct localization of EVs (mainly liver) and their miRNA cargo (mainly brain and kidney) [89]. Another likely reasoning could be an unequal uptake of different EV subpopulations within colostrum. EVs in milk and colostrum are mainly derived by two independent secretory pathways. Firstly by the classic endosomal multivesicular body pathways common to all exosome secretion and secondly by a Golgi-endoplasmic reticulum-derived pathway shared with milk fat globule secretion [90,91]. Due to the high overlap of milk exosomal proteomes with other exosomes independent from their tissue of origin [90], we chose two proteins (BTN1A1, MFGE8) as indicators for milk specificity that while present in exosmal proteomes are highly enriched in milk fat globule membranes. It stands to reason that the dissimilar uptake of EV cargo could also stem from a disparate distribution in exosomal EVs (miRNA) and EVs derived from the Golgi-endoplasmic reticulum pathway (BTN1A1, MFGE8). Based on our experiments we cannot rule out the possibility that miRNAs from colostrum EVs were either not taken up at all, remained in the intestinal epithelium, or were directly transported to recipient tissues without extended circulation in the blood. Furthermore, we cannot exclude the possibility that our sampling points (1h, 3h, 6h and 9-12h), might have missed the point of maximum colostral EV uptake in general or exosomal uptake in particular. Further in-depth studies on kinetics of colostral EVs uptake will be of great help in defining optimal sampling time points. To discern final destinations of miRNAs, further investigations including sampling from intestinal tissues as well as likely recipient organs such as liver or kidneys are needed.

Supporting information

S1 Fig. Morphology of colostral and blood EVs by transmission electron microscopy. Images are representative for three separate biological replicates per sample group. No differences were observed for postprandial time points in calf blood EV.
(TIFF)

S1 Table. Particle size and concentrations of EV suspensions as determined by NTA analysis. Measurements are summarized as mean values and standard deviations within a sampling group and size distributions are further characterized by giving the most frequent particle size.
(DOCX)

S2 Table. Distribution of raw read counts on small ncRNA species and inappropriate length classes. Short: smaller than 16 nt, No Adaptor: 50 nt long with no detectable adaptor sequence at 5'-end. Read numbers are given as mean values for each sample group.
(DOCX)

S3 Table. log2 fold-changes of significantly regulated canonical miRNAs in colostral and postprandial calf blood EV compared to pre-feeding calf blood EVs. Green background indicates up-regulation compared to calf EV 0h samples while red background highlights down-regulation. Expression changes without significancy are denoted by n.s.
(DOCX)

S4 Table. Raw read counts of significantly regulated canonical miRNAs in colostral and postprandial calf blood EV compared to pre-feeding calf blood EVs. Dam, calf pairs are denoted by (A)-(C); sample types are abbreviated as follows B = unfractionated blood, BC = blood cells, EV = extracellular vesicles, M = unfractionated colostrum, MC = colostrum cells; time points are denoted as 0h-9-12h.
(XLSX)

S5 Table. Gene set enrichment of the top 20 KEGG pathways based on significantly up-regulated canonical miRNA in postprandial time points. Analysis was performed on targets of human homologous miRNAs obtained from miRTarBase supported by strong experimental evidence (Reporter assay or Western blot).
(DOCX)

S1 Raw images.
(PDF)

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