**TUM School of Life Sciences**

# The role of microRNAs and extracellular vesicles in the detection of autologous blood doping

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**„In der Wissenschaft gleichen wir alle nur den Kindern, die am Rande des Wissens hie und da einen Kiesel aufheben, während sich der weite Ozean des Unbekannten vor unseren Augen erstreckt.“**

~ Isaac Newton ~

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# Summary

Blood transfusions, especially transfusions of erythrocyte concentrates, are applied in hospitals around the globe to treat anemia or blood loss during surgeries by increasing total hemoglobin mass and oxygen capacity. As this procedure also enhances the performance of exercising humans, it is considered as doping and prohibited by the World Anti-Doping Agency to assure equality and fairness in competitive sports. Though the transfusion of homologous erythrocytes can already be uncovered using accredited techniques, there is still no reliable method to detect autologous transfusions. The current gold standard procedure of longitudinally monitoring hematological markers is promising; however, it has been demonstrated that many cases of blood doping remain undetected due to the lack of unequivocal verification procedures, or unpunished due to conservative athlete sanctioning.

Recent findings substantiate the potential of microRNAs, which are already under intense investigation as diagnostic and prognostic disease marker, in doping detection. Interestingly, the total microRNA profile of erythrocyte concentrates changes significantly during storage, but whether this altered signature can be observed after transfusion *in vivo* has not yet been investigated. Therefore, we conducted a randomized controlled human study including 30 healthy, recreationally active men and performed supervised blood doping with two different volumes of autologous erythrocyte concentrates. Multiple blood samples collected before and after blood donation as well as after erythrocyte transfusion were subjected to hematological profiling and microRNA sequencing. Comprehensive multivariate data analyses for single microRNAs and pattern recognition were performed to reveal discriminating biomarkers for the doped and non-doped conditions. Finally, the classification performance of resulting microRNA signatures was compared to the data from hematological profiling.

In addition to inducing specific changes in total microRNA profiles, erythrocyte storage also stimulates the release of extracellular vesicles, which most likely mirror the altered microRNA cargo of their parent cells and are also re-transfused. Remarkably, extracellular vesicles, their cargo, and biodistribution have been examined in therapeutics and biomarker studies in which a trans-renal release of circulating vesicles was ascertained. Since urine samples are routinely taken in doping controls, urinary extracellular vesicles and their microRNA cargo might represent another promising analyte in doping detection. However, a lack of consensus on appropriate separation methods for urinary extracellular vesicles required a proof of principle. Thus, we evaluated five different strategies of urinary extracellular vesicle separation for their feasibility in and applicability for downstream microRNA sequencing.

In essence, we could successfully establish a whole blood microRNA fingerprint (miR-144-3p and miR-320d) that has potential to identify subjects who underwent autologous blood doping with erythrocyte concentrates. Even though the obtained classification performance only reached a sensitivity of up to 11 % and was thus inferior to the sensitivity of hematological profiling (44 %), the window of detection could be slightly extended based on the microRNA signature. Interestingly, the previously ascertained storage-induced microRNA signature was not confirmed after re-transfusion *in vivo*. In the feasibility study on urinary extracellular vesicle-derived microRNAs, all five approaches allowed downstream microRNA sequencing. However, different microRNA patterns and levels of purity in the vesicle preparations were observed. This emphasizes the need for comprehensive characterization and proper reporting of biophysical and transcriptional results to facilitate reproducibility and comparability. Once an appropriate method is selected based on our findings, longitudinal monitoring of both whole blood and urinary extracellular vesicle microRNA profiles might be implemented in routine doping controls in the future to verify their true applicability in the detection of autologous blood doping. Such an approach could prove even useful in the presence of other drugs and masking agents that could not have been controlled for in this present study.

# Zusammenfassung

Bluttransfusionen, und dabei die Transfusion von Erythrozytenkonzentraten im Besonderen, werden in der klinischen Routine hauptsächlich zur Behandlung von Anämien und größerem Blutverlust während Operationen eingesetzt. Durch den damit einhergehenden sofortigen Anstieg von Hämoglobin wird gleichermaßen die Sauerstoffkapazität erhöht, welche außerdem in Zusammenhang mit erhöhter Ausdauer und Leistungsfähigkeit bei Sportlern steht. Aus diesem Grund werden Bluttransfusionen jeglicher Blutbestandteile von der weltweiten Anti-Doping Agentur als Doping eingestuft und sind somit verboten, um die Gleichberechtigung und Fairness während sportlicher Wettkämpfe sicherzustellen. Zwar kann die Transfusion von allogenen Erythrozytenkonzentraten mittlerweile zuverlässig durch ein anerkanntes Verfahren bestätigt werden, doch für den Nachweis von autologen Transfusionen fehlt derzeit noch ein valides Konzept. Der aktuelle Goldstandard, welcher auf der individuellen Langzeitmessung von hämatologischen Markern basiert, ist vielversprechend. Dennoch bleiben aufgrund fehlender eindeutiger Nachweisverfahren viele Dopingfälle unerkannt oder aufgrund konservativer Sanktionierung der Sportler unbestraft.

Neuere Erkenntnisse belegen das Potenzial von microRNAs, welche bereits als diagnostische und prognostische Biomarker bekannt sind, zum Nachweis von Doping. Interessanterweise verändert sich das microRNA-Profil von Erythrozytenkonzentraten während deren Lagerung signifikant, allerdings wurde bisher noch nicht untersucht, ob dieses Lagerungsmuster nach einer Transfusion auch *in vivo* beobachtet werden kann. Daher haben wir eine randomisierte, kontrollierte Humanstudie mit 30 gesunden, sportlichen Männern durchgeführt, in welcher ein überwachtes Blutdoping mit autologen Erythrozytenkonzentraten in zwei unterschiedlichen Volumina durchgeführt wurde. Mehrere Blutproben wurden vor und nach der Blutspende sowie nach der Erythrozyten-Transfusion entnommen, bevor sie der Analyse verschiedener hämatologischer Parameter und einer microRNA-Sequenzierung unterzogen wurden. Umfassende multivariate Datenanalysen für einzelne microRNAs sowie zur Mustererkennung wurden durchgeführt, um diskriminierende Biomarker für die Unterscheidung zwischen gedopten und nicht-gedopten Bedingungen aufzuzeigen. Schließlich wurden die erzielten prädiktiven Werte der microRNA Signatur mit den Ergebnissen der hämatologischen Analyse verglichen.

Zusätzlich zu den lagerungsbedingten Veränderungen im microRNA Profil fördert die Lagerung von Erythrozytenkonzentraten auch die Ausschüttung extrazellulärer Vesikel, welche vermutlich das veränderte microRNA-Muster ihrer Ursprungszellen widerspiegeln und ebenfalls transfundiert werden. Bei Untersuchungen von extrazellulären Vesikeln, ihrer Bestandteile und Verteilung im Körper zur Anwendung als Biomarker bzw. Therapeutika konnte zudem

festgestellt werden, dass zirkulierende Vesikel die Niere passieren können. Da Urinproben routinemäßig in Dopingkontrollen gesammelt werden, bilden extrazelluläre Vesikel und die darin enthaltene microRNA eine weitere aussichtsreiche Ebene bei der Detektion von Doping. Bisher konnte allerdings keine allgemein anerkannte Methode für die Isolation von extrazellulären Vesikeln aus Urin etabliert werden, weswegen wir zuerst eine Machbarkeitsstudie durchgeführt haben. In dieser wurden fünf verschiedene Strategien zur Isolation von extrazellulären Vesikeln aus Urin in Bezug auf biophysikalische Charakteristika der jeweiligen Vesikelpräparationen sowie die nachgelagerte microRNA Sequenzierung verglichen.

Im Wesentlichen zusammengefasst konnten wir eine auf Vollblut basierte microRNA-Signatur (miR-144-3p und miR-320d) ermitteln, welche da Potential hatte, Eigenblutdoping mit Erythrozytenkonzentraten zu identifizieren. Zwar wurde im Vergleich zur hämatologischen Analyse (44 %) eine geringere Sensitivität von maximal 11 % erreicht, doch konnte das Detektionsfenster mittels der microRNA-Signatur etwas erweitert werden. Das früher ermittelte lagerungsabhängige microRNA-Profil konnte allerdings nicht nach Re-Transfusion *in vivo* bestätigt werden. Hinsichtlich der Machbarkeitsstudie zur microRNA-Analyse aus extrazellulären Vesikeln im Urin erlaubten alle fünf getesteten Methoden eine microRNA-Sequenzierung. Es wurden jedoch unterschiedliche microRNA-Muster sowie Reinheitsgrade der Vesikel festgestellt. Dies unterstreicht die Notwendigkeit einer umfassenden Charakterisierung und ordnungsgemäßen Protokollierung aller biophysikalischen und transkriptionellen Ergebnisse, um die Reproduzierbarkeit und Vergleichbarkeit zu ermöglichen. Sobald basierend auf unseren Erkenntnissen eine geeignete Methode ausgewählt ist, könnte die Langzeitüberwachung von microRNA-Profilen sowohl aus Vollblut als auch aus extrazellulären Vesikeln im Urin in Zukunft in Routine-Dopingkontrollen implementiert werden, um ihre tatsächliche Anwendbarkeit bei der Erkennung von Eigenblutdoping zu verifizieren, insbesondere wenn andere Medikamente und Maskierungsmittel, die in dieser Studie nicht kontrolliert werden konnten, eingesetzt werden.

# Abbreviations

## A

ABP ..... Athlete Biological Passport
ABT ..... autologous blood transfusion
AFM ..... atomic force microscopy
AGO2 ..... Argonaute 2
ANOVA ..... analysis of variance
ARF6 ..... ADP-ribosylation factor 6

## C

circRNA ..... circular RNA
cryo-EM ..... cryogenic electron microscopy

## D

DGCR8 ..... DiGeorge syndrom critical region 8
DLS ..... dynamic light scattering

## E

EC ..... erythrocyte concentrate
ELISA ..... enzyme-linked immunosorbent assay
EPO ..... erythropoietin
ESCRT ..... endosomal sorting complex required for transport
EV ..... extracellular vesicle

## F

FCM ..... flow cytometry

## H

HDL ..... high-density lipoprotein
HIF ..... hypoxia-inducible transcription factor

## I

IB ..... immunoblotting
IFCM ..... imaging flow cytometry
ILV ..... intraluminal vesicle
ISEV ..... International Society for Extracellular Vesicles

## L

LC-MS ..... liquid chromatography mass spectrometry
lncRNA ..... long non-coding RNA

## M

MIQE ..... minimal information for publication of real-time qPCR experiments
miRNA ..... microRNA
MISEV ..... minimal information for studies of extracellular vesicles
mRNA ..... messenger RNA
MRPS ..... microfluidic resistive pulse sensing
MS ..... mass spectrometry
MS/MS ..... tandem mass spectrometry

MVB ..... multivesicular body

## N

NFCM ..... nanoflow cytometry
NGS ..... next-generation sequencing
nt ..... nucleotides
NTA ..... nanoparticle tracking analysis

## O

OPLS-DA ..... orthogonal projections of latent structures discriminant analysis

## P

piRNA ..... piwi-interacting RNA
pre-miRNA ..... precursor microRNA
pri-miRNA ..... primary microRNA

## Q

qPCR ..... quantitative polymerase chain reaction

## R

rhEPO ..... recombinant human erythropoietin
RISC ..... RNA-induced silencing complex
rRNA ..... ribosomal RNA

## S

SEM ..... scanning electron microscopy
siRNA ..... small interfering RNA
SNARE ..... soluble N-ethylmaleimide-sensitive factor attachment protein receptors
snoRNA ..... small nucleolar RNA
snRNA ..... small nuclear RNA
SP-IRIS ..... Single-particle interferometric reflectance imaging sensing
sPLS-DA ..... sparse partial least square discriminant analysis

## T

TEM ..... transmission electron microscopy
TRBP ..... transactivating response RNA-binding protein
tRNA ..... transfer RNA
TRPS ..... tunable resistive pulse sensing
TSG101 ..... tumor susceptibility gene 101

## W

WADA ..... World Anti-Doping Agency
WB ..... Western Blot

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# 1. Introduction

## 1.1. Doping – win at any price

### 1.1.1. Historical perspective and classification

As early as antiquity, numerous ways and means were found to outperform competitors and achieve victory in all sorts of recreational and athletic endeavors [1]. Herbal mixtures and bovine testicles were probably used for this purpose back then, matching the original meaning of "to dope", which has its etymology in Afrikaans and referred to any potation with stimulating effects [2]. Later on, amphetamines and anabolic steroids were deliberately administered to soldiers to increase their alertness and prisoners of war to promote convalescence of exhausted and malnourished captives, respectively [1]. In 1963, doping was finally defined as the application of foreign substances in physically abnormal ways for the purpose of unfair performance enhancement by the Council of Europe [3]. However, this vague scope raised confusion since naturally occurring hormones, such as testosterone, were not covered by the definition, probably leading to its heavy misuse then [4]. Shockingly, the former German Democratic Republic even enforced state-sponsored doping campaigns to improve its worldwide image and shine as the third best sports nation [4, 5]. It even did not shy away from performing unethical human subject research, which resulted in an earlier mortality age for those affected. In order to centralize all ongoing efforts to protect an athlete's health and likewise ensure equality among competitors, which heretofore had happened on the national levels, the World Anti-Doping Agency (WADA) was founded in 1999 as consequence of the first worldwide anti-doping conference. One of its first actions was to adopt "The Code" which initially came into force in 2009 and was recently updated in 2021 [6]. This comprehensive and harmonizing document comprises not only a more extensive definition of doping, but also thoroughly lists rules and sanctions that athletes who violate the regulations have to consider. With this regularizing and widely accepted document, doping is classified as the occurrence of one or more violations of any of the stated anti-doping rules, some of which are whereabouts failure, evading to submit collected samples, the presence of a prohibited substance or its metabolites in an athlete's sample, (attempted) use, trafficking, possession and/or administration of any prohibited substance or method, even when complicity. Prohibited substances and methods that violate the spirit of sports, represent a health risk, have the evidenced or expected potential to enhance sports performance, or mask the use of such alone or in combination, are detailed and updated regularly in the WADA's "Prohibited List" [7]. These include anabolic agents, growth factors, beta-2 agonists, hormone modulators, narcotics, gene doping, and the manipulation of blood and blood components, amongst others [7].

### 1.1.2. Stamina boost by different forms of blood doping

Speaking of blood manipulation, the use of erythropoietin (EPO) stimulants, synthetic oxygen carriers, blood substitutes, or blood transfusions is generally referred to as blood doping and prohibited at all times, meaning in- and out-of-competition [7]. The overall aim of blood doping is to enhance oxygen delivery, e.g. by increasing the blood hemoglobin mass or the oxygen transfer capacity, as this is the limiting factor during aerobic exercise and is thus mainly but not exclusively attractive to endurance athletes [8].

The peptide hormone EPO, for instance, is produced in the kidneys under the control of hypoxia-inducible transcription factors (HIFs), and stimulates erythropoiesis in the bone marrow [9]. By the use of HIF stabilizers or recombinant human EPO (rhEPO) – actually intended to treat anemia caused by chronic kidney diseases, cancer-related chemotherapy, or autoimmune diseases – erythrocyte production can be accelerated disproportionally in healthy people [9]. With varying half-life times around 25 – 160 hours, depending on the respective improved biosimilar (“biobetter”), rhEPO has to be repeatedly injected with a lead time of about one month before a competition to benefit from the peak hemoglobin concentration, as erythropoiesis requires several weeks [10, 11]. Apart from the enhanced oxygen capacity, which was already demonstrated by a significant increase of maximal oxygen uptake and running performance by 6 – 7 % [12, 13], rhEPO misuse has two more consequences. On the one hand, endogenous EPO production is paused to maintain homeostasis, on the other hand, the risk of thromboembolic complications is elevated due to increased blood viscosity [9].

As hemoglobin is the key compound for oxygen transfer, the application of pure hemoglobin might seem straightforward to immediately increase circulating hemoglobin concentration and oxygen supply. However, free hemoglobin is very unstable and can even induce toxic effects to the kidney prior to its rapid metabolization by the liver [14]. To overcome these issues, synthetic oxygen carriers with the ability to ferry oxygen, which are either based on biochemically cross-linked and modified hemoglobin or on perfluorocarbons and feature a prolonged half-life around 12 - 24 hours, were generated [15, 16]. The actual clinical indication for the use of synthetic oxygen carriers was to substitute blood whenever the availability of blood products for transfusions is limited [14]. When administered to a healthy athlete shortly before a competition, an improved aerobic capacity is assumed [15]. However, more recent studies refute the asserted performance-enhancing effect, demonstrating just how speculative this purported effect still is [17]. Although the biological mechanism for the performance-enhancing effect of synthetic oxygen carriers remains unclear, the adverse effects of hemoglobin-based oxygen substitutes are meanwhile well-studied and include hypertension, induced by nitric oxide scavenging, and renal toxicity [14, 17].

Beyond the clinical intention of increasing hemoglobin levels in anemia patients, blood transfusions also represent a substantial area of blood doping [18]. Building on early practices in which whole blood was withdrawn and transfused, more evolved blood doping rather focuses on the concentrated administration of relevant blood fractions in order to reduce volume load of blood vessels [19]. In this process, blood compartments are separated after withdrawal and only the parts of interest are transfused. Regarding the enhancement of oxygen capacity, erythrocytes correspond to the functional unit, which can be stored in appropriate solutions at 4 °C for 49 days or cryopreserved for up to 30 years according to current regulations [20]. Prior to a competition, stored erythrocyte concentrates (ECs) are transfused, leading to an immediate increase in circulating erythrocytes and thus hemoglobin mass that lasts for a maximum of 70 days, which corresponds to the normal life span of erythrocytes of 120 days minus storage time. Not being biological inert structures, however, erythrocytes age during storage and around 25 % are already removed from the circulation within 24 hours after transfusion [21]. While EC transfusions significantly improve physical performance by around 15 % [22], there are also adverse effects that have to be recognized. Comparable to rhEPO injections, increased hematocrit levels lead to a rise in blood viscosity and thereby promote the risk of stroke, heart attack, and venous thromboembolism [23]. Especially in case of homologous or allogenic transfusions, in which blood donor and recipient are different persons, immunological reactions have to be considered as well, which could presumably be neglected in case of autologous transfusions, meaning that blood donor and recipient are the same individuum.

### 1.1.3. Detection of blood doping and its limitations

In 2009, the Athlete Biological Passport (ABP) was established by WADA to longitudinally monitor athletes and evaluate their individual physiological range by applying Bayesian inference, which predicts the probability for a marker to fall within an individual's normal range [24]. The ABP consists of three modules, hematological, steroidal, and endocrinological, with the hematological module being thought to uncover any form of blood doping. The included test parameters as well as sampling procedures, storage, transport conditions, and reporting are tightly regulated in the WADA's ABP operating and sample collection guidelines [24, 25]. The primary markers of the hematological module include hemoglobin concentration and the OFF-hr score, which is computed based on hemoglobin concentration and reticulocyte percentage [26]. Once an atypical passport finding is reported – which is triggered by one primary marker exceeding an individual's 99 % probability threshold as determined by the adaptive model of Bayesian inference – an expert panel reviews the results and considers additional available information, such as longitudinal profiles of secondary (reticulocyte percentage and Abnormal Blood Profile Score) and confounding marker (hematocrit, age, hydration levels, whereabouts, etc.) [24]. Finally, the experts' decision can be one of the following: "normal",

“suspicious”, “likely doping”, “likely medical condition”. Even in case of a “likely doping” decision, the longitudinal results are repeatedly reviewed, and the respective athlete is allowed to submit his or her explanation of the event prior to any proceedings. With this very conservative procedure, WADA implicitly aims at protecting innocent athletes from aspersions as sanctions could irreversibly and disadvantageously affect their social as well as financial situation. However, this morale might get off many actually doped athletes without punishment unless they are not justifiably unmasked by a test procedure other than the ABP.

In this context, more conclusive evidence for the misuse of prohibited substances or methods can indeed be achieved by direct or indirect screening for exogenous substances or metabolites thereof. In case of the illicit use of rhEPO, the hematological module of the ABP can only substantiate an increase in erythropoiesis, whilst the reason for its stimulation remains unknown; however, isoelectric focusing visualized by immunoblotting can clearly reveal the recombinant origin of the hormone in urine-based testing and was officially approved as a detection method [27]. Likewise, hemoglobin-based oxygen-carriers can be detected reliably by the combination of chromatography and mass spectrometry, a testing strategy which was implemented in 2004 [28]. Perfluorocarbons remain unaltered in the body and can therefore be directly detected in exhaled air or blood by gas chromatography-mass spectrometry [29, 30]. For the detection of blood or EC transfusions, it makes a difference whether the transfusion was homologous or autologous. 360 distinct erythrocyte antigens have been discovered to date, and their grouping varies between individuals, especially regarding the minor antigens [31]. Therefore, homologous transfusions can be clearly detected by flow cytometric analysis, which readily distinguishes the specific antigen set of the donor from the distinct antigen grouping of the recipient [32]. However, this approach is not possible in case of autologous blood transfusions (ABT) in which donor and recipient are one and the same person.

Promising approaches to detect ABT that are based on the measurement of the total hemoglobin mass applying the carbon monoxide rebreathing procedure were described by Gore and coworkers [33-35]. Although an increase in total hemoglobin mass after ABT, allowing for its detection with high sensitivity and specificity, was ascertained, the method to measure total hemoglobin mass itself presents the bottleneck for effective implementation of this parameter in the ABP, as it can easily be distorted by the athlete and, moreover, carbon monoxide is potentially harmful, impacting both an athlete’s performance and health [35]. As another approach, blood bag plasticizers and their metabolites were proven to migrate from the bag to the blood during storage and could thus be detected by liquid chromatography-mass spectrometry in urine after transfusion, even if plasticizer-free bags, which result in lower concentrations, are used [36, 37]. Nevertheless, anti-doping societies raised concerns about the specificity of this testing strategy, as they are of the opinion that plasticizer uptake via food,

cosmetics, and other potential sources cannot be definitely excluded [36]. Interestingly, studies focusing on the analysis of hepcidin, an important protein in iron metabolism, as well as immature reticulocytes detected by mass spectrometry in dried blood spots revealed promising results to recognize ABT [38, 39]. However, there is still no approved method to unequivocally identify ABT. Though the general utility of hematological parameters included in the ABP to observe ABT-dependent changes has already been demonstrated, genetic variations, altitude training, the use of masking agents, and missing the appropriate window of detection can heavily influence hematological profiles [22, 40-46]. This results in potentially high numbers of unknown cases that remain undetected, as underpinned by the recently concluded trial of "Operation Aderlass" (English: "Operation Bloodletting") [47]. During this raid in 2019, police seized several blood bags and appropriate equipment for blood transfusion after a whistleblower accused the physician of an Austrian cycling team of performing blood doping. In January 2021, he was convicted of 26 cases of anti-doping rule violations [47]. While ultimately successful, this anti-doping effort was not prompted by conspicuous test results and would not have been possible without insider information. Thus, it is exceedingly obvious that reliable test methods to prove ABT are urgently needed.

### 1.1.4. Transcriptomics as the new kid on the block

From the variety of "omic"-based research that is oftentimes applied to detect, monitor, and prognose diseases [48], transcriptomics also proved highly attractive in the anti-doping field. By whole blood mRNA profiling, Durussel and coworkers identified various genes affected by rhEPO microdosing and suggested transcriptomics as a new facet in the ABP [49]. Similarly, messenger RNA (mRNA) expression changes were observed in T-lymphocytes after ABT [50]. Of note, not only the coding but also the non-coding transcriptome was studied in the context of blood doping. For instance, Leuenberger *et al.* detected promising changes in the levels of some microRNAs (miRNAs) after the use of erythropoiesis-stimulating agents [51]. Remarkably, several miRNAs were already associated with changes in erythropoiesis induced by ABT [52, 53]. Based on these initial results and the facts that (1) erythrocytes are enriched with miRNAs mirroring the whole blood miRNA profile [54, 55], and (2) erythrocytes display highly significant changes in miRNA patterns upon storage [56, 57], miRNA signatures screened *in vivo* after transfusion of stored ECs could potentially be the key in ABT detection.

## 1.2. microRNAs – small but mighty epigenetic regulators

### 1.2.1. The family of non-coding RNAs

The non-coding RNA fraction makes up more than 98 % of the human genome and comprises a huge variety of different RNA subclasses that carry genetic information and contribute to the cellular infrastructure [58]. In this respect, Y RNAs interrelate with euchromatin, while circular RNAs (circRNA) were thought to act as miRNA sponge [59, 60]. Alongside ribosomal RNAs (rRNA) and transfer RNAs (tRNA), which participate in the translation of mRNA, small nuclear RNAs (snRNA) perform functions in splicing, while small nucleolar RNAs (snoRNA) are involved in rRNA modification, but can also target other RNAs, such as snRNA or mRNA [61]. Likewise, long non-coding RNAs (lncRNA), piwi-interacting RNAs (piRNA), small interfering RNAs (siRNA), and miRNAs assume a powerful function in the regulation of gene expression on both the transcriptional and post-transcriptional level widely known as the RNA silencing phenomenon [61-63]. By comprehensively interacting with each other as well as impacting epigenetic mechanisms such as histone modification and DNA methylation [61], these RNA subclasses build a complex regulatory network, the surface of which we have only scratched so far and are still trying to elucidate. Consistent with this, miRNAs – although only accounting for a minimal proportion of less than 0.02 % of total RNA by mass – were predicted to regulate about 60 % of mRNAs in a highly conserved fashion [64, 65]. In this miRNA-mediated modulation of gene expression, one particular miRNA can target up to several hundred mRNAs, while one mRNA can be targeted by multiple miRNAs, creating a complex web of interactions [66, 67]. Though these predictions were mainly based on bioinformatics, experimental proofs render more and more evidence to its biological validity as exemplified by Wu *et al.* [68]. Further, Plotnikova *et al.* revealed a negative correlation of the number of targeted mRNAs with the abundance of the corresponding miRNA [69]. Interestingly, the expression state of miRNAs can adapt and shift between relatively high and low expressed depending on the cellular environment [69]. Even though the functional significance of these alternations seems to be unclear, it highlights the vast regulatory impact and adaptability of miRNAs in an astonishingly sophisticated and still heavily investigated network.

### 1.2.2. Focusing on microRNA biogenesis

Since the first miRNA has been identified in 1993 [70], the number of detected miRNAs with putative functional activity has risen enormously, nowadays amounting to up to 48,860 mature sequences in 271 different organisms (miRBase [71], release 22.1). More than 2,600 mature sequences are annotated in the human genome; however, only one third of them could be backed up with ‘high confidence’ sequences so far. The typical mature miRNA sequence appears with a size range that centers on 22 nucleotides (nt) and originates mainly from introns

of both protein-coding genes and non-coding transcription units [72, 73]. Hence, miRNA expression could depend on the host genes' promotors but they are not necessarily linked to each other in terms of biological relevance [74]. By contrast, clustered miRNAs that are transcribed in a polycistronic way and subsequently further processed into individual miRNAs indeed oftentimes share similar biological functions [75]. Both post-transcriptional and post-translational modifications of miRNA biogenesis factors, such as the impact of RNA-binding proteins or non-coding RNAs as well as changes in phosphorylation or ubiquitylation, seem to be involved in the adjustment of miRNA biogenesis [74]; however, the underlying mechanisms of initiation of miRNA transcription remain poorly understood. Remarkably, miRNA expression might also be regulated by DNA methylation status, as an astonishing 50 % of miRNAs are located adjacent to CpG islands [76, 77].

In the canonical pathway of mammalian miRNA biogenesis (Figure 1), the miRNA gene is transcribed by RNA polymerases II and III into its primary miRNA sequence (pri-miRNA) that forms a hairpin structure constituted of 60 to 70 nt with long flanking regions [78-80]. Further processing from the pri-miRNA to the precursor miRNA (pre-miRNA) is performed by the so-called microprocessor complex comprised of Drosha, an RNase III endonuclease, and the double-stranded RNA binding domain DiGeorge syndrome critical region 8 (DGCR8), leaving a 5' phosphate and a short 2 nt 3' overhang by trimming the flanking regions [81, 82]. After the pre-miRNA is actively transported from the nucleus into the cytoplasm via the Exportin-5 receptor and Ran-GTP, a further RNase III endonuclease called Dicer cuts the remaining stem loop in concert with the human immunodeficiency virus-1 transactivating response RNA-binding protein (TRBP) and the Argonaute 2 protein (AGO2) [79, 83, 84]. The resulting miRNA duplex is made up of two complementary sequences of similar size with approx. 22 nt; one of which is handled as the guide strand, then called mature miRNA, and gets loaded into the AGO2-associated RNA-induced silencing complex (RISC) [85]. Its complementary sequence, on the other hand, is handled as the passenger strand and subsequently cleaved by AGO2 [85]. Interestingly, only mediocre impairments in miRNA maturation were detected in knockout experiments that separately depleted key enzymes and receptors of the canonical pathway, implying the existence of other albeit less marked non-canonical routes independent of Drosha or Dicer cleavage activities [86].

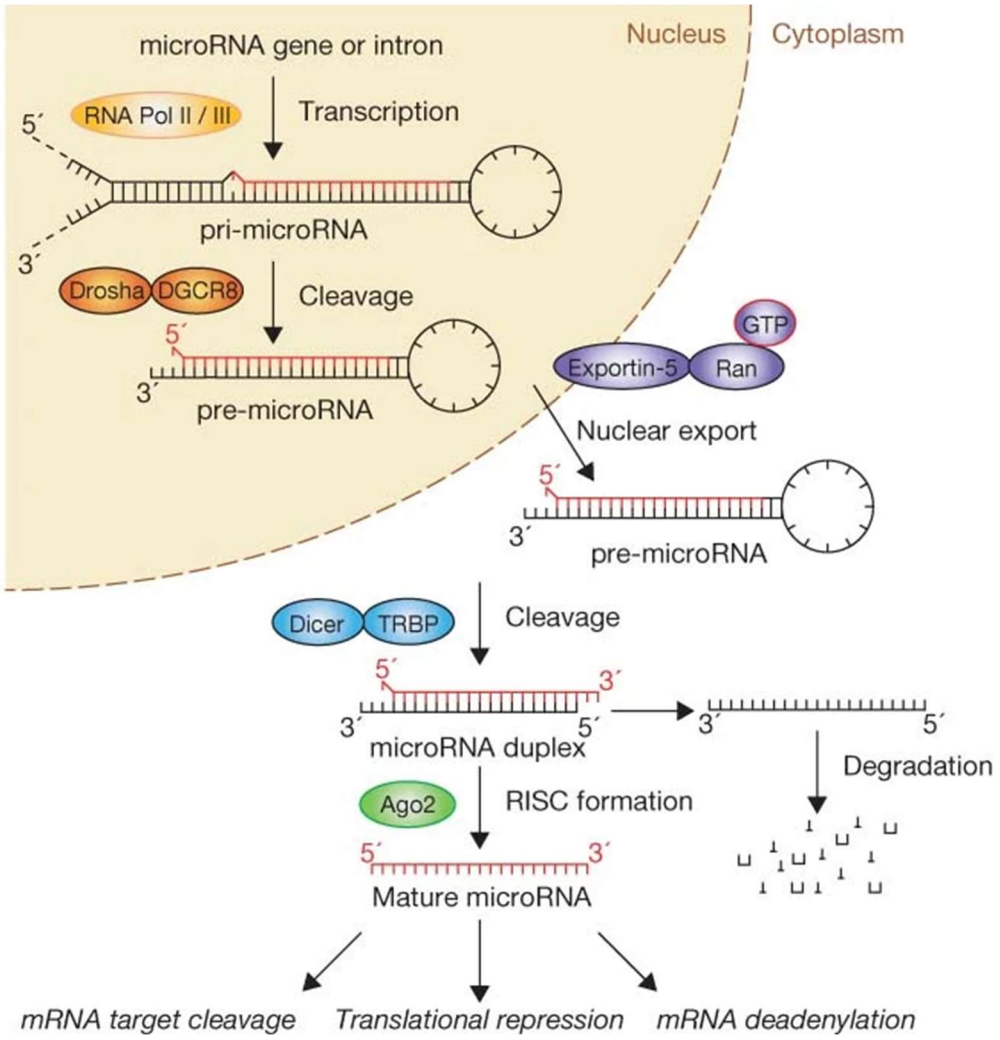


**Figure 1:** Canonical biogenesis of mammalian microRNAs. Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Cell Biology, Many roads to maturity: microRNA biogenesis pathways and their regulation, Winter et al., © 2009 Macmillan Publishers Limited [87]. RNA Pol II/III: RNA polymerase II/III, pri-microRNA: primary microRNA, pre-microRNA: precursor microRNA, DGCR8: DiGeorge syndrome critical region 8, TRBP: transactivating response RNA-binding protein, RISC: RNA-induced silencing complex, Ago2: Argonaute 2, mRNA: messenger RNA.

The miRNA duplex is unwound during AGO2-associated RISC assembly with the help of chaperones prior to energy-dependent RISC loading with the guide strand [88, 89]. The decision of which of the two miRNA arms, the miRNA strand stemming from the 5' region (-5p) or the strand stemming from the 3' region (-3p) of the precursor, is integrated into RISC and exerts a regulatory impact was previously assumed to result from thermodynamic base pair stability and asymmetry of the 5' end, leading to the preferential selection of one of the two arms [90]. However, we now know that both strands can be biologically functional, and that miRNA strand selection is an active, highly dynamic, and adaptive process that depends on a variety of features, including pathological conditions as well as tissue and species specificity [91-93]. Finally, the incorporated miRNA arm directs the RISC to its specific target site(s) to carry out regulatory activities.

### 1.2.3. Target recognition and regulatory impact of microRNAs

It is well-established that the miRNA seed region, which is the highly conserved stretch from the second to the eighth nt position from a miRNA's 5' end, is essential for target recognition and Watson-Crick pairing [94, 95]. Notably, the eighth position and a flanking adenosine opposite of position one are dispensable for seed matching, but they both contribute to higher specificity of Watson-Crick pairing [66]. There is also evidence of a second binding site at positions 13 to 16 nt of the guide miRNA that can be supplementary or complementary to a pairing at the seed region, compensating for mismatches and/or bulges in the seed region, and improves miRNA targeting, just as an AU-rich sequence context does [94, 96]. Usually, the miRNA seed sequence binds to its complementary sequence in the 3' untranslated region of an mRNA, but more than one fifth of miRNA target sites are predicted to lie in coding sequences or 5' untranslated regions [97]. However, it remains to be investigated whether and to what extent the alternative miRNA binding affects downstream biological functions.

The main and most commonly known biological action of miRNAs is downregulation of gene expression transmitted via AGO2-associated RISC [72, 98]. Depending on the complementarity of the guide miRNA and the target mRNA, RISC initiates either a translational repression or mRNA cleavage [99]. Imperfect complementarity still sufficient for miRNA:mRNA binding induces translational repression that occasionally appears to be reversible [100], and is thought to interfere with translational initiation by preventing initiation factors from recognizing the targeted mRNA's 5'- cap or impeding ribosome assembly, potentially leading to polysomal shifts [101-103]. In addition, several studies proposed that translational repression also takes place post-initiation, affecting elongation steps, for instance by the recruitment of proteolytic enzymes or premature termination of translation and ribosome drop-off [104, 105]. Despite ongoing research, the underlying mechanisms are still not fully understood yet. Perfect or almost perfect complementarity between a miRNA and its target, on the other hand, predominantly leads to enzymatic degradation of the target mRNA with AGO2 as the key element [106]. In combination with GW182, which has evolved as an indispensable and highly conserved component of the RISC, 3'-5' exonucleases for deadenylation are recruited upon base pairing [107, 108]. Deadenylation can also take place concurrently with ongoing translation, suggesting that its onset even precedes translational repression [109]. Interestingly, the miRNA remains intact after mRNA decay and may enter multiple rounds of targeting, while repressed or degraded mRNAs are reposited in P-bodies, which are specific cytosolic aggregates enriched with proteins of the RNA decay machinery [99, 110].

In addition to the well-established mechanisms of miRNA-induced gene silencing, more recent research also reports miRNAs to activate translation. Vasudevan *et al.* figured out a cell cycle-dependent oscillation of miRNA activity as ascertained by translational repression during cell

proliferation, whereas an AU-rich element-associated activation of mRNA translation was induced in quiescent cells [111]. Likewise, the expression of ribosomal proteins was upregulated during starvation by miRNA interaction with the corresponding 5' untranslated regions [112]. Although these examples might not generalize the stimulatory impact of miRNAs on gene expression, they significantly expand horizons in the huge network of RNA-induced regulation, thus challenging researchers to consider postulated (patho-)physiological pathways in all their aspects.

Remarkably, the regulatory impact of miRNAs is augmented by the presence of miRNA isoforms, also known as isomiRs, which arise from alternative processing of pri-miRNAs and result in highly heterogeneous variations at the 5' end, the 3' end, or both ends [113-115]. Given that changes at the 5' end can lead to a shift in nt positions and thus alter the seed sequence, different target sites might be addressed by different isoforms of the same miRNA, leading to alterations in the affected biological functions. By contrast, variations at the 3' end are assumed to expose the same seed sequence as cognate miRNAs, which suggests a similar target selection, but changed miRNA stability [116]. Tan and coworkers confirmed the tissue-specific functionality of isomiRs and conceded them to convey additional targeting in a tightly regulated evolutionary context [117]. The presence of miRNA editing and single nucleotide polymorphisms in miRNA genes adds another layer of biological variability [118]. However, Cloonan *et al.* observed a concurrent targeting of canonical miRNAs and their variants to a shared set of mRNAs and postulated an increase in specificity and efficacy while reducing off-target effects [119].

### 1.2.4. Relevance of circulating microRNAs

Interestingly, intracellular miRNAs are also actively or passively exported into the extracellular milieu where they can reach recipient cells. Although the exact processes of extracellular RNA stabilization and transport are still largely unknown, several RNA-binding proteins such as AGO2, high-density lipoproteins (HDL), or nucleophosmin 1 have been suggested to shuttle miRNAs in the extracellular milieu [120-122]. The uptake of HDL-bound miRNAs was demonstrated to happen via the scavenger receptor BI, which releases miRNAs into the recipient cell's cytoplasm [120]. The mechanisms behind the cellular uptake of miRNAs bound to other RNA-binding proteins remains to be elucidated but internalization might not even be required for biological effects. As Fabbri and coworkers have shown, miRNAs are able to transfer information by triggering intracellular signaling cascades via Toll-like receptors on the surface of recipient cells [123]. Vesicle-mediated export and extracellular transfer of miRNAs were also studied based on the intracellular proximity of RISC and the sites at which intraluminal vesicles are generated [124]. Some of these vesicles, a fraction of which is said to carry miRNAs, can be exported into the extracellular milieu, presumably delivering bioactive cargo to recipient

cells [125]. Shielded by these various shuttle systems, extracellular miRNAs are protected from degradation by RNases and were also shown to be extremely stable upon multiple cycles of freezing and thawing and long-term storage [126, 127]. Given their stability and their minimal invasive accessibility in liquid biopsies derived from biofluids, such as serum, urine, bile, ascites, and sputum, amongst others, circulating miRNAs were already comprehensively studied as powerful diagnostic and prognostic biomarkers in multiple pathological conditions [128-130]. Interestingly, the biospecimen-specific expression of miRNAs is even exploited in forensic analyses [131]. Moreover, the therapeutic application of miRNA mimetics or miRNA inhibitors, either after chemical modification or encapsulated in delivery vectors, such as extracellular vesicles, substantiated the crucial role of miRNAs in disease states and their appeal as drug targets [132].

## 1.3. Extracellular vesicles – multifarious communicators

### 1.3.1. Generic classification

Formerly known as the “trash bin of the cell” and a mere mechanism to eliminate unwanted material, these days extracellular vesicles (EVs) are well established as highly conserved, lipid bilayer-enclosed nanoparticles that are unable to replicate, sequestered from assumingly all cells, and take functional action in cell-to-cell communication [133]. Featuring said lipid membrane, EVs protect their cargo and are highly stable upon storage [134, 135]. Over the past decade, EVs have been investigated in many biofluids, including different blood compartments, urine, saliva, milk, cerebrospinal fluid, ascites, and semen [136-140] (Appendix II). Three main classes of vesicles are covered by the umbrella term ‘EV’: exosomes (< 150 nm), microvesicles (< 1,000 nm), and apoptotic bodies (< 5,000 nm) (Figure 2). Other names based on the vesicles’ origin, such as prostasomes, oncosomes, or ectosomes can also be found in literature, which leads to confusion and low comparability between studies [141]. Though the different types of vesicles vary widely in size, density, and composition, they might also broadly overlap, leaving their biogenesis as their only reliable discriminator. Since the vesicles’ intracellular origin can only be ascertained by life tracking the vesicles just in the moment of their release, which is still challenging, the International Society for Extracellular Vesicles (ISEV) endorses the generic use of the term “EV” [141].

Due to the increasing interest in EVs and the lack of appropriate standardization in this rather young field of research, a tremendous number of experimental studies with just as many approaches for EV isolation have been published [142]. Moreover, the protocols utilized in many of these studies not only tended to lack detailed description of the applied methodologies, but

also to omit proper characterization of EV preparations resulting in minimal comparability and reproducibility of study results. Consequently, the EV community agreed on a set of essential steps to improve rigor and consistency and published this consensus in the minimal information for studies of EVs (MISEV) guidelines in 2014, which were extensively updated in 2018 to implement newly accrued lessons from this fast-paced field [141, 143].

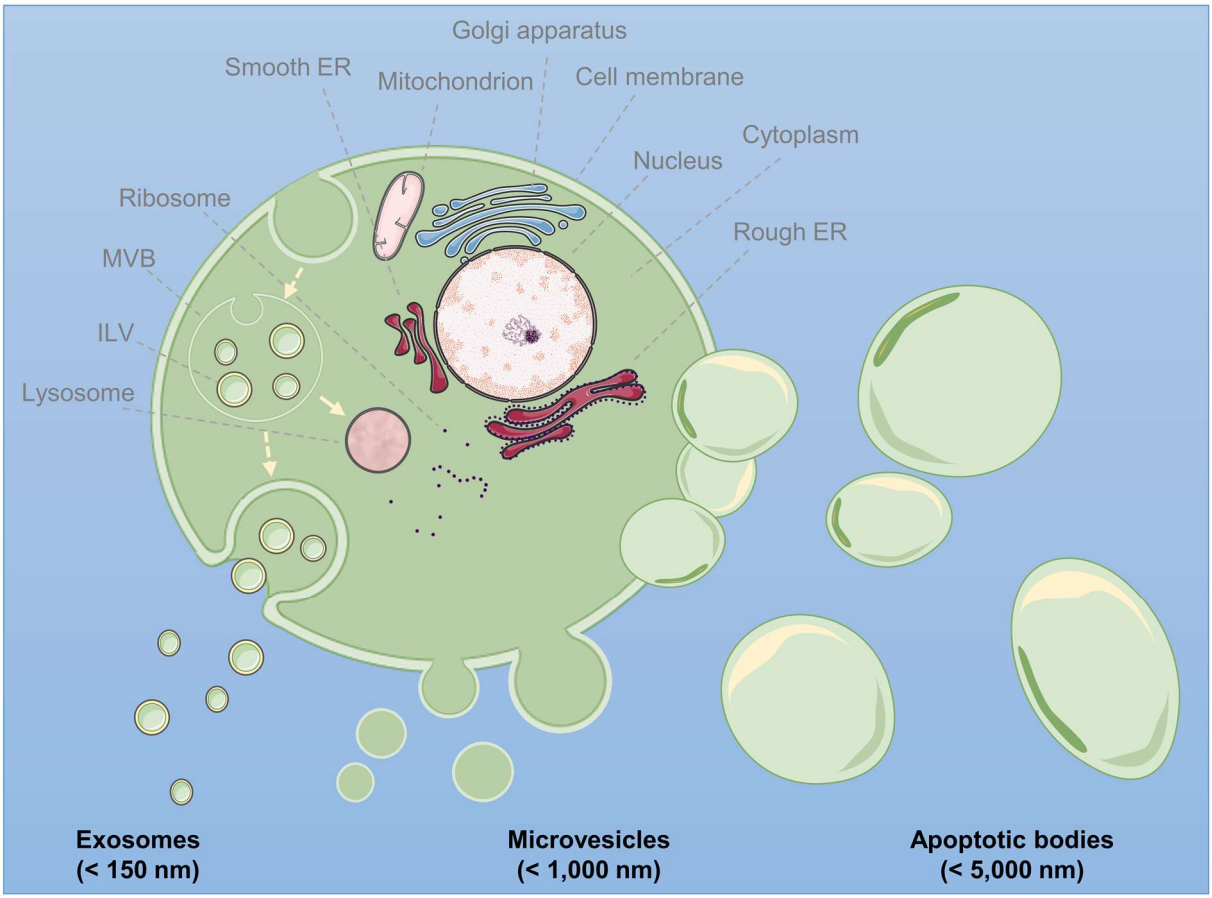


**Figure 2:** Outline of the different main subclasses of extracellular vesicles. Reprinted by permission from Elsevier: Advanced Drug Delivery Reviews, Separation, characterization, and standardization of extracellular vesicles for drug delivery applications, Buschmann et al. © 2021 Elsevier B.V. [144] (Appendix III). ER: endoplasmic reticulum, MVB: multivesicular body, ILV: intraluminal vesicle.

### 1.3.2. Biogenesis of extracellular vesicles

Exosomes were first discovered by Johnstone and coworkers in the 1980s during their studies of reticulocyte maturation [145]. They observed that cultured reticulocytes formed vesicles in the endosomal network and released them into the extracellular milieu via fusion of multivesicular bodies (MVBs) with the plasma membrane. The endosomal sorting complex required for transport (ESCRT) with its different subunits was allotted a pivotal role in MVB generation [146]. While ESCRT-0 is supposed to recruit and sequester ubiquitinated proteins thought to be recycled or degraded and other content that clustered into trans-membranous

microdomains, ESCRT-I and ESCRT-II initiate local budding of endosomal membranes. Finally, ESCRT-III participates in de-ubiquitination of sorted proteins and induces pinching off intraluminal vesicles (ILVs) into the endosome, which is now called an MVB. These ILVs, which contain cytosolic material, are named exosomes once they are released into the extracellular space by fusion of the MVB membrane with the cell's plasma membrane [145]. Whenever the MVBs fate is not to reach the membrane for exosome release, they usually fuse with lysosomes for cargo degradation [147]. Of note, there is also evidence for an ESCRT-I and -II -independent pathway for exosome release, which can be prompted by ceramides but is especially requiring CD63 as trigger [148, 149]. Exactly how and under which circumstances one or the other pathway will be activated is still mostly unknown, but it seems to be largely dependent on the presence of various components involved in the endosomal network. In this context, exosome secretion tends to be promoted via an orchestrated interplay of syndecan, syntenin, and ALIX, the latter of which is also known as Programmed cell death 6-interacting protein [150]. Moreover, microdomains of tetraspanins – CD9, CD63, and CD81, in particular – and members of the Rab small GTPase family were functionally associated with MVB and ILV formation, while soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) mediate final exosome secretion [151-155].

By contrast, microvesicles originate from direct outward budding of the plasma membrane [156]. Though this process is less studied than the biogenesis of exosomes, a complex interplay of phospholipid domains and cytoskeletal features has been ascertained so far. In this context, neutral and acid sphingomyelinases, which promote the conversion of mostly clustered membranous sphingomyelin to ceramide, were demonstrated to be central but not essential to vesicle budding [157, 158]. In addition, calcium-induced stimulation of phospholipid changes in the plasma membrane by translocases, which shift phosphatidylserine from the inner to the outer leaflet, induces energy-dependent actomyosin contraction and actuates vesicle shedding [159, 160]. Interestingly, the recruitment of ESCRT proteins, tumor susceptibility gene 101 (TSG101), as well as the small GTP-binding protein ADP-ribosylation factor 6 (ARF6) to the plasma membrane were also shown to be involved in membrane scission [159, 161].

In contrast to exosomes and microvesicles, both of which are shed from living cells, apoptotic bodies are generated during the programmed cell death [162, 163]. Commenced with chromatin condensation, plasma membrane blebbing and pinching off of cellular content in membranous vesicles is stimulated prior to and during phagocytic removal of dying cell material. Although some mechanisms of apoptotic body biogenesis, such as the actin and microtubule-independent formation of beaded apoptopodia, are known, more precise knowledge has to be generated [164, 165].

### 1.3.3. Extracellular vesicle's cargo

Remarkably, EVs are packed with highly diverse cargo, which usually mirrors the conditions of their originating cells; however, it may also happen that the EVs' content differs from that of their parental cell [166, 167]. This implies highly selective cargo sorting, which has been associated with different steps during biogenesis but is not yet fully understood [168, 169]. Nevertheless, the implications of specific sorting raised great interest in EVs in a clinical perspective and initiated deep profiling studies of EVs, as differentially loaded EVs might cause different biological responses at their target sites [170, 171].

Proteomic profiling of different EV subclasses separated based on their sedimentation speed and buoyant density established CD9, CD63, and CD81 as the most prominent marker proteins for small EVs, while CD9 and CD63 were also present in EV fractions that sediment at lower speed [172]. Indeed, most of the proteins, such as flotillin-1 and major histocompatibility complex class I and class II, were commonly detected in all investigated fractions. However, endosomal proteins, such as syntenin and TSG101, and proteins associated with the plasma membrane were enriched, or even exclusively detected in smaller EVs [172]. By contrast, mitochondrial and proteasomal proteins were mainly assigned to the larger EV subtypes.

Even though less studied than the EV protein content, nucleic acids also represent a substantial payload. In their groundbreaking 2007 study, Valadi *et al.* revealed that EVs are loaded with mRNA and miRNA and, moreover, that these are horizontally transferred to other cells [125]. Interestingly, especially small EVs were observed to be enriched in miRNAs compared to their parental cells [173]. Other classes of non-coding RNA, such as lncRNA, as well as DNA were detected to be shuttled by EVs [166, 174]. While large vesicles of prostate cancer origin were demonstrated to be enriched in DNA reflecting the genetic makeup of their parental cells, this was not true for smaller vesicles [175].

Due their lipid-bilayered nature, a substantial amount of lipids represents another class of biomolecules integral to EVs, although their lipid composition highly differs from that of the cell membrane [176]. Due to their endosomal origin this is especially true for exosomes [148, 176]. Deep profiling also confirmed differences in lipid composition in EV subclasses assigning glycolipids and free fatty acids mainly to exosomes, while ceramides and sphingomyelins appear enriched in microvesicles [177].

### 1.3.4. Extracellular vesicles taking action

After the "garbage bin" hypothesis has been increasingly discounted, intercellular communication and cargo transfer are now deemed the major functions of EVs [133]. Targeting and biodistribution of EVs to their recipient cells are highly dependent on their originating cells and their cargo composition [178]. Once finally reaching their addressed targets, three modes of

transmitting information, which mostly take place concurrently, have been described for EVs. First, vesicles were observed to directly fuse with the plasma membrane of acceptor cells [179]. Second, EVs can transmit signals by interacting with surface structures of the recipient cell to active intracellular signaling cascades [180]. Third, they can be internalized by the recipient cell via macropinocytosis, phagocytosis, or endocytosis, whereby the latter can be mediated by clathrins, calveolin, or lipid-rafts [181-183]. The route of EV internalization results in EVs being trapped in endosomes and needing to escape from lysosomal degradation to come to release their cargo and exert their intracellular functions [184].

The supposed physiological as well as pathological functions of EVs are myriad, although it is hardly feasible to attribute particular functions to specific EV subtypes because one cell can release multiple types of EVs at once, resulting in a highly heterogenous set of vesicles that is present in a particular biofluid at any given time. Nevertheless, there are exceptions in which specific types of EVs could be linked to distinct functions, such as EVs with tissue factor on their surface exhibiting coagulant properties [185]. Moreover, EVs demonstrated immunomodulatory competencies with leukocyte-derived EVs promoting anti-inflammatory signaling, while macrophage-derived EVs activate pro-inflammatory features upon microbial infection [186, 187]. In addition, cancer cells also release EVs which not only promote angiogenesis and tumor-growth, but also make the vesicles – and their shuttled miRNAs, in particular – especially attractive in clinical research as prognostic or diagnostic biomarkers [188, 189]. The fact that patients suffering from type 2 diabetes mellitus or cardiovascular diseases exhibit higher concentrations and distinct compositional changes of EVs compared to healthy people underpins the potential of EVs as powerful biomarkers [190, 191].

Besides their use in biomarker research, EVs are also explored in therapeutic contexts, either as therapeutic agents themselves or as drug delivery system [144] (Appendix III). The EVs' inherent abilities of crossing barriers, protecting their cargo from early degradation, tissue tropism as well as their low immunogenicity make them favorable drug carriers. The endogenous origin and the absence of nuclear or mitochondrial DNA confer a high biocompatibility to erythrocytic EVs, in particular [192]. Moreover, the fact that large quantities of erythrocytic EVs are produced during blood bag storage might make them superior to EVs originating from other sources, such as mesenchymal stem cells, which have to be laboriously produced in bioreactors [193-195]. Interestingly, the increased vesicularization of erythrocytes was already discovered in the 80s as marker of storage lesions [196]. This effect is based on an intracellular drop in ATP concentration, which in turn leads to destabilized cell membranes and increased permeability followed by a rise in intracellular calcium, all of which promote vesicle release [197, 198]. Thus, the transfusion of ECs, and the vesicles contained therein, induces an immediate increase of circulatory EVs with a peak two hours after transfusion [199]. The majority of

transfused EVs is rapidly removed from the circulation and degraded by macrophages in the spleen [200]. Although a trans-renal release for circulatory EVs was also demonstrated [201], the exact details of their clearance are not yet fully understood. When loaded with siRNA, antisense oligonucleotides, guide RNA, or even mRNA using electroporation, erythrocyte-derived EVs were already efficiently and safely applied to reduce the progression of breast cancer and acute myelogenous leukemia in mice [202].

## 1.4. Aim of this work

The overall aim of the present thesis was to devise novel strategies to detect autologous blood doping with ECs, for which conclusive tools remain to be established. As storage of ECs was demonstrated to significantly alter whole blood miRNA profiles and elevate the release of EVs with altered miRNA cargo [56, 193, 203], whole blood and EV-associated miRNAs were explored *in vivo* for their putative biomarker potential in the uncovering of ABT.

Thus, we performed supervised blood donations and autologous EC re-transfusions and conducted small RNA sequencing of whole blood samples to both verify the storage-dependent miRNA signature *in vivo* and to identify further potentially dysregulated miRNAs that might not be brought into the context of blood doping or hematological regulation before. The thereby obtained predictive classification performances were compared to those resulting from longitudinal hematological profiling implemented in the ABP, which is currently the only approved method to detect autologous blood doping but shows only limited sensitivity.

Furthermore, we hypothesized that the storage-dependent increase in EV concentration results in an increase in circulatory EVs, which would in turn promote faster EV turnover and urinary excretion after re-transfusion. Moreover, these urinary EVs are thought to demonstrate storage-dependent and transfusion-dependent differences in miRNA cargo. To prove this line of hypotheses, we first had to establish an appropriate urinary EV separation method enabling downstream miRNA profiling. Building on the results of our proof-of-principle study, we furthermore prepared a review of available EV separation and characterization methods and their advantages and disadvantages.

# 2. Methods

## 2.1. Study protocols

### 2.1.1. Autologous blood doping [204] (Appendix I)

Thirty healthy males with recreational activity were included in this study to assess the relevance of miRNAs in the detection of autologous blood doping (Figure 3). The study was approved by the local ethics committee and conducted in accordance with the 'Declaration of Helsinki' [205]. Written informed consent was obtained from all participants before they were randomly assigned to one of the three study groups. Participants in group 1 and 2 performed a standard whole blood donation one week after baseline measurements (-6w), group 2 donated a second time two weeks later (-4w), while the control group did not donate any blood. Subsequently, donated whole blood was instantly processed into ECs and stored at 4 °C prior to autologous re-transfusion after four (-4w) or six weeks of storage (-6w), respectively. Each volunteer was sampled once weekly for three consecutive weeks to record individual baseline levels (-9w, -8w, -7w). Additional, further samples were drawn shortly before re-transfusion (t=0) and at several hours and days during the week after re-transfusion to monitor doping-dependent changes (+3h, +6h, +1d, +2d, +3d, +4d, +7d). Downstream examination of blood samples encompassed the comprehensive assessment of hematological markers as included in the ABP as well as thorough analyses of miRNA profiles obtained by small RNA sequencing.

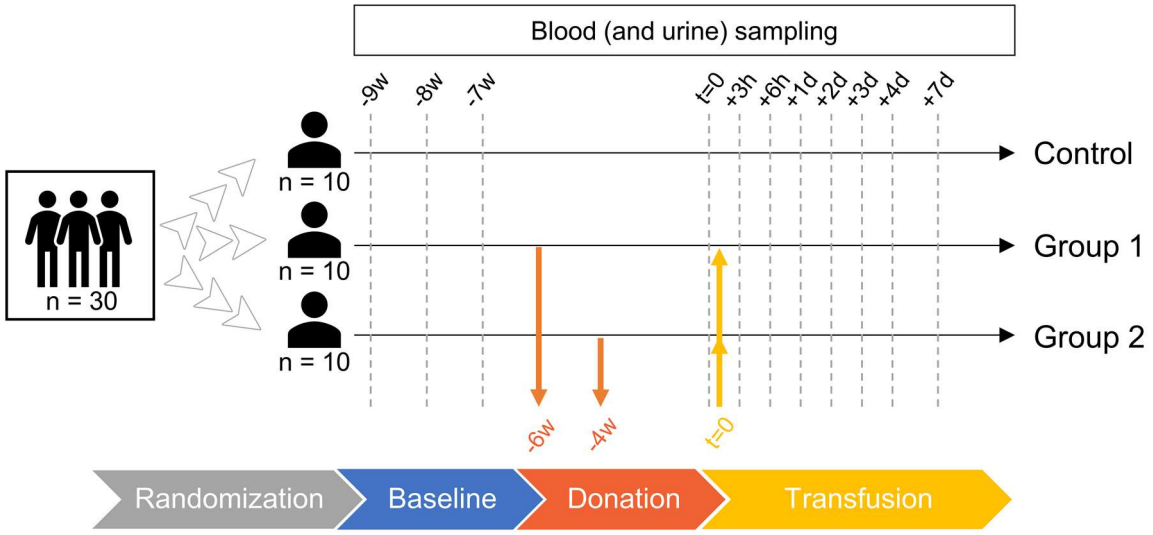


**Figure 3:** Underlying study design to monitor changes in hematological parameters and the whole blood microRNA profile after blood withdrawal and re-transfusion of autologous erythrocyte concentrates. Urine samples were collected at the same time points as blood and safely stored until future processing.

### 2.1.2. Urinary extracellular vesicle characterization [140] (Appendix II)

Prior to using urinary EVs in biomarker research, an appropriate method to separate them from other biofluid components has to be carefully selected from the huge variety of available techniques, all of which bare advantages and disadvantages [144] (Appendix III). For this reason, urine samples from six healthy participants, who also volunteered for the autologous blood doping study, were collected at baseline, processed, and characterized with reference to the current MISEV 2018 guidelines to evaluate their potential for miRNA investigations [141]. Equal volumes of cell-free urine were applied to five different EV separation methods, all of which rely on different principles (Figure 4). The isolation process was conducted in duplicates, one of which was used for further characterization of urinary EVs by western blotting (WB), transmission electron microscopy (TEM), and both scattering and fluorescence nanoparticle tracking analysis (NTA). Total RNA was isolated from the other portion of EV samples and directed at miRNA screening via small RNA sequencing.

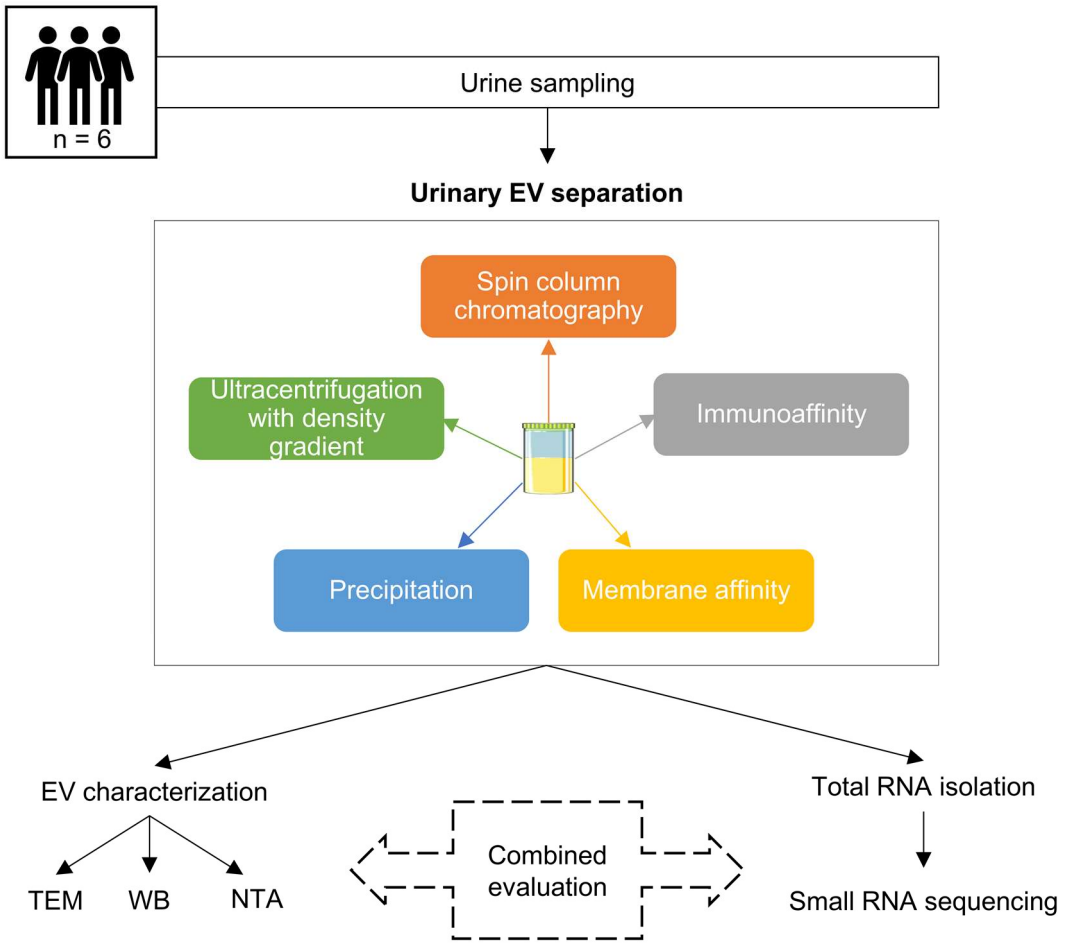


**Figure 4:** Study protocol for the evaluation of five different urinary extracellular vesicle (EV) separation methods with downstream characterization by transmission electron microscopy (TEM), Western blot (WB), and nanoparticle tracking analysis (NTA) (scattering and fluorescence) for a combined evaluation with microRNA profiles obtained by small RNA sequencing.

## 2.2. Experimental workflow and data analyses

### 2.2.1. Study subjects

In- and exclusion criteria can be accessed in the supplemental information of Mussack *et al.* [204] (Appendix I) with detailed information on the participants' baseline characteristics in Mussack *et al.* [204] (Appendix I) and Mussack *et al.* [140] (Appendix II). In summary, the included volunteers were athletic and healthy males without any medication and vaccination four weeks prior to and during their participation. On average, they were included with 27 (± 4) years, 182 (± 5) cm height, 80 (± 7) kg weight, and recreational activity for 5 (± 2) hours per week.

### 2.2.2. Preparation and transfusion of erythrocyte concentrates

Whole blood bags were processed and EC transfusion performed as described in Mussack *et al.* [204] (Appendix I). In brief, around 500 ml blood were withdrawn from an antecubital vein, e.g. the median cubital vein, collected in the sterile Composelect T3984-23 system (Fresenius; Germany), and mixed instantly on a horizontal shaker. After four hours of storage at room temperature, whole blood was separated into its components by lymphocyte filtration and centrifugation. The resulting EC was then stored in PAGGS-M buffer, comprising a volume of approx. 250 ml, at 4 °C for either four or six weeks. Each study subject signed every bag of the Composelect system and verified his signature prior to re-transfusion, thereby assuring the re-transfusion of autologous ECs. Moreover, bedside tests were conducted to additionally approve blood group compatibility prior to autologous EC transfusion via peripheral venous access. All steps were performed in compliance with official and in-house regulatory guidelines.

### 2.2.3. Sample collection and processing

Blood samples from an antecubital vein were collected in PAXgene Blood RNA tubes (PreAnalytiX; Switzerland) for downstream miRNA analyses and stored at -20 °C according to the manufacturer's instructions as described by Mussack *et al.* [204] (Appendix I). Additional blood was sampled into EDTA, citrate and serum monovettes (Sarstedt; Germany) and mixed by inverting to facilitate the subsequent measurements of various hematological markers. For the comparison of different urinary EV separation methods, urine was saved in sterile containers and processed into cell-free urine via differential centrifugation prior to storage at -80 °C, as stated in Mussack *et al.* [140] (Appendix II). A total urine aliquot was retained for immediate urinary creatinine measurement and to assess general urinary health.

### 2.2.4. Longitudinal hematological profiling

To assess the classification performance of whole blood miRNA profiles in the detection of ABT and their superiority compared to the current gold standard in blood doping detection, clinical data was first evaluated according to the hematological module of the ABP [24], as already published by Mussack *et al.* [204] (Appendix I). For this reason, EDTA, citrate, and serum samples obtained during blood sampling of the present human study were immediately analyzed for the following parameters in local accredited laboratories: hemoglobin, reticulocyte percentage, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, platelets, erythrocyte count, white blood cell count, red cell distribution width, EPO, iron, ferritin, transferrin, transferrin receptor, and transferrin saturation. In addition, OFF-hr Score and Abnormal Blood Profile Score were calculated as described by Gore *et al.* and Sottas *et al.* [26, 206]. Intra-individual and inter-individual variabilities were assessed, and individual critical ranges to identify unphysiologically high or low concentrations were ascertained by longitudinal profiling of each single parameter based on the adaptive model of Bayesian inference [24, 207].

### 2.2.5. Urinary extracellular vesicle isolation and characterization

Mussack *et al.* [140] (Appendix II) particularized the comparative assessment of five different EV separation methods for their suitability in miRNA-based biomarker research as well as the downstream characterization of isolated urinary EVs by TEM, WB, and scattering and fluorescence NTA. In a nutshell, applied separation techniques were based on spin column chromatography (Urine Exosome Purification and RNA Isolation Midi Kit; Norgen Biotek; Norway), immunoaffinity (Exosome Isolation Kit Pan, human; Miltenyi Biotec; Germany), membrane affinity (exoRNeasy Serum/Plasma Midi Kit; Qiagen; Germany), precipitation (miRCURY Exosome Isolation Kit – Cells, urine and CSF; Exiqon; Denmark), and ultracentrifugation with density gradient (Optima LE-80 K; Beckman Coulter; USA; and OptiPrep; Merck; Germany). Amicon centrifugal filter units (100K, Ultracel membrane of regenerated cellulose; Merck; Germany) were utilized to concentrate cell-free urine to recommend input volumes, if required. Then, obtained EV isolates were vacuum-concentrated to equal volumes prior to any downstream characterization. The morphology of the resulting EV preparations was visualized with TEM via negative staining using aqueous uranyl acetate (Zeiss EM 900 instrument; Zeiss; Germany). Further, the absence of impurities as well as the presence of EV-specific protein markers were ascertained by WB with appropriate controls (XCell SureLock Mini-Cell Electrophoresis System; Thermo Fisher Scientific; USA). NTA examinations of intact EV preparations were performed in the scattering and the fluorescence mode of the ZetaView PMX110 instrument (Particle Metrix; Germany) using the CellMask Orange Plasma Membrane Stain (Invitrogen; USA) to allow a differentiation between biological and non-biological particles. Particle

concentrations were normalized to urinary creatinine levels, which were determined by an external lab based on the Jaffé method [208], as they were shown to strongly correlate with urinary EV numbers and can therefore be used to account for urine dilution variations [209]. Then, significant differences were assessed via one-way analysis of variance (ANOVA) with Tukey post-hoc test adjusting for multiple comparisons. Adjusted P values < 0.05 were rated as significant. At the request of the ISEV as mentioned in the MISEV guidelines [141], methodological details were disclosed on the EV-TRACK platform (EV-TRACK ID: EV190007) [142].

### 2.2.6. Total RNA isolation and small RNA library preparation

The PAXgene Blood miRNA Kit (PreAnalytiX; Switzerland) was deployed to isolate whole blood total RNA from PAXgene samples followed by RNA quality control and quantification as stated in Mussack *et al.* [204] (Appendix I). By contrast, total RNA from urinary EVs was purified using the miRNeasy Mini Kit (Qiagen; Germany), quality controlled, completely vacuum-evaporated and resuspended in equal volumes of nuclease-free water [140] (Appendix II). Small RNA library preparations were conducted applying NEBNext Multiplex Small RNA Library Prep Sets for Illumina (New England BioLabs; USA) as previously published [140, 204] (Appendix I, Appendix II).

### 2.2.7. Small RNA sequencing and data evaluation

Small RNA sequencing of libraries size-selected for miRNAs was performed on the HiSeq 2500 (Illumina; USA) and obtained raw data was processed as previously described [140, 204] (Appendix I, Appendix II, Figure 5). Raw trimmed data was deposited in the European Nucleotide Archive (accession numbers PRJEB30403 and PRJEB38354).

### 2.2.8. Validation of differentially regulated miRNAs

A selection of miRNAs that appeared dysregulated after blood doping according to small RNA sequencing results was orthogonally validated using quantitative real-time polymerase chain reaction (qPCR) as described in Mussack *et al.* [204] (Appendix I). More detailed information with reference to the minimal information for publication of real-time PCR experiments (MIQE) guidelines [210] is provided in Mussack *et al.* [211]. In brief, quality-controlled total RNA was reverse transcribed using 5'-poly T universal primers, amplified using miRNA-specific primers (miRCURY LNA miRNA PCR system; Qiagen; Germany), and quantified. Appropriate controls were included in all experiments. Moreover, primer efficiencies, potential PCR inhibition, and unspecific PCR amplifications were controlled by performing calibration curve and melting curve analyses. Reference candidates for normalization were pre-selected based on sequencing results and confirmed by qPCR based on the NormFinder and geNorm algorithms [212, 213]. Finally, data normalization and analyses were performed based on the 2^(-△△Cq)

method [214]. Statistical investigations comprised outlier detection, testing for normal distribution, and correlation analysis between log2 fold changes obtained by small RNA sequencing and qPCR.

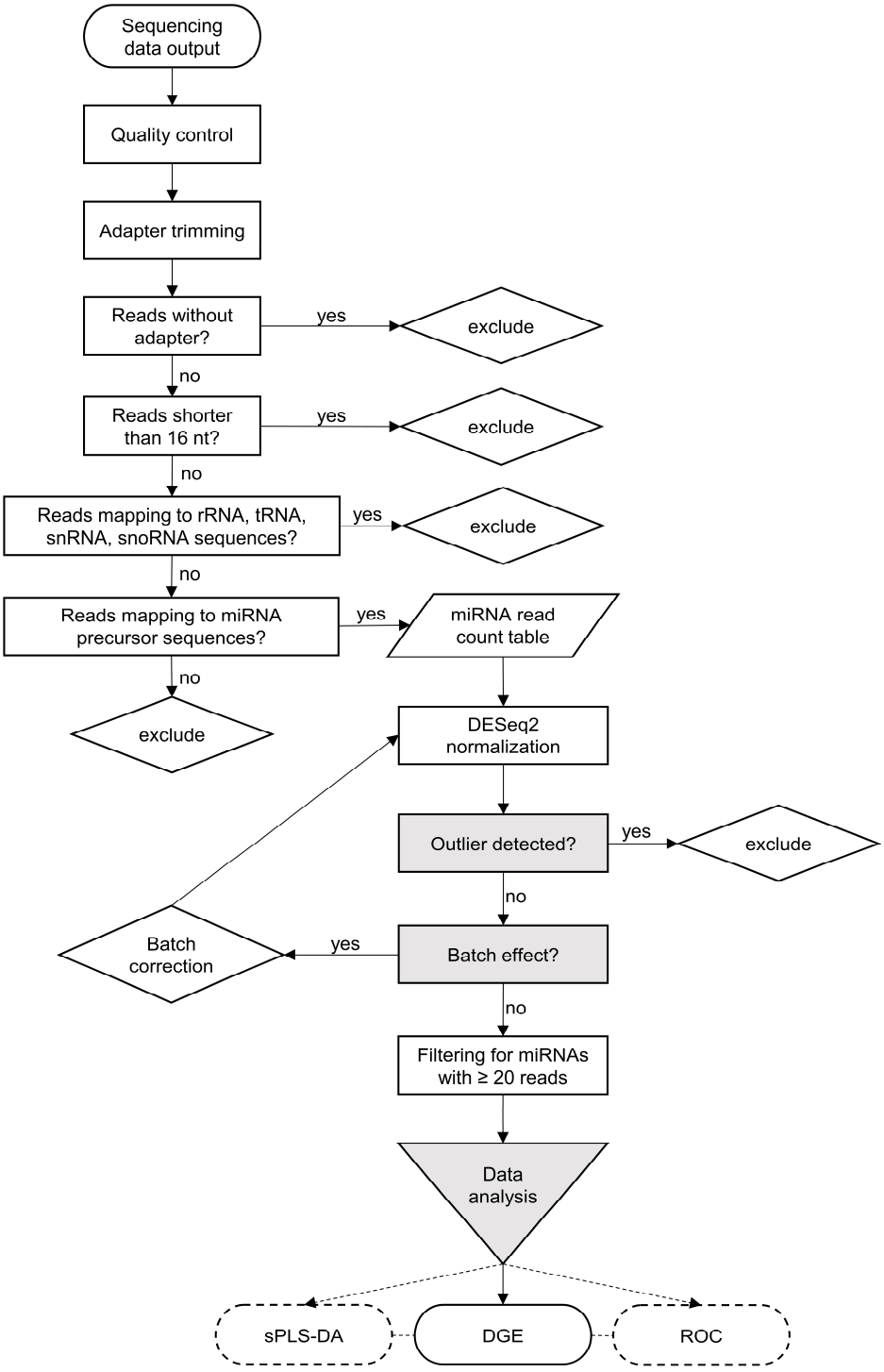


**Figure 5:** Flow diagram of the sequencing data processing pipeline. Dashed boxes were only conducted in microRNA (miRNA) analyses of whole blood samples of the autologous blood doping study. Filled boxes included exploratory data analyses, such as principal component analysis, hierarchical clustering, and heatmap analysis. Computation was performed in the statistical environment of R applying appropriate packages for analysis and visualization [215]. nt: nucleotides, rRNA: ribosomal RNA, tRNA: transfer RNA, snRNA: small nuclear RNA, snoRNA: small nucleolar RNA, sPLS-DA: sparse partial least square discriminant analysis, DGE: differential gene expression, ROC: receiver operating characteristics.

# 3. Results

## 3.1. “On the trail of blood doping – microRNA fingerprints to monitor autologous blood transfusions in vivo” [204] (Appendix I)

*Summary*

The detection of the misuse of autologous blood transfusions in high-performance athletes continues to be difficult. Direct detection methods focusing on exogenous substances administered to the body are not well applicable. Instead, reliable indirect detection is urgently needed to meet the WADA’s mission statement of assuring health and equality. Up to now, per-individual longitudinal profiling of hematological parameters as specified in the ABP appears as the sole possibility of uncovering any form of blood doping, including ABT. However, it can be assumed that a substantial number of cases remains unidentified as shown by the many athletes that were convicted only based on evidence provided by whistleblowers during the recent “Operation Aderlass” trial. As miRNAs have already proven their potential in unveiling other forms of doping, such as the misuse of anabolic agents, and were shown to change significantly upon erythrocyte storage, the present study aimed at examining their ability to identify human subjects that received autologous transfusions of ECs. For this reason, whole blood was sampled from 30 healthy males at three consecutive weeks to assess inter-individual and intra-individual reference ranges of total miRNA profiles. Two thirds of study participants donated whole blood, either once or twice, and were transfused with stored autologous ECs, whereas the remaining third group served as an untreated control. To investigate transfusion-dependent changes in total circulating miRNA profiles, multiple whole blood samples were collected in the week after transfusion or the respective time frame in the non-treated group. Holistic small RNA sequencing was conducted to screen total miRNA profiles and evaluated by complex multivariate statistics, including differential gene expression analysis, supervised clustering, and ROC curve analytics. Finally, the classification performance of a promising miRNA fingerprint (miR-144-3p, miR-320d) was compared to the sensitivity and specificity measures that were obtained by applying a Bayesian adaptive model for longitudinal profiling as described by the ABP to the hematological parameters monitored in the same study cohort. Overall results demonstrated a true potential of miRNAs in the detection of autologous blood doping, particularly six hours after transfusion, with ≤ 11 % sensitivity at 100 % specificity. While this could lead to an extension of the window of detection, classification performance of miRNA patterns was inferior to that obtained by the current gold standard ABP, which yielded higher sensitivity rates of ≤ 44 % at 100 % specificity, highest at two days after transfusion.

Considering the very controlled study environment, which might not be comparable to the reality in which cheating athletes might also use an unknown combination of masking agents, elaborated miRNA signatures could be included in usual anti-doping tests to verify their true utility in the combat against blood doping.

*Contribution*

Veronika Mussack was involved in conceptualizing and executing the study. Veronika Mussack recruited study subjects, organized and monitored all visits, samplings, and transfusions. Veronika Mussack performed all experiments and data analyses, created all figures and tables, wrote the manuscript, and revised it according to the reviewer comments.

**Mussack V**, Wittmann G, Pfaffl MW. On the trail of blood doping—microRNA fingerprints to monitor autologous blood transfusions in vivo. Am J Hematol. 2021;96:338–353. https://doi.org/10.1002/ajh.26078

## 3.2. “Comparing small urinary extracellular vesicle purification methods with a view to RNA sequencing—Enabling robust and non-invasive biomarker research” [140] (Appendix II)

*Summary*

Despite an enormous increase in publications from various subfields, EV research is still in its infancy and has just begun realizing the pressing need for thorough characterization of any separated sample fraction to provide extensive understanding and contextualization of resulting outcomes. A variety of different methods claiming to isolate EVs or subpopulations thereof were commercialized; however, comparison studies already revealed differences in blood EV miRNA biomarker signatures depending on the selected isolation method. Furthermore, literature often fails to state methodological details, thus limiting the comparability and reproducibility of results. As urine can be easily and non-invasively accessed and provides specific information on the health status of the urogenital tract, the comparability of different urinary EV separation approaches was assessed in the context of biomarker research. Specifically, we focused on the question of whether the obtained EV and RNA yield would be sufficient for downstream small RNA sequencing with the goal of establishing a sound basis for transcriptomic biomarkers. For this reason, the present study applied five different EV separation methods to urine samples from six healthy subjects, and extensively characterized the purified fractions by WB, standard and fluorescence NTA, TEM, and small RNA sequencing. The separation strategies in this study were chosen based on their broad application in the EV field, applicability in routine diagnostics, costs and time required for separation, and comprised spin column chromatography, immunoaffinity, membrane affinity, precipitation, and ultracentrifugation combined with density gradient centrifugation. The average size of biological particles separated by each of the five methods was fairly comparable, but particle concentrations varied significantly between the applied EV separation protocols. Further, WB and TEM analyses confirmed that while each method was indeed able to separate EVs, both the yield and purity of vesicle preparations differed significantly. All five approaches revealed enough vesicle-associated RNA to be sequenced; however, differences were observed in terms of library sizes, mapping statistics, and miRNA transcript abundance as well as diversity. Overall degrees of comparison of miRNA signatures were assessed by exploratory data analyses, indicating a broad overlap of results obtained by immunoaffinity, membrane affinity, precipitation, and ultracentrifugation followed by a density gradient, but also highlighted the distinctness of miRNA patterns in EVs separated by spin column chromatography. Summed up, these findings emphasized the fact that different separation methods result in distinct EV subpopulations with

discrete miRNA profiles, which in turn stresses the importance of proper EV characterization and reporting thereof to ensure a standardized scientific environment that allows for reproducibility and comparability.

*Contribution*

Veronika Mussack designed the experimental setup, performed all experiments and data analyses, created all figures and tables, wrote the manuscript, and revised it according to the reviewer comments.

**Mussack V**, Wittmann G, Pfaffl MW. Comparing small urinary extracellular vesicle purification methods with a view to RNA sequencing—Enabling robust and non-invasive biomarker research. Biomol Detect Quantif. 2019; 17:100089. https://doi.org/10.1016/j.bdq.2019.100089

## 3.3. “Separation, characterization, and standardization of extracellular vesicles for drug delivery applications” [144] (Appendix III)

*Summary*

Drug discovery and development continuously thrive to devise new medications that address novel treatment areas and improve therapeutic effectiveness while assuring patients’ safety by reducing the risk of adverse side effects. As EVs are common and naturally occurring shuttle and communication systems within the body, they are assumed to exhibit high biocompatibility and low immunogenicity when administered exogenously. Additionally, other inherent features such as their ability to penetrate barriers, the fact that they can be loaded with small molecules, and the cargo protection offered by their lipid bilayer envelope make them attractive candidates as therapeutics and drug delivery systems. Furthermore, the possibilities of not only producing EVs in large-scale bioreactors, but also engineering them to fit a specific purpose, render EVs a promising tool kit for therapeutic applications. For instance, EVs can be loaded actively or passively with drugs or other bioactive compounds, genetically modified to express certain markers, and decorated with surface structures that increase their circulation time or enhance their tissue targeting capacity while reducing off-target effects. Due to these manifold advantageous characteristics, EVs may outperform other nanoscale vehicles in the development of drug delivery applications. Although more and more information on EVs has been generated, EV research is still in a fledgling stage and lacks standardization. In this context, there is also no consensus in the scientific community about which of the many available EV separation methods, including differential ultracentrifugation, chromatography, filtration, and affinity-based methods, should be favored to prepare vesicles to be used for drug delivery. While some approaches seem superior to others due to the achievement of higher EV yield or purity, others shine because of their high scalability and time efficiency. Nevertheless, thorough decision-making on how to most appropriately separate EVs – while considering source material and therapeutic target – is required. Once a suitable separation method, or combination of methods, has been selected, the thereby obtained EV sample demands stringent characterization. This is not only recommended to achieve high consistency in EV production, but also to control for the absence of potential impurities and the presence of engineered or naturally occurring features that are eminently important for the appropriate choice of administration route, EV targeting, and pharmacokinetics, such as EV distribution and clearance. At the present, researchers can pick from a wide variety of characterization techniques, all of which focus on different aspects – be it enumeration, sizing, morphology, phenotyping, or cargo profiling, including lipidomics, proteomics, and genomics. Comprehensive profiling thus calls for complementary methods to shine a light on all aspects of the complex molecular makeup of

vesicles. Summed up, there is no doubt about the existence of distinct EV features that can be exploited for therapeutic applications. However, the selection of EV separation methods should be carefully considered and adapted to the respective therapeutic aim. In addition, very thorough characterization of EV samples appears imperative especially at the beginning of setting up an EV production pipeline, whereas using fewer and less sophisticated techniques could be sufficient for in-process quality control of a well-established system.

*Contribution*

Veronika Mussack was involved in conceptualization of the manuscript outline, performed thorough literature research, and wrote the complete third paragraph including tables. Veronika Mussack created all figures included in the manuscript. Veronika Mussack proof-read and revised the manuscript according to the reviewer comments.

Buschmann D, **Mussack V**, Byrd JB. Separation, characterization, and standardization of extracellular vesicles for drug delivery applications. Adv Drug Deliv Rev. 2021;174: 348-368. https://doi.org/10.1016/j.addr.2021.04.027

# 4. Discussion

## 4.1. Thoughts on the study protocol of a blood doping study

### 4.1.1. Employing the right study population

Ongoing research to establish new test strategies and enhance existing methods in the combat against doping in high-performance sports is highly required to convict cheating athletes, who perpetually seem to be one step ahead. Interestingly, blood doping by ABT is not an exceedingly relevant issue in equestrian sports or dog racing, as both horses and dogs store immense portions of their total erythrocytes in the spleen and release them immediately upon exercise as kind of a natural oxygen booster [216, 217]. Moreover, results from animal studies might not be well transferable to humans, thus, it appears unavoidable to conduct human studies. An important ethical aspect of human (but also animal) studies is the choice of an appropriate sample size. Basically, the smallest possible number of participants based on which detection of effects can be assumed should be included [205]. However, inclusion of too few study participants may lead to overestimation of effect sizes and low reproducibility. To steer clear of this issue, we performed a power analysis using current results of doping research as the basis for sample size calculations and obtained a power of 95 % at a beta error rate of 5 % when including 30 study subjects divided into three groups of ten participants each [204] (Appendix I).

Another important consideration when selecting the most appropriate study population in the context of transcriptomic-based ABT detection is the fact that smoking can heavily influence miRNA profiles [218]. The hypoxic environment at high-altitudes can also alter the miRNA profile of erythrocytes and hematological marker [219]. Thus, only non-smokers were included in the present study and any prolonged stay (> one week) in the high mountains in the past three months was protocolled [204] (Appendix I). Moreover, the menstrual cycle of females affects hematological parameters included in the ABP [220]. Therefore, women should be excluded, as well. Although a study population only composed of males would be unfavorable in an application study, excluding females is a necessary step in this exploratory study to ensure that monitored ABT-dependent changes were not distorted or even masked by hormonal fluctuations. Apart from the sex, the included study population has to be selected based on the athletic community that stands to benefit most from the type of doping to be tested. High-performance athletes participating in official competitions are usually subjected to the rules of WADA or national anti-doping regulations, and thus are not allowed to perform ABT. Therefore, we included highly recreationally active volunteers (five hours of activity per week on average), not

participating in any regulated competitions [204] (Appendix I). In comparison to other biomarker studies, e.g. to detect a certain disease, where the study population represents the later clinical patients quite well, any result of anti-doping studies can be rather seen as an approximation to the outcome that might be observed in competing high-performance athletes and thus urgently requires subsequent field test validation.

### 4.1.2. Creating the sampling and dosing schedule

As exemplified for hematocrit levels (Figure 6), the population-based reference range for a "normal" hematocrit value is defined too broadly to identify an individual's unphysiological value [204] (Appendix I). Moreover, hematological parameters varied widely between individuals, which could lead any individual outlier being masked by the average differences. For these reasons, longitudinal monitoring of individual profiles and the creation of individual reference ranges are imperative. While there is no information available on the actual condition (doped or non-doped) in "real world" doping controls, exploratory studies benefit from the presence of a known non-doped condition, which allows the generation of valid individual physiological ranges, a more sensitive doping detection, and the evaluation of a certain biomarker in its magnitude of change. According to Aikin *et al.*, three samples collected during three to four weeks are enough to monitor intra-individual variations of doping biomarkers caused by external factors such as food, fluid balance, and exercise [221]. As for all discovery trials in doping detection, the window of detection of the applied doping strategy is an important factor that has to be assessed. In theory, ABT would be detectable for up to 120 days depending on the maximum lifetime of erythrocytes. However, 25 % of erythrocytes are already degraded within one day after re-transfusion and the performance enhancing effect of ABT was shown to last only a few days [21, 222]. Therefore, the most likely window of detection for ABT was assumed to be somewhen within one week post-transfusion. Hence, several samples were collected during the week after ABT in the present study so as not to miss the sampling time frame with highest sensitivity and specificity [204] (Appendix I). However, a potential reduction of effect size by this strategy has to be considered, as a large proportion of the transfused EC volume is withdrawn again during the week of post-ABT sampling.

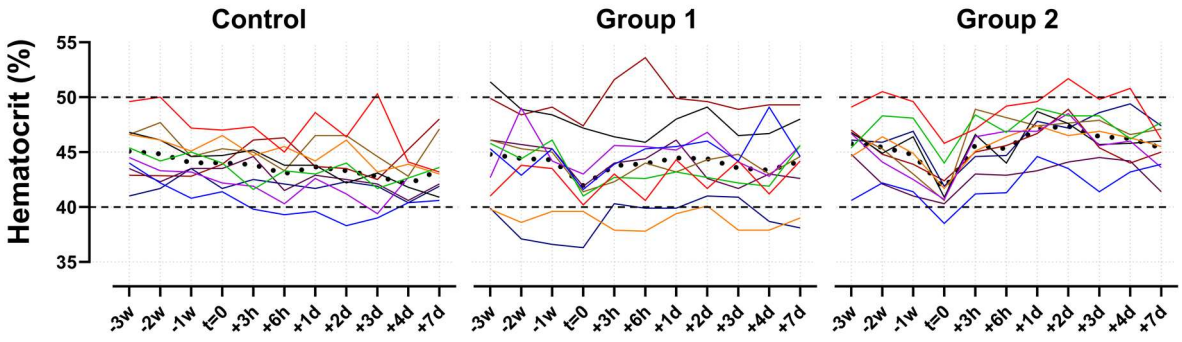


**Figure 6:** Individual hematocrit levels obtained from subjects included in our blood doping study. Dotted lines represent the group means; dashed lines represent the population-based reference range of hematocrit for recreationally active males [223]. Reprinted and modified by permission from John Wiley and Sons: American Journal of Hematology, On the trail of blood doping—microRNA fingerprints to monitor autologous blood transfusions in vivo, Mussack et al. © 2020 The Authors [204] (Appendix I).

To investigate only ABT-dependent effects, a sufficient recovery from blood donation before transfusion is essential. This should be reached within four to six weeks after blood donation [41, 224]. Though we exploited almost the maximum storage time for ECs and performed transfusion four or six weeks after blood donations, we observed insufficient recovery in most study subjects [204] (Appendix I). This led to the analysis of miRNA profiles which might represent both ongoing compensation for the blood loss and the onset of ABT-dependent reactions. Encouragingly, the additional sampling right before re-transfusion (t=0) allowed us to identify any ongoing donation effect and account for it in downstream analyses. The observed conflated donation and transfusion effect might have been prevented by cryopreserving ECs instead of storing them at 4 °C. As cryopreserved ECs can be stored for more than ten years, there would be enough time to recover from any preceding blood withdrawal [20]. Indeed, the use of cryopreserved ECs in the doping scene was at least partly substantiated during the "Operation Aderlass" [47]. However, preparing ECs for freezing is highly laborious and expensive, requiring specialized equipment and trained personnel. The immense effort needed and the fact that frozen ECs are only prepared under exceptional circumstances in Germany caused refrigerated ECs to be transfused in the present study [204, 225] (Appendix I).

The volume of donated blood and transfused EC also has to be elaborated thoroughly. The standard volumes of one blood bag and EC unit are around 500 ml and 235 ml, respectively [20]. However, micro-dosing of ABT, as already well-known in rhEPO misuse, may be also performed by cheating athletes aiming at maintaining their hematological parameters within their physiological ranges [226]. Low-volume transfusions of less than 150 ml EC were already proven to increase cycling performance, but a correlation between EC transfusion volume and aerobic capacity was not ascertained [227]. Nevertheless, in avoidance of missing any ABT-dependent changes in miRNA profiles, for which a potential dose-response

relationship cannot be excluded, we decided to transfuse 235 ml ECs obtained from standard blood bags [204] (Appendix I). In addition, we performed a second standard-volume donation and transfusion, which was anticipated to generate an increased ergogenic aid resulting in more conclusive differences in miRNA profiles.

### 4.1.3. Choosing an appropriate sample matrix

Currently, serum, EDTA-blood, and urine are the only approved sample matrices collected during doping controls, with their collection, transportation, analysis, and storage being highly regulated [25]. The samples' stability during transport and storage as well as a low risk for manipulation are particularly important. Moreover, sampling shall be possible by minimally invasive procedures. In this context, novel sample matrices such as dried blood spots are currently under review as additional test material due to their easy sampling and resilience towards transport-induced alterations [39]. As whole blood miRNA profiles sampled in PAXgene Blood RNA tubes are exceedingly stable and minimally affected by transportation and temperature (see manufacturer's specifications), they could also serve as new test material in doping controls. For any retrospective detection of blood doping using ABT, novel detection methods have to be based on the already collected matrices, which is why we also collected urine samples and intended to screen them for ABT-dependent changes in the EV-associated miRNA profiles in addition to the whole blood miRNA profiles [204] (Appendix I).

### 4.1.4. Selecting suitable screening technologies and data analysis pipelines

Selecting a comprehensive screening technology to obtain thorough insights into biological processes that are induced by an intervention is essential in discovery trials. Thus, we applied small RNA sequencing, which requires no *a priori* knowledge and even allows for detection of novel transcripts [204] (Appendix I). Owing to the requirements for experienced staff, specialized equipment, and potential technical biases, any ABT-dependent miRNA signature established by small RNA sequencing should be subsequently validated by and implemented in a more convenient and cost-efficient technique [228]. In such a validation study special focus should be given to lower and, if applicable, upper limits of detection as well as to analytical measurement errors. Regarding small RNA sequencing, the method of choice for the implementation of ABT-related miRNA patterns as routine diagnostics in anti-doping testing would be qPCR. Although RNA quantification is not yet an approved method in WADA's accredited laboratories, there is no obvious reason why this should not change in the future, as qPCR is a highly standardizable technique already used in clinical laboratories and is feasible with low cost and personnel expenditures [229].

The large bulk of miRNA data generated by small RNA sequencing requires appropriate handling that allows to filter for the most relevant miRNA candidates while benefiting from the

presence of individual baseline and longitudinal measurements. Mixed-effects regression models appear very suitable to analyze longitudinal data and might also be applied to identify potential confounding factors of biomarkers [230]. However, biostatistical tools allowing for pattern recognition based on discriminant analysis, e.g. (sparse) partial least square discriminant analysis ((s)PLS-DA) or orthogonal projections of latent structures discriminant analysis (OPLS-DA), are superior to regression models as they can additionally reveal a more complex and thus probably more robust doping-dependent signature [204, 231, 232]. In the following, a reasonably pruned list of miRNA candidates should be selected and validated by assessing specificity and sensitivity, which are important measures to gauge a biomarker's performance in anti-doping. In this context, specificity levels of 100 % are particularly crucial to ensure that clean athletes are not accused by mistake.

## 4.2. Evaluation of the ABT-induced miRNA fingerprint in blood

The number of studies investigating the miRNA biomarker potential for blood doping is small (Table 1). Only two of these examined the application of ABT of stored refrigerated or cryopreserved ECs or blood [52, 53]. Though these studies underpinned the possibility of including miRNAs into the ABP, their failure to establish adequate baseline measurements and to control for sufficient recovery from the foregoing blood donation are reasons to treat the results with caution. Moreover, Gasparello *et al.* only investigated the miRNA change 15 days after transfusion [52], which might be too late to observe major ABT-induced effects during competitive events for two reasons. First, the most pronounced impact of ABT on exercise performance and hence on the athlete's miRNA profile is assumed to happen within the first two to three days after transfusion [222]. Second, cheating athletes are said to receive EC transfusion on the day of a competition and could donate blood shortly afterwards to cast off the hematological add-on effect before their next doping test [233].

**Table 1:** List of anti-doping studies investigating the potential of microRNAs as biomarker for blood doping. ABT: autologous blood transfusion, EPO: erythropoietin, HIF: hypoxia-inducible transcription factor.

| Reference | Type of blood doping | Sample matrix | Regulated miRNAs |
|---|---|---|---|
| Gasparello *et al.* [52] | ABT | Human plasma | up: miR-197-3p |
| Leuenberger *et al.* [53] | ABT | Human plasma | up: let-7d, miR-26b, miR-30c, miR-30b, miR-142-3p, miR-103, let-7g, miR-26a, let-7b, miR-339-5p |
| Leuenberger *et al.* [51] | EPO stimulating agents | Human plasma | up: miR-144, miR-19b, miR-106b, miR-93, miR-101, miR-185, miR-19a, miR-140-3p, miR-142-5p, miR-25, miR-29c, miR-92a, miR-17; down: miR-874 |
| Marchand *et al.* [234] | HIF stabilizer | Rat plasma | up: miR-130a and miR-21 |

While accounting for the above-mentioned factors and choosing appropriate sampling time points (as described in section 4.1), Mussack *et al.* was able to establish an ABT-induced miRNA fingerprint (miR-144-3p and miR-320d) based on holistic miRNA screening by small RNA sequencing [204] (Appendix I). Assuming that the miRNAs of the miR-320 family, which share the same seed sequence, act similarly, both miRNAs of the fingerprint were already described in the context of erythropoiesis, emphasizing their biological relevance in blood doping-dependent adaptions [235, 236]. The upregulation of miR-144 after treatment with EPO-stimulating agents, which was observed by Leuenberger *et al. (2011)* [51], supports this relationship. Interestingly, none of the miRNAs differentially regulated upon ABT identified by Gasparello *et al*. and Leuenberger *et al. (2013)* could be confirmed in our study [52, 53, 204] (Appendix I). This might be a consequence of investigating different biofluids, as cellular and extracellular miRNAs seem partially blood compartment-specific [237]. While the research groups mentioned above examined circulating miRNA profiles in plasma, we studied whole blood to explore both the total circulating and cellular miRNAs and to verify storage-dependent variations in the miRNA pattern. However, none of the miRNAs that were significantly altered during EC storage at 4 °C were detected to be changed *in vivo* after transfusion [56, 204] (Appendix I). Remarkably, miR-144-3p had significantly different levels when comparing samples obtained from venipuncture right before processing of blood into ECs with samples obtained from the freshly prepared ECs [56]. This implies that the blood transition into a blood bag and its processing into ECs for storage is the more relevant issue for doping detection than the storage itself. Nevertheless, the eventual re-transfusion of stored ECs induced a

detectable change in the miRNA profiles of miR-144-3p and miR-320d, which was exceeding the individuals' 99 % confidence interval six hours after transfusion irrespective of having received one or two EC bags (Figure 7) [204] (Appendix I). Thereby, the detection window was extended to an earlier stage compared to the ABP (ABT detection window: one to two days after EC re-transfusion). Moreover, most of the hematological parameters included in the ABP responded highly sensitively to the preceding blood donations. This impact was not present when applying the miRNA fingerprint to screen for ABT, indicating a certain specificity for ABT. Nevertheless, the classification performance of the ABP with a maximum sensitivity of 40 % is still higher than the maximum sensitivity obtained by the whole blood miRNA fingerprint (11 %, at 100 % specificity), which is why the miRNA profile should be used as a supplement rather than a replacement to the ABP.

Another factor that needs to be considered is that the selection of a controlled study population and investigational environment is beneficial to observe any ABT-induced effects, but that cheating athletes are not likely to use only one well-defined doping method. Instead, they might apply a cocktail of doping techniques and substances, for instance by combining ABT with plasma expanders, rhEPO, and other drugs or masking agents, the impact of all of which was not studied by Mussack *et al.* [204] (Appendix I). However, such distortions can hamper the ABT detection rate enormously. In particular, plasma expanders can dilute hematocrit levels, which are elevated by ABT of ECs mainly one to three days after transfusion, and shift the detectable concentration back into the individuals' physiological ranges [41]. In addition, plasma volume and hematocrit levels are also influenced by exercise itself, which, on the one hand initiates a short-term reduction of plasma volume [238], and, on the other hand, induces a redistribution of circulating leukocytes [239]. In addition to this cellular shift that might also influence circulating miRNA profiles, they are also impacted by exercise [240, 241]. Though these studies revealed no exercise-related effect on specifically miR-144-3p and miR-320d levels, Fernandez-Sanjurjo and coworkers indeed observed an up- and down-regulation of miR-320d and miR-144-3p, respectively, after marathon running [242]. Thus, further investigation of putative confounding factors is highly required to assess the robustness of the whole blood miRNA fingerprint in anti-doping testing.

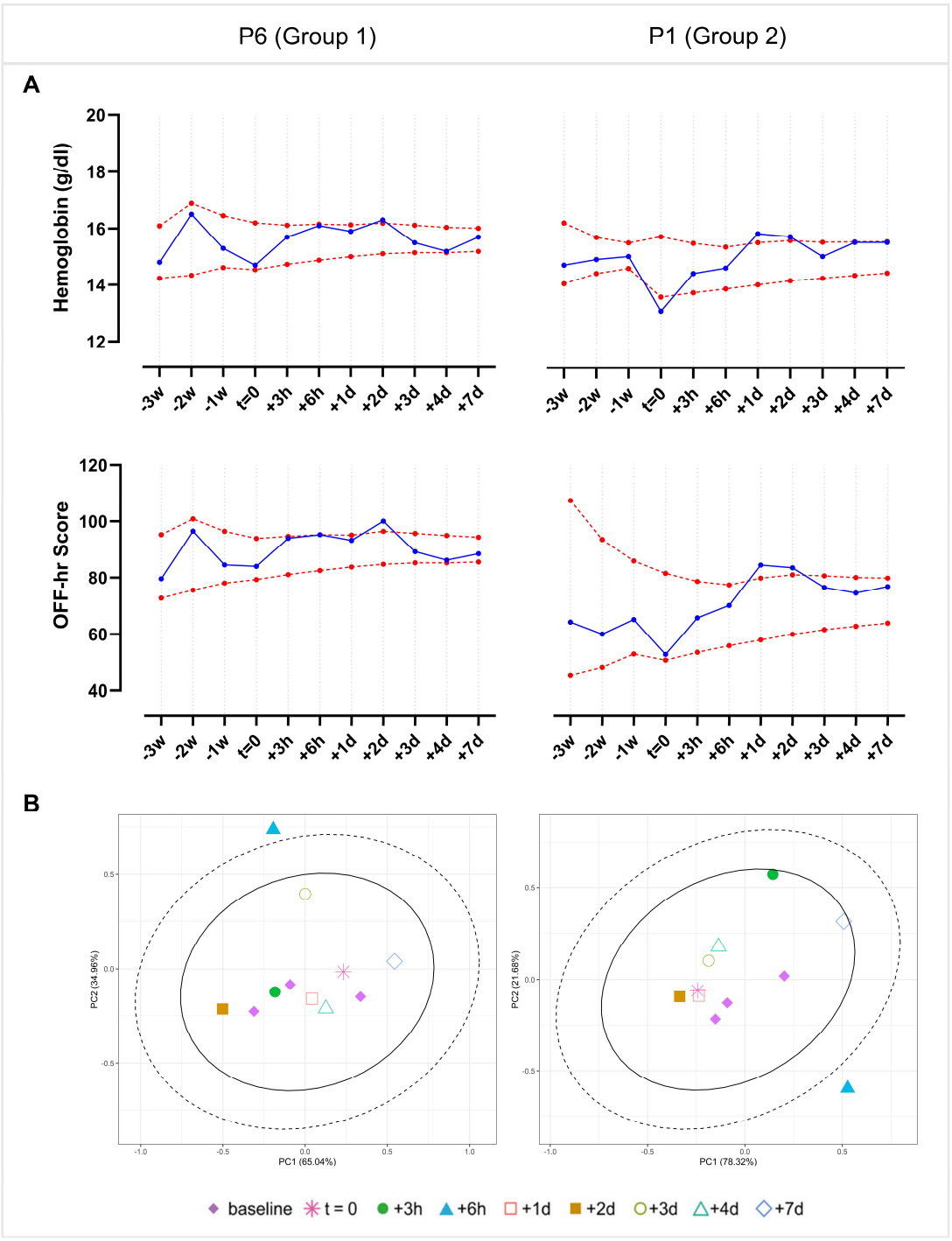


**Figure 7:** Exemplary juxtaposition of the longitudinal profile and detection window of autologous blood doping of two study subjects (P6 and P1) applying **(A)** the primary hematological markers hemoglobin and OFF-hr score included in the athlete biological passport (ABP) with the dotted red lines marking the 99 % confidence interval of the individual's critical range and the solid blue lines indicating the respective measured value; and **(B)** the microRNA fingerprint (miR-144-3p and miR-320d), visualized by principal components (PC) and the individual's 95 % (solid line) and 99 % (dashed line) confidence interval. Reprinted and modified by permission from John Wiley and Sons: American Journal of Hematology, On the trail of blood doping—microRNA fingerprints to monitor autologous blood transfusions in vivo, Mussack et al. © 2020 The Authors [204] (Appendix I).

## 4.3. The rationale of using miRNA patterns from urinary EVs for blood doping detection

As shown by OFF-hr score and Abnormal Blood Profile Score, which are both calculated values based on several factors and as such are included in the ABP, the combination of multiple readouts may enhance doping detection [26, 206]. In search of further data that could be included in the ABP to improve the detection of ABT apart from hematological markers and total blood miRNAs, urine presents a neglected biospecimen. Urine samples are routinely collected in doping controls and are approved for the detection of rhEPO [27]. With respect to ABT, however, urine has only been analyzed for residues of blood bag plasticizers exogenously administered during blood or EC transfusions [36, 37]. Given that ABT might affect a multitude of biological pathways, it does not seem far-fetched to assume that it will also be reflected in urine. Transfusion of ECs immediately increases total blood volume and thus the volume load on the blood vessels, which leads to a timely compensatory reaction via increased fluidic excretion stimulated by the atrial natriuretic peptide [243]. The kidney seems to be a pivotal sensor in the physiological response to ABT, as it is the organ where not only blood filtration and urine production, but also EPO synthesis are located, and is thus directly linked to the regulation of hematocrit levels [244].

As already mentioned, exercise and the overall physical condition affect blood volume and might heavily affect total blood circulating miRNA profiles by exercise-induced leukopedesis [239]. However, urinary miRNA patterns should remain largely unaffected by this cellular shift. Moreover, urinary EV-associated miRNAs are highly stable [245, 246], putatively rendering them robust biomarkers for the detection of ABT. The particular interest in urinary EV-associated miRNA signatures to uncover ABT originates from the facts that cold storage of ECs leads to both an increase in EV concentration and significant changes in miRNA expression levels in just these EVs [193, 203]. Moreover, the fast increase of circulatory EVs after transfusion and the rapid clearance within one day suggest an involvement of elevated urinary excretion of a small but non-negligible amount of these transfused vesicles besides their primary metabolization in the spleen [199, 200]. In theory, EVs are thought to be too large to be filtrated by the nephron in healthy humans [247, 248]. Nevertheless, a trans-renal release of labeled EVs previously introduced into the circulation was observed in mice [201]. Moreover, a significant increase in urinary EV concentrations shortly after re-transfusion of ECs was observed by Mussack *et al.* in humans [249], strengthening the hypothesis of urinary excretion of transfused EVs. So far, it has been assumed that urinary EVs mainly originate from the urogenital tract [250]. Interestingly, recent studies of Mussack *et al.* showed that at least parts

of urinary EVs indeed carry surface markers that are indicative of an hematological origin [251]. Moreover, tissue-specific proteomes of more distant sites were also detected in urinary vesicle preparations investigated in the context of lung cancer and Parkinson's disease [252, 253]. Despite these promising findings, however, studying urinary EVs for their biomarker potential is accompanied by biological as well as technical challenges that can affect reproducibility as well as comparability and should therefore be well considered.

## 4.4. Challenges in urinary EV research

### 4.4.1. Facing a complex and heterogeneous sample matrix

Urine is non-invasively available in large volumes, but both urine volume and composition depend on diverse variables including sampling time (morning void vs. spontaneous void vs. 24-hour void), fluid intake, age, and sex of the subject [247, 254, 255]. To account for said variations, different correction strategies were discussed [247]. Although not ranked as the optimal strategy, estimating the absolute excretion rate by normalizing to urinary creatinine seems favorable [256]. In addition, a positive correlation of urinary creatinine levels with urinary EV concentration was ascertained, affirming the applicability of this concept in vesicle-related biomarker research [257]. Nevertheless, urinary creatinine is a waste product of muscle metabolism, and the effect of differences in the muscle mass of high-performance athletes during exercise on the utility of urinary creatinine as normalizer remains to be established and might require additional corrective actions in investigating urinary EVs [258].

Furthermore, it is advisable to assess urine contamination with bacteria, as these also release RNA-containing vesicles which may be co-separated and thus impair downstream analyses [259, 260]. An initial dipstick analysis of urine prior to further downstream processing could provide introductory information on both the general urine health and the presence of bacteria [140] (Appendix II). Moreover, the presence of the highly abundant Tamm-Horsfall protein, which is also known as uromodulin and is synthesized in the loop of Henle, can confound urinary EV analyses by trapping the vesicles and thereby reducing vesicle yield [261]. Even though the use of reducing agents, such as dithiothreitol, might improve EV recovery by breaking the polymeric structure, their overall benefit on the concentration of EVs and vesicle-associated RNA is limited [262].

Based on the rising interest in EV research, many techniques have lately been adapted or even specifically designed to separate EVs from various sample matrices [142]. However, the presence of a heterogeneous assortment of EVs challenges each separation method. All cells

of the urogenital tract mainly simultaneously release various subtypes of EVs that largely differ in their cargo, size, and buoyancy, but may share characteristics not only with each other but also with other non-vesicular features and impurities [144, 255] (Appendix III). The specificity to separate EVs and/or EV subtypes while depleting non-vesicular material might thus be at the expense of total vesicle yield, whereas higher vesicle yields most likely result in reduced purity [144] (Appendix III). Thus, there is no consensus for any biofluid on the one and only EV separation method and, pending novel methods with increased selectivity, there might always be a trade-off between purity and yield [142]. Concerningly, the use of different methodologies to separate EVs from human sera resulted in highly distinct miRNA patterns [263]; a finding that can most likely be extrapolated to EVs from other biofluids including urine. When separating urinary EVs, some methods are even clearly unsuitable for downstream mRNA sequencing [264]. Consequently, feasibility but also reproducibility and comparability of miRNA profiling from different urinary EV preparations were questioned and assessed by Mussack *et al.* as described hereafter [140] (Appendix II).

### 4.4.2. The pros and cons of EV separation methods

The most frequently used approach for EV separation is based on differential ultracentrifugation [265], which is why it is oftentimes used as benchmark. Here, EVs are separated based on their sedimentation. In this process, however, non-vesicular structures with similar sedimentation speed are pelleted as well [263]. Hence, obtained pellets can be subsequently applied to sucrose or iodixanol density gradients, enabling further separation of EVs from unwanted material based on their different densities [172]. Once the expenditure on an ultracentrifuge has been made, large sample volumes can be processed in an inexpensive manner. This is especially beneficial for urine samples because more diluted urine might require larger input volumes. However, only a few samples can be processed in parallel, and the separation is very time-consuming – especially with the addition of density gradient centrifugation, which usually requires more than 12 hours of runtime. Moreover, the huge centrifugation forces may possibly damage EVs, and large proportions of vesicles could adhere to the tube walls, both of which could negatively impact EV yield [266, 267].

Another commonly applied strategy to enrich EVs is based on precipitation. Polyethylene glycol is currently the volume excluding polymer of choice, precipitating EVs from solution by changing their solubility [268]. The problem with this is the very unspecific alteration of solubility that leads to high amounts of non-vesicular material in the precipitate [263]. However, this method is very fast, simple to use, cost-efficient, and allows substantial upscaling of samples, all of which probably contribute to the reason for its vast usage though [144] (Appendix III). Other strategies are also widely accepted. Spin column chromatography, e.g. based on a silicon carbide resin, is a centrifugation-independent system and thus an easy and rapid

technique [262]. Often combined with membrane affinity binding, these approaches allow for the release of intact vesicles and yield high concentrations, however they are not specific for EVs and might co-separate a questionable number of impurities [144, 269] (Appendix III).

The aforementioned strategies are all based on the principle of negative selection, which generates high EV yields, but also bares an aggravated risk for unspecific co-separation of non-vesicular particles that could heavily impact downstream applications and distort obtained miRNA profiles. In contrast thereto, immunoaffinity-based EV separation rests upon positive selection. In this approach, capturing antibodies, that target specific surface proteins, are immobilized on beads and isolate bound structures from unlabeled material, e.g. by magnetic separation [140] (Appendix II). Though this ensures elevated levels of purity, obtained EV yields might be greatly reduced because there is no surface marker protein that is valid for all EVs, intrinsically biasing affinity capture to pre-determined EV populations [172]. Only subfractions of EVs exposing the respective epitope of nominated marker proteins will be recognized by the selected antibodies. However, the combined targeting of the three tetraspanins CD9, CD63, and CD81 seems promising in specifically capturing large quantities of EVs, as these surface markers were already identified as key proteins in their biogenesis [149, 270]. Interestingly, syntenin-1 was very recently reported as a universal marker for exosomes [271]. Regardless of the respective target protein, the conventional attachment of capture antibodies to magnetic beads facilitates a fast and simple procedure. However, these magnetic beads remain bound to the EVs, unless relatively harsh buffers are used to detach them, and can thus interfere with downstream experiments or functional analyses.

### 4.4.3. Understanding the means for EV characterization

Finally, to evaluate the success and specificity of EV separation from urine and other biofluids, the MISEV 2018 guidelines provide a list of minimal as well as optional EV properties that should be characterized, including the particle number, size distribution, presence of EV-specific protein marker, and absence of non-EV markers and putative impurities [141]. A variety of methods has been employed to achieve thorough EV characterization (Figure 8), the underlying principles of which were already extensively discussed, with each of the stated methods demonstrating its own strengths and limitations [144] (Appendix III). While microscopic devices enable a detailed morphological assessment of vesicle surfaces and inner-vesicular structures, only a subset of EVs is captured thereby [144] (Appendix III). Furthermore, the results obtained by techniques utilizing scattered light for sizing and enumerating EVs can be skewed towards larger particles in case of polydisperse solutions, as larger particles scatter more light thereby masking the less scattered light of smaller particles [272]. Nevertheless, they provide a fast and simple measurement of EV preparations. In addition, most techniques were not initially designed to investigate EVs and, thus, not only limits in the detectable size range and

refractory index have to be considered, but also the putative characterization of both vesicular and non-vesicular particles, unless any labeling has been performed to increase specificity [144] (Appendix III). Several approaches are based on bulk analysis, which calls for a pure sample preparation, but enables a quick multiparametric assessment of EV preparations, whereas especially the more recently developed technologies are focusing on the investigation of single-EVs with improved resolution and specificity [144] (Appendix III). Depending on the available sample volumes, it is recommended to conduct various EV characterization methods – the more, the better – and merge the outcomes thereof to increase the significance and validity of obtained results [141]. As demonstrated by Mussack *et al.* [140] (Appendix II), the combinational application of TEM, WB, and fNTA prior to miRNA sequencing allows for a comprehensive evaluation of urinary EVs on both the bulk and the single-vesicle level.

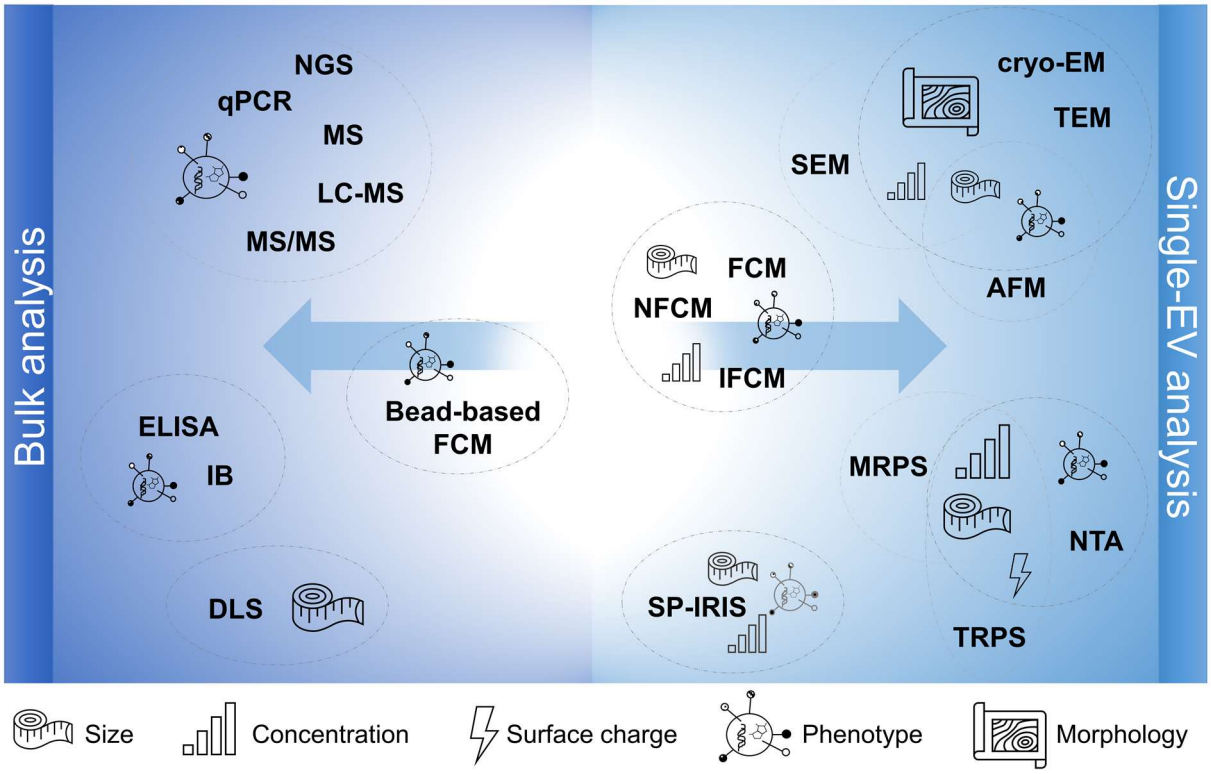


**Figure 8:** Graphical summary of techniques used to characterize extracellular vesicle preparations. Reprinted by permission from Elsevier: Advanced Drug Delivery Reviews, Separation, characterization, and standardization of extracellular vesicles for drug delivery applications, Buschmann et al. © 2021 Elsevier B.V. [144] (Appendix III). AFM: atomic force microscopy, cryo-EM: cryogenic electron microscopy; DLS: dynamic light scattering, ELISA: enzyme-linked immunosorbent assay, EV: extracellular vesicle, FCM: flow cytometry; IB: immunoblotting; IFCM: imaging flow cytometry; LC-MS: liquid chromatography mass spectrometry; MRPS: microfluidic resistive pulse sensing; MS: mass spectrometry; MS/MS: tandem mass spectrometry; NFCM: nanoflow cytometry; NGS: next-generation sequencing; NTA: nanoparticle tracking analysis; qPCR: quantitative polymerase chain reaction; SEM: scanning electron microscopy; SP-IRIS: Single-particle interferometric reflectance imaging sensing; TEM: transmission electron microscopy; TRPS: tunable resistive pulse sensing.

## 4.5. Assessment of different urinary EV separation methods regarding their utility in miRNA-based biomarker research

As recommended by the ISEV, methodological details of our studies were disclosed on the EV-TRACK platform (EV-TRACK ID: EV190007) [141, 142], where the protocols of Mussack *et al.* [140] (Appendix II) received EV-METRICs of 75 % and 100 % and thereby ranked among the top five entries investigating human urinary EVs. The EV-METRIC was created to assess the thoroughness of reporting experimental findings and methodological details that allow for appropriate interpretation and reproducibility of results [142]. In our study comparing different separation methods for urinary EVs, the highest metric of 100 % was obtained for ultracentrifugation combined with density gradient centrifugation and further downstream characterization, while all other separation methods combined with the same subsequent characterization received scores of 75 % [140] (Appendix II). Remarkably, all of the selected separation methods were generally suitable for downstream miRNA sequencing, and the resulting miRNA patterns were mostly congruent [140] (Appendix II). Only spin column chromatography resulted in a set of urinary vesicle-associated miRNAs distinct from those obtained by the other methods. This disparity was also observed to be true for mRNA profiles by Barreiro *et al.* [264] and may be problematic for generating reproducible results in EV biomarker research whenever methods of low comparability are used. The remaining four separation methods clearly overlapped in their miRNA patterns, even though a noticeable impurity with Tamm-Horsfall protein was detected in the EV preparations obtained by membrane affinity and precipitation [140] (Appendix II). This suggests that purity of urinary EV preparations is not a decisive prerequisite for comparable biomarker studies when investigating miRNAs. In therapeutic approaches, however, purity is of major importance to comply with good manufacturing and clinical practices and to avoid adverse events [144] (Appendix III). Expecting that any anti-doping decision can be brought to law, it might be advisable to strive for very high purity EV preparations to prevent miRNAs linked to unattended non-vesicular structures or other signal distorters from interfering with transcriptomic analyses, thereby weaking the validity of an established ABT-dependent urinary EV miRNA fingerprint.

Based on the general technical conditions, which were revisited earlier (see section 4.4 and Appendix III [144] ), and the combined evaluation of both biophysiochemical and transcriptomic features of urinary EVs and EV-associated miRNAs, which were assessed in Mussack *et al.* [140] (Appendix II), a competitive analysis of methods was performed (Figure 9). By assessing each factor individually and calculating an overall grade, spin column chromatography was rated as the least suitable and immunoaffinity as the most suitable urinary EV separation method to study miRNA profiles as biomarkers in the context of anti-doping. However, it has

to be emphasized that there is no “one-fits-all” solution and that the decision for or against a specific separation strategy still strongly depends on the initial research question and should be well contemplated.

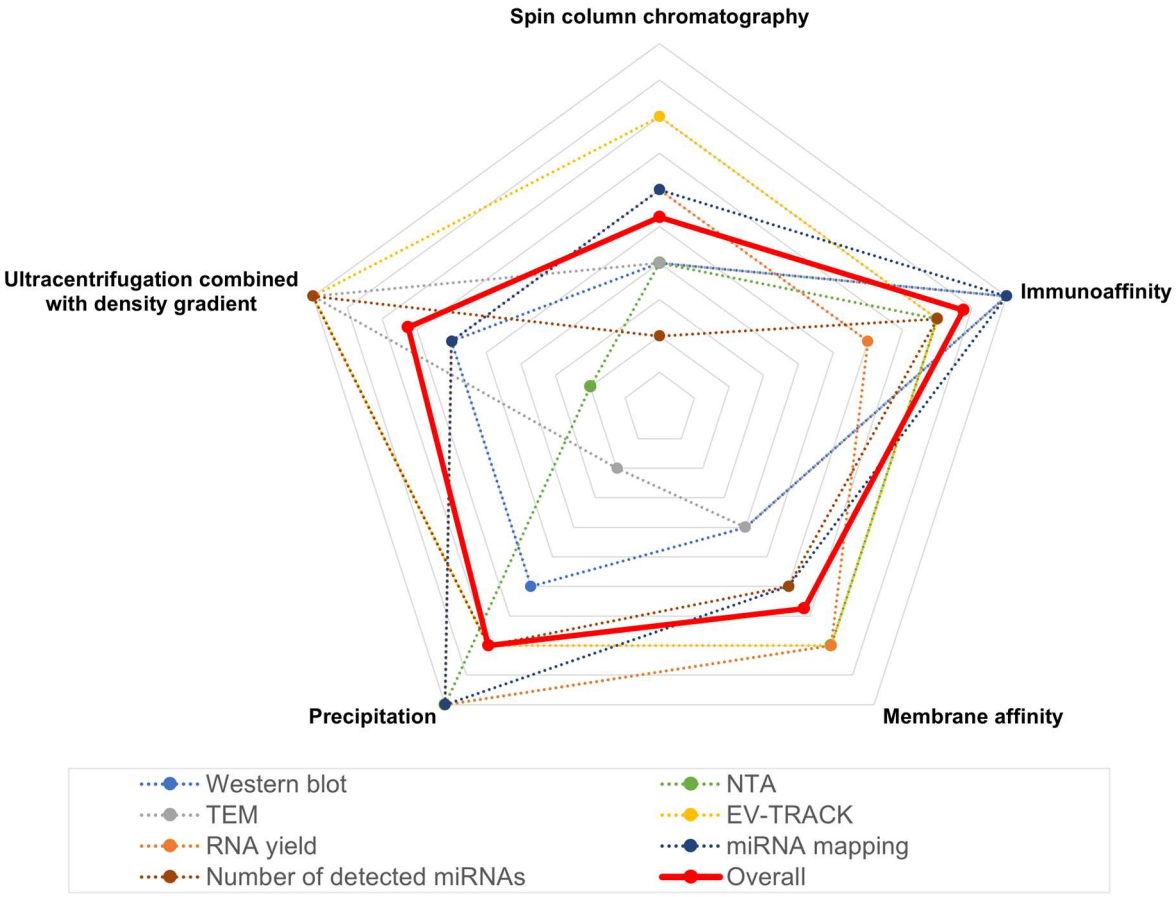


**Figure 9:** Spider plot illustrating the performance of five different methods to separate extracellular vesicles (EVs) from urine. Each method obtained grades in different characterization disciplines with the figure’s center indicating the lowest/worst level and the outer ring indicating the highest/best grade. miRNA: microRNA, NTA: nanoparticle tracking analysis, TEM: transmission electron microscopy. The overall grade represents the average rate over all disciplines.

## 4.6. Conclusion and future perspectives

Ever since humans started engaging in physical competition, and certainly since being an athlete began opening avenues to make a living from professional sports, doping has been part of the equation. While substances and methods changed over time, from consuming exotic mushrooms and herbal potions to cocktails of steroids and stimulants, the goal remained the same: to gain a competitive advantage over one’s competitors in the race for recognition, records, price money, and sponsorships. The goal of anti-doping efforts has therefore always

been clear. Not only detecting and preventing doping but also safeguarding the rights of clean athletes and promoting a spirit of health and equality in sports are at the core of the anti-doping notion.

What this present research amounts to is that a well-wrought human study providing longitudinal data of both hematological markers and blood total miRNA profiles of subjects undergoing ABT was performed. The comprehensive raw data generated in our experiments was generally made accessible and can thus be used by the anti-doping community in subsequent studies. Although a previously reported storage-dependent miRNA signature in ECs could not be verified *in vivo*, we establish an elaborated ABT-dependent miRNA fingerprint. This set of miRNAs is an auspicious complementary marker to the currently applied ABP and could be added in doping controls in order to screen for the illicit application of ABT shortly after transfusion. This is a time frame that is both highly relevant to athletic competitive events and a weakness of the ABP as unphysiological variations of hematological parameters might not yet have changed noticeably. In contrast to its promising detection kinetics, the overall detection rate of the miRNA fingerprint is rather low compared to the ABP and requires ultimate verification in anti-doping field-tests and, in particular, in a more diversified population.

Even though, on the face of it, the success rate of devising a universally applicable marker for ABT appears humbling, continued anti-doping research is indispensable to show presence in the combat against doping and intimidate fraudulent athletes and potential dopers. Increased screening for blood doping intends not only to uncover cheating athletes, but also to evoke a recoil from illicit strategies for performance enhancement and a shift to less efficient doping strategies. Moreover, continuously improving existing detection strategies and elaborating novel biomarkers may help to understand the cause-effect relationship and enforce better anti-doping decisions.

To continue on the path of investigating advanced anti-doping approaches, urinary EV-associated miRNAs present a promising resource to detect ABT. To pave the way for a robust transcriptomic analysis of these urinary vesicular miRNAs, we performed a comprehensive comparison study evaluating the pros and cons of different EV separation methods and discussed available methodologies for their characterization. Based on the thereby gained knowledge, the positive selection of urinary EVs by immunoaffinity-based separation seems to be one of the more favorable strategies. Future experiments could use this technique to screen for an ABT-dependent signature compiled of novel and established components. Beyond doubt, the combination of transcriptomic markers with other “omic” data is highly conceivable and might reach higher detection sensitivities of ABT.

Juxtaposing the development of more sophisticated and potent strategies to enhance athletic performance and the battle against their utilization and propagation, it is obvious that detection methods for some forms of doping, autologous blood doping chiefly among then, remain suboptimal. Judging by the enormous progress the past few years of anti-doping efforts have witnessed, realizing the evident potential of miRNAs and EVs in this context seems feasible. But many challenges will have to be overcome for miRNAs to be integrated into routine anti-doping testing. With the advent of improved devices, experimental methods, and algorithms for data analysis and integration, however, current detection strategies are likely to be outperformed by novel biomarkers and combinations thereof, with miRNAs among the most promising candidates to step onto the podium.

# References

[1] Y.O. Schumacher, M. Ashenden, Doping with artificial oxygen carriers: an update, Sports Med 34(3) (2004) 141-50.

[2] G. Lippi, G. Guidi, [Doping and sports], Minerva Med 90(9) (1999) 345-57.

[3] C. Prokop, Die Grenzen der Dopingverbote, Nomos-Verlag-Ges.2000.

[4] K.N. Latzel, Lutz, Hormone und Hochleistung: Doping in Ost und West, Köln: Böhlau, 2008.

[5] G. Spitzer, Doping and doping control in Europe: Performance enhancing drugs, elite sports and leisure time sport in Denmark, Great Britain, East and West Germany, Poland, France, Italy, Meyer & Meyer, Aachen, 2006.

[6] World Anti-Doping Agency, World Anti-Doping Code, 2021. https://www.wada-ama.org/sites/default/files/resources/files/2021_wada_code.pdf. 2021).

[7] World Anti-Doping Agency, Prohibited List 2021, 2021. https://www.wada-ama.org/sites/default/files/resources/files/2021list_en.pdf. (Accessed 12.04.2021 2021).

[8] D.R. Bassett, Jr., E.T. Howley, Limiting factors for maximum oxygen uptake and determinants of endurance performance, Med Sci Sports Exerc 32(1) (2000) 70-84.

[9] W. Jelkmann, Physiology and pharmacology of erythropoietin, Transfus Med Hemother 40(5) (2013) 302-9.

[10] D. Padhi, L. Ni, B. Cooke, R. Marino, G. Jang, An extended terminal half-life for darbepoetin alfa: results from a single-dose pharmacokinetic study in patients with chronic kidney disease not receiving dialysis, Clin Pharmacokinet 45(5) (2006) 503-10.

[11] S. Fishbane, A. Pannier, X. Liogier, P. Jordan, F.C. Dougherty, B. Reigner, Pharmacokinetic and pharmacodynamic properties of methoxy polyethylene glycol-epoetin beta are unaffected by the site of subcutaneous administration, J Clin Pharmacol 47(11) (2007) 1390-7.

[12] K.I. Birkeland, J. Stray-Gundersen, P. Hemmersbach, J. Hallen, E. Haug, R. Bahr, Effect of rhEPO administration on serum levels of sTfR and cycling performance, Med Sci Sports Exerc 32(7) (2000) 1238-43.

[13] J. Durussel, E. Daskalaki, M. Anderson, T. Chatterji, D.H. Wondimu, N. Padmanabhan, R.K. Patel, J.D. McClure, Y.P. Pitsiladis, Haemoglobin mass and running time trial performance after recombinant human erythropoietin administration in trained men, PLoS One 8(2) (2013) e56151.

[14] R. Palaparthy, H. Wang, A. Gulati, Current aspects in pharmacology of modified hemoglobins, Adv Drug Deliv Rev 40(3) (2000) 185-98.

[15] G.S. Hughes, Jr., E.P. Yancey, R. Albrecht, P.K. Locker, S.F. Francom, E.P. Orringer, E.J. Antal, E.E. Jacobs, Jr., Hemoglobin-based oxygen carrier preserves submaximal exercise capacity in humans, Clin Pharmacol Ther 58(4) (1995) 434-43.

[16] S.F. Flaim, Pharmacokinetics and side effects of perfluorocarbon-based blood substitutes, Artif Cells Blood Substit Immobil Biotechnol 22(4) (1994) 1043-54.

[17] M.J. Ashenden, Y.O. Schumacher, K. Sharpe, E. Varlet-Marie, M. Audran, Effects of Hemopure on maximal oxygen uptake and endurance performance in healthy humans, Int J Sports Med 28(5) (2007) 381-5.

[18] W. Jelkmann, Autologous Red Blood Cell Transfusions in Clinics and their Misuse in Sports, German Journal of Sports Medicine/Deutsche Zeitschrift fur Sportmedizin 71(3) (2020).

[19] N. Kohli, S. Bhaumik, S. Jagadesh, R.K. Sales, I. Bates, Packed red cells versus whole blood transfusion for severe paediatric anaemia, pregnancy-related anaemia and obstetric bleeding: an analysis of clinical practice guidelines from sub-Saharan Africa and evidence underpinning recommendations, Trop Med Int Health 24(1) (2019) 11-22.

[20] Bundesärztekammer, Richtlinie Hämotherapie, Gesamtnovelle 2017, mit Erratum/Anpassungen, in: Dezernat 6–Wissenschaft Forschung und Ethik (Ed.) 2019.

[21] M. Luten, B. Roerdinkholder-Stoelwinder, N.P. Schaap, W.J. de Grip, H.J. Bos, G.J. Bosman, Survival of red blood cells after transfusion: a comparison between red cells concentrates of different storage periods, Transfusion 48(7) (2008) 1478-85.

[22] C.B. Malm, N.S. Khoo, I. Granlund, E. Lindstedt, A. Hult, Autologous Doping with Cryopreserved Red Blood Cells - Effects on Physical Performance and Detection by Multivariate Statistics, PLoS One 11(6) (2016) e0156157.

[23] C.D. Oliveira, A.V. Bairros, M. Yonamine, Blood doping: risks to athletes' health and strategies for detection, Subst Use Misuse 49(9) (2014) 1168-81.

[24] World Anti-Doping Agency, Athlete Biological Passport Operating Guidelines, 2019. https://www.wada-ama.org/sites/default/files/resources/files/guidelines_abp_v71.pdf. (Accessed 24.08.2020 2020).

[25] I.S.f.T.a. Investigation, 2021 ISTI Guidelines for Sample Collection, 2021. https://www.wada-ama.org/sites/default/files/resources/files/isti_sample_collection_guidelines_en_final_2_feb_2021_0.pdf. (Accessed 03.06.2021.

[26] C.J. Gore, R. Parisotto, M.J. Ashenden, J. Stray-Gundersen, K. Sharpe, W. Hopkins, K.R. Emslie, C. Howe, G.J. Trout, R. Kazlauskas, A.G. Hahn, Second-generation blood tests to detect erythropoietin abuse by athletes, Haematologica 88(3) (2003) 333-44.

[27] F. Lasne, J. de Ceaurriz, Recombinant erythropoietin in urine, Nature 405(6787) (2000) 635.

[28] M. Thevis, R.R. Ogorzalek Loo, J.A. Loo, W. Schanzer, Doping control analysis of bovine hemoglobin-based oxygen therapeutics in human plasma by LC-electrospray ionization-MS/MS, Anal Chem 75(14) (2003) 3287-93.

[29] T.H. Shaffer, R. Foust, 3rd, M.R. Wolfson, T.F. Miller, Jr., Analysis of perfluorochemical elimination from the respiratory system, J Appl Physiol (1985) 83(3) (1997) 1033-40.

[30] M. Audran, M.P. Krafft, J. De Ceaurriz, J.C. Maturin, M.T. Sicart, B. Marion, G. Bougard, F. Bressolle, Determination of perfluorodecalin and perfluoro-N-methylcyclohexylpiperidine in rat blood by gas chromatography-mass spectrometry, J Chromatogr B Biomed Sci Appl 745(2) (2000) 333-43.

[31] J.R. Storry, F.B. Clausen, L. Castilho, Q. Chen, G. Daniels, G. Denomme, W.A. Flegel, C. Gassner, M. de Haas, C. Hyland, J. Yanli, M. Keller, C. Lomas-Francis, N. Nogues, M.L. Olsson, T. Peyrard, E. van der Schoot, Y. Tani, N. Thornton, F. Wagner, C. Weinstock, S. Wendel, C. Westhoff, V. Yahalom, International Society of Blood Transfusion Working Party on Red Cell Immunogenetics and Blood Group Terminology: Report of the Dubai, Copenhagen and Toronto meetings, Vox Sang 114(1) (2019) 95-102.

[32] M. Nelson, H. Popp, K. Sharpe, M. Ashenden, Proof of homologous blood transfusion through quantification of blood group antigens, Haematologica 88(11) (2003) 1284-95.

[33] T. Pottgiesser, M. Umhau, C. Ahlgrim, S. Ruthardt, K. Roecker, Y.O. Schumacher, Hb mass measurement suitable to screen for illicit autologous blood transfusions, Med Sci Sports Exerc 39(10) (2007) 1748-56.

[34] C.J. Gore, P.C. Bourdon, S.M. Woolford, L.M. Ostler, A. Eastwood, G.C. Scroop, Time and sample site dependency of the optimized co-rebreathing method, Med Sci Sports Exerc 38(6) (2006) 1187-93.

[35] T. Pottgiesser, T. Echteler, P.E. Sottas, M. Umhau, Y.O. Schumacher, Hemoglobin mass and biological passport for the detection of autologous blood doping, Med Sci Sports Exerc 44(5) (2012) 835-43.

[36] N. Leuenberger, L. Barras, R. Nicoli, N. Robinson, N. Baume, N. Lion, S. Barelli, J.D. Tissot, M. Saugy, Urinary di-(2-ethylhexyl) phthalate metabolites for detecting transfusion of autologous blood stored in plasticizer-free bags, Transfusion 56(3) (2016) 571-8.

[37] N. Monfort, R. Ventura, P. Platen, T. Hinrichs, K. Brixius, W. Schanzer, M. Thevis, H. Geyer, J. Segura, Plasticizers excreted in urine: indication of autologous blood transfusion in sports, Transfusion 52(3) (2012) 647-57.

[38] N. Leuenberger, L. Barras, R. Nicoli, N. Robinson, N. Baume, N. Lion, S. Barelli, J.D. Tissot, M. Saugy, Hepcidin as a new biomarker for detecting autologous blood transfusion, Am J Hematol 91(5) (2016) 467-72.

[39] H.D. Cox, G.D. Miller, A. Lai, D. Cushman, D. Eichner, Detection of autologous blood transfusions using a novel dried blood spot method, Drug Test Anal 9(11-12) (2017) 1713-1720.

[40] T. Pottgiesser, P.E. Sottas, T. Echteler, N. Robinson, M. Umhau, Y.O. Schumacher, Detection of autologous blood doping with adaptively evaluated biomarkers of doping: a longitudinal blinded study, Transfusion 51(8) (2011) 1707-15.

[41] R. Damsgaard, T. Munch, J. Morkeberg, S.P. Mortensen, J. Gonzalez-Alonso, Effects of blood withdrawal and reinfusion on biomarkers of erythropoiesis in humans: Implications for anti-doping strategies, Haematologica 91(7) (2006) 1006-8.

[42] S. Iljukov, S. Bermon, Y.O. Schumacher, Application of the Athlete's Performance Passport for Doping Control: A Case Report, Front Physiol 9 (2018) 280.

[43] L. Malcovati, C. Pascutto, M. Cazzola, Hematologic passport for athletes competing in endurance sports: a feasibility study, Haematologica 88(5) (2003) 570-581.

[44] J. Morkeberg, K. Sharpe, B. Belhage, R. Damsgaard, W. Schmidt, N. Prommer, C.J. Gore, M.J. Ashenden, Detecting autologous blood transfusions: a comparison of three passport approaches and four blood markers, Scand J Med Sci Sports 21(2) (2011) 235-43.

[45] P.E. Sottas, N. Robinson, O. Rabin, M. Saugy, The athlete biological passport, Clin Chem 57(7) (2011) 969-76.

[46] V. Pialoux, R. Mounier, J.V. Brugniaux, Hemoglobin and hematocrit are not such good candidates to detect autologous blood doping, Int J Hematol 89(5) (2009) 714-5.

[47] Cyclingnews, Operation Aderlass doctor Mark Schmidt jailed for almost five years, 2021. https://www.cyclingnews.com/news/operation-aderlass-doctor-mark-schmidt-jailed-for-almost-five-years/. (Accessed 16.05.2021.

[48] A.A. Monte, V. Vasiliou, K.J. Heard, Omics Screening for Pharmaceutical Efficacy and Safety in Clinical Practice, J Pharmacogenomics Pharmacoproteomics S5 (2012).

[49] J. Durussel, D.W. Haile, K. Mooses, E. Daskalaki, W. Beattie, M. Mooses, W. Mekonen, N. Ongaro, E. Anjila, R.K. Patel, N. Padmanabhan, M.W. McBride, J.D. McClure, Y.P. Pitsiladis, Blood transcriptional signature of recombinant human erythropoietin administration and implications for antidoping strategies, Physiol Genomics 48(3) (2016) 202-9.

[50] T. Pottgiesser, Y.O. Schumacher, H. Funke, K. Rennert, M.W. Baumstark, K. Neunuebel, S. Mosig, Gene expression in the detection of autologous blood transfusion in sports--a pilot study, Vox Sang 96(4) (2009) 333-6.

[51] N. Leuenberger, N. Jan, S. Pradervand, N. Robinson, M. Saugy, Circulating microRNAs as long-term biomarkers for the detection of erythropoiesis-stimulating agent abuse, Drug Test Anal 3(11-12) (2011) 771-6.

[52] J. Gasparello, N. Lamberti, C. Papi, I. Lampronti, L.C. Cosenza, E. Fabbri, N. Bianchi, C. Zambon, F. Dalla Corte, M. Govoni, R. Reverberi, F. Manfredini, R. Gambari, A. Finotti, Altered erythroid-related miRNA levels as a possible novel biomarker for detection of autologous blood transfusion misuse in sport, Transfusion 59(8) (2019) 2709-2721.

[53] N. Leuenberger, Y.O. Schumacher, S. Pradervand, T. Sander, M. Saugy, T. Pottgiesser, Circulating microRNAs as biomarkers for detection of autologous blood transfusion, PLoS One 8(6) (2013) e66309.

[54] J.F. Doss, D.L. Corcoran, D.D. Jima, M.J. Telen, S.S. Dave, J.T. Chi, A comprehensive joint analysis of the long and short RNA transcriptomes of human erythrocytes, BMC Genomics 16 (2015) 952.

[55] S.Y. Chen, Y. Wang, M.J. Telen, J.T. Chi, The genomic analysis of erythrocyte microRNA expression in sickle cell diseases, PLoS One 3(6) (2008) e2360.

[56] A. Haberberger, B. Kirchner, I. Riedmaier, R. Henschler, C. Wichmann, R. Buhmann, M.W. Pfaffl, Changes in the microRNA expression profile during blood storage, BMJ Open Sport Exerc Med 4(1) (2018) e000354.

[57] M. Kannan, C. Atreya, Differential profiling of human red blood cells during storage for 52 selected microRNAs, Transfusion 50(7) (2010) 1581-8.

[58] J.S. Mattick, Non-coding RNAs: the architects of eukaryotic complexity, EMBO Rep 2(11) (2001) 986-91.

[59] E. Kheir, T. Krude, Non-coding Y RNAs associate with early replicating euchromatin in concordance with the origin recognition complex, J Cell Sci 130(7) (2017) 1239-1250.

[60] T.B. Hansen, T.I. Jensen, B.H. Clausen, J.B. Bramsen, B. Finsen, C.K. Damgaard, J. Kjems, Natural RNA circles function as efficient microRNA sponges, Nature 495(7441) (2013) 384-8.

[61] J.S. Mattick, I.V. Makunin, Non-coding RNA, Hum Mol Genet 15 Spec No 1 (2006) R17-29.

[62] L. Statello, C.J. Guo, L.L. Chen, M. Huarte, Gene regulation by long non-coding RNAs and its biological functions, Nat Rev Mol Cell Biol 22(2) (2021) 96-118.

[63] C. Wang, H. Lin, Roles of piRNAs in transposon and pseudogene regulation of germline mRNAs and lncRNAs, Genome Biol 22(1) (2021) 27.

[64] A.F. Palazzo, E.S. Lee, Non-coding RNA: what is functional and what is junk?, Front Genet 6 (2015) 2.

[65] R.C. Friedman, K.K. Farh, C.B. Burge, D.P. Bartel, Most mammalian mRNAs are conserved targets of microRNAs, Genome Res 19(1) (2009) 92-105.

[66] B.P. Lewis, C.B. Burge, D.P. Bartel, Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets, Cell 120(1) (2005) 15-20.

[67] M. Selbach, B. Schwanhausser, N. Thierfelder, Z. Fang, R. Khanin, N. Rajewsky, Widespread changes in protein synthesis induced by microRNAs, Nature 455(7209) (2008) 58-63.

[68] S. Wu, S. Huang, J. Ding, Y. Zhao, L. Liang, T. Liu, R. Zhan, X. He, Multiple microRNAs modulate p21Cip1/Waf1 expression by directly targeting its 3' untranslated region, Oncogene 29(15) (2010) 2302-8.

[69] O. Plotnikova, A. Baranova, M. Skoblov, Comprehensive Analysis of Human microRNA-mRNA Interactome, Front Genet 10 (2019) 933.

[70] R.C. Lee, R.L. Feinbaum, V. Ambros, The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14, Cell 75(5) (1993) 843-54.

[71] A. Kozomara, M. Birgaoanu, S. Griffiths-Jones, miRBase: from microRNA sequences to function, Nucleic Acids Res 47(D1) (2019) D155-D162.

[72] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell 116(2) (2004) 281-97.

[73] A. Rodriguez, S. Griffiths-Jones, J.L. Ashurst, A. Bradley, Identification of mammalian microRNA host genes and transcription units, Genome Res 14(10A) (2004) 1902-10.

[74] T. Treiber, N. Treiber, G. Meister, Regulation of microRNA biogenesis and its crosstalk with other cellular pathways, Nat Rev Mol Cell Biol 20(1) (2019) 5-20.

[75] S.P. Kabekkodu, V. Shukla, V.K. Varghese, D.S. J, S. Chakrabarty, K. Satyamoorthy, Clustered miRNAs and their role in biological functions and diseases, Biol Rev Camb Philos Soc 93(4) (2018) 1955-1986.

[76] Z. Wang, H. Yao, S. Lin, X. Zhu, Z. Shen, G. Lu, W.S. Poon, D. Xie, M.C. Lin, H.F. Kung, Transcriptional and epigenetic regulation of human microRNAs, Cancer Lett 331(1) (2013) 1-10.

[77] O. Glaich, S. Parikh, R.E. Bell, K. Mekahel, M. Donyo, Y. Leader, R. Shayevitch, D. Sheinboim, S. Yannai, D. Hollander, Z. Melamed, G. Lev-Maor, G. Ast, C. Levy, DNA methylation directs microRNA biogenesis in mammalian cells, Nat Commun 10(1) (2019) 5657.

[78] Y. Lee, M. Kim, J. Han, K.H. Yeom, S. Lee, S.H. Baek, V.N. Kim, MicroRNA genes are transcribed by RNA polymerase II, EMBO J 23(20) (2004) 4051-60.

[79] Y. Lee, K. Jeon, J.T. Lee, S. Kim, V.N. Kim, MicroRNA maturation: stepwise processing and subcellular localization, EMBO J 21(17) (2002) 4663-70.

[80] G.M. Borchert, W. Lanier, B.L. Davidson, RNA polymerase III transcribes human microRNAs, Nat Struct Mol Biol 13(12) (2006) 1097-101.

[81] Y. Lee, C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim, V.N. Kim, The nuclear RNase III Drosha initiates microRNA processing, Nature 425(6956) (2003) 415-9.

[82] R.I. Gregory, K.P. Yan, G. Amuthan, T. Chendrimada, B. Doratotaj, N. Cooch, R. Shiekhattar, The Microprocessor complex mediates the genesis of microRNAs, Nature 432(7014) (2004) 235-40.

[83] E. Lund, S. Guttinger, A. Calado, J.E. Dahlberg, U. Kutay, Nuclear export of microRNA precursors, Science 303(5654) (2004) 95-8.

[84] T.P. Chendrimada, R.I. Gregory, E. Kumaraswamy, J. Norman, N. Cooch, K. Nishikura, R. Shiekhattar, TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing, Nature 436(7051) (2005) 740-4.

[85] C. Matranga, Y. Tomari, C. Shin, D.P. Bartel, P.D. Zamore, Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes, Cell 123(4) (2005) 607-20.

[86] Y.K. Kim, B. Kim, V.N. Kim, Re-evaluation of the roles of DROSHA, Export in 5, and DICER in microRNA biogenesis, Proc Natl Acad Sci U S A 113(13) (2016) E1881-9.

[87] J. Winter, S. Jung, S. Keller, R.I. Gregory, S. Diederichs, Many roads to maturity: microRNA biogenesis pathways and their regulation, Nat Cell Biol 11(3) (2009) 228-34.

[88] P.B. Kwak, Y. Tomari, The N domain of Argonaute drives duplex unwinding during RISC assembly, Nat Struct Mol Biol 19(2) (2012) 145-51.

[89] S. Iwasaki, M. Kobayashi, M. Yoda, Y. Sakaguchi, S. Katsuma, T. Suzuki, Y. Tomari, Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes, Mol Cell 39(2) (2010) 292-9.

[90] D.S. Schwarz, G. Hutvagner, T. Du, Z. Xu, N. Aronin, P.D. Zamore, Asymmetry in the assembly of the RNAi enzyme complex, Cell 115(2) (2003) 199-208.

[91] S.C. Li, Y.L. Liao, M.R. Ho, K.W. Tsai, C.H. Lai, W.C. Lin, miRNA arm selection and isomiR distribution in gastric cancer, BMC Genomics 13 Suppl 1 (2012) S13.

[92] S.C. Li, W.C. Chan, M.R. Ho, K.W. Tsai, L.Y. Hu, C.H. Lai, C.N. Hsu, P.P. Hwang, W.C. Lin, Discovery and characterization of medaka miRNA genes by next generation sequencing platform, BMC Genomics 11 Suppl 4 (2010) S8.

[93] S. Griffiths-Jones, J.H. Hui, A. Marco, M. Ronshaugen, MicroRNA evolution by arm switching, EMBO Rep 12(2) (2011) 172-7.

[94] D.P. Bartel, MicroRNAs: target recognition and regulatory functions, Cell 136(2) (2009) 215-33.

[95] B.P. Lewis, I.H. Shih, M.W. Jones-Rhoades, D.P. Bartel, C.B. Burge, Prediction of mammalian microRNA targets, Cell 115(7) (2003) 787-98.

[96] A. Grimson, K.K. Farh, W.K. Johnston, P. Garrett-Engele, L.P. Lim, D.P. Bartel, MicroRNA targeting specificity in mammals: determinants beyond seed pairing, Mol Cell 27(1) (2007) 91-105.

[97] J.P. Broughton, M.T. Lovci, J.L. Huang, G.W. Yeo, A.E. Pasquinelli, Pairing beyond the Seed Supports MicroRNA Targeting Specificity, Mol Cell 64(2) (2016) 320-333.

[98] H. Guo, N.T. Ingolia, J.S. Weissman, D.P. Bartel, Mammalian microRNAs predominantly act to decrease target mRNA levels, Nature 466(7308) (2010) 835-40.

[99] G. Hutvagner, P.D. Zamore, A microRNA in a multiple-turnover RNAi enzyme complex, Science 297(5589) (2002) 2056-60.

[100] S.N. Bhattacharyya, R. Habermacher, U. Martine, E.I. Closs, W. Filipowicz, Relief of microRNA-mediated translational repression in human cells subjected to stress, Cell 125(6) (2006) 1111-24.

[101] B. Wang, A. Yanez, C.D. Novina, MicroRNA-repressed mRNAs contain 40S but not 60S components, Proc Natl Acad Sci U S A 105(14) (2008) 5343-8.

[102] G. Mathonnet, M.R. Fabian, Y.V. Svitkin, A. Parsyan, L. Huck, T. Murata, S. Biffo, W.C. Merrick, E. Darzynkiewicz, R.S. Pillai, W. Filipowicz, T.F. Duchaine, N. Sonenberg, MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F, Science 317(5845) (2007) 1764-7.

[103] R. Thermann, M.W. Hentze, Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation, Nature 447(7146) (2007) 875-8.

[104] C.P. Petersen, M.E. Bordeleau, J. Pelletier, P.A. Sharp, Short RNAs repress translation after initiation in mammalian cells, Mol Cell 21(4) (2006) 533-42.

[105] S. Nottrott, M.J. Simard, J.D. Richter, Human let-7a miRNA blocks protein production on actively translating polyribosomes, Nat Struct Mol Biol 13(12) (2006) 1108-14.

[106] G. Meister, M. Landthaler, A. Patkaniowska, Y. Dorsett, G. Teng, T. Tuschl, Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs, Mol Cell 15(2) (2004) 185-97.

[107] I. Behm-Ansmant, J. Rehwinkel, T. Doerks, A. Stark, P. Bork, E. Izaurralde, mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes, Genes Dev 20(14) (2006) 1885-98.

[108] M.R. Fabian, G. Mathonnet, T. Sundermeier, H. Mathys, J.T. Zipprich, Y.V. Svitkin, F. Rivas, M. Jinek, J. Wohlschlegel, J.A. Doudna, C.Y. Chen, A.B. Shyu, J.R. Yates, 3rd, G.J. Hannon, W. Filipowicz, T.F. Duchaine, N. Sonenberg, Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation, Mol Cell 35(6) (2009) 868-80.

[109] T.H. Beilharz, D.T. Humphreys, J.L. Clancy, R. Thermann, D.I. Martin, M.W. Hentze, T. Preiss, microRNA-mediated messenger RNA deadenylation contributes to translational repression in mammalian cells, PLoS One 4(8) (2009) e6783.

[110] U. Sheth, R. Parker, Decapping and decay of messenger RNA occur in cytoplasmic processing bodies, Science 300(5620) (2003) 805-8.

[111] S. Vasudevan, Y. Tong, J.A. Steitz, Switching from repression to activation: microRNAs can up-regulate translation, Science 318(5858) (2007) 1931-4.

[112] U.A. Orom, F.C. Nielsen, A.H. Lund, MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation, Mol Cell 30(4) (2008) 460-71.

[113] B. Kim, K. Jeong, V.N. Kim, Genome-wide Mapping of DROSHA Cleavage Sites on Primary MicroRNAs and Noncanonical Substrates, Mol Cell 66(2) (2017) 258-269 e5.

[114] H. Wu, C. Ye, D. Ramirez, N. Manjunath, Alternative processing of primary microRNA transcripts by Drosha generates 5' end variation of mature microRNA, PLoS One 4(10) (2009) e7566.

[115] R. Fukunaga, B.W. Han, J.H. Hung, J. Xu, Z. Weng, P.D. Zamore, Dicer partner proteins tune the length of mature miRNAs in flies and mammals, Cell 151(3) (2012) 533-46.

[116] T. Katoh, Y. Sakaguchi, K. Miyauchi, T. Suzuki, S. Kashiwabara, T. Baba, T. Suzuki, Selective stabilization of mammalian microRNAs by 3' adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2, Genes Dev 23(4) (2009) 433-8.

[117] G.C. Tan, E. Chan, A. Molnar, R. Sarkar, D. Alexieva, I.M. Isa, S. Robinson, S. Zhang, P. Ellis, C.F. Langford, P.V. Guillot, A. Chandrashekran, N.M. Fisk, L. Castellano, G. Meister, R.M. Winston, W. Cui, D. Baulcombe, N.J. Dibb, 5' isomiR variation is of functional and evolutionary importance, Nucleic Acids Res 42(14) (2014) 9424-35.

[118] W. Yang, T.P. Chendrimada, Q. Wang, M. Higuchi, P.H. Seeburg, R. Shiekhattar, K. Nishikura, Modulation of microRNA processing and expression through RNA editing by ADAR deaminases, Nat Struct Mol Biol 13(1) (2006) 13-21.

[119] N. Cloonan, S. Wani, Q. Xu, J. Gu, K. Lea, S. Heater, C. Barbacioru, A.L. Steptoe, H.C. Martin, E. Nourbakhsh, K. Krishnan, B. Gardiner, X. Wang, K. Nones, J.A. Steen, N.A. Matigian, D.L. Wood, K.S. Kassahn, N. Waddell, J. Shepherd, C. Lee, J. Ichikawa, K. McKernan, K. Bramlett, S. Kuersten, S.M. Grimmond, MicroRNAs and their isomiRs function cooperatively to target common biological pathways, Genome Biol 12(12) (2011) R126.

[120] K.C. Vickers, B.T. Palmisano, B.M. Shoucri, R.D. Shamburek, A.T. Remaley, MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins, Nat Cell Biol 13(4) (2011) 423-33.

[121] K. Wang, S. Zhang, J. Weber, D. Baxter, D.J. Galas, Export of microRNAs and microRNA-protective protein by mammalian cells, Nucleic Acids Res 38(20) (2010) 7248-59.

[122] J.D. Arroyo, J.R. Chevillet, E.M. Kroh, I.K. Ruf, C.C. Pritchard, D.F. Gibson, P.S. Mitchell, C.F. Bennett, E.L. Pogosova-Agadjanyan, D.L. Stirewalt, J.F. Tait, M. Tewari, Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma, Proc Natl Acad Sci U S A 108(12) (2011) 5003-8.

[123] M. Fabbri, A. Paone, F. Calore, R. Galli, C.M. Croce, A new role for microRNAs, as ligands of Toll-like receptors, RNA Biol 10(2) (2013) 169-74.

[124] D.J. Gibbings, C. Ciaudo, M. Erhardt, O. Voinnet, Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity, Nat Cell Biol 11(9) (2009) 1143-9.

[125] H. Valadi, K. Ekstrom, A. Bossios, M. Sjostrand, J.J. Lee, J.O. Lotvall, Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells, Nat Cell Biol 9(6) (2007) 654-9.

[126] P.R. Matias-Garcia, R. Wilson, V. Mussack, E. Reischl, M. Waldenberger, C. Gieger, G. Anton, A. Peters, A. Kuehn-Steven, Impact of long-term storage and freeze-thawing on eight circulating microRNAs in plasma samples, PLoS One 15(1) (2020) e0227648.

[127] X. Chen, Y. Ba, L. Ma, X. Cai, Y. Yin, K. Wang, J. Guo, Y. Zhang, J. Chen, X. Guo, Q. Li, X. Li, W. Wang, Y. Zhang, J. Wang, X. Jiang, Y. Xiang, C. Xu, P. Zheng, J. Zhang, R. Li, H. Zhang, X. Shang, T. Gong, G. Ning, J. Wang, K. Zen, J. Zhang, C.Y. Zhang, Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases, Cell Res 18(10) (2008) 997-1006.

[128] F. Fricke, V. Mussack, D. Buschmann, I. Hausser, M.W. Pfaffl, J. Kopitz, J. Gebert, TGFBR2dependent alterations of microRNA profiles in extracellular vesicles and parental colorectal cancer cells, Int J Oncol 55(4) (2019) 925-937.

[129] E. Hulstaert, A. Morlion, F. Avila Cobos, K. Verniers, J. Nuytens, E. Vanden Eynde, N. Yigit, J. Anckaert, A. Geerts, P. Hindryckx, P. Jacques, G. Brusselle, K.R. Bracke, T. Maes, T. Malfait, T. Derveaux, V. Ninclaus, C. Van Cauwenbergh, K. Roelens, E. Roets, D. Hemelsoet, K. Tilleman, L. Brochez, S. Kuersten, L.M. Simon, S. Karg, A. Kautzky-Willers, M. Leutner, C. Nohammer, O. Slaby, R.W. Prins, J. Koster, S. Lefever, G.P. Schroth, J. Vandesompele, P. Mestdagh, Charting Extracellular Transcriptomes in The Human Biofluid RNA Atlas, Cell Rep 33(13) (2020) 108552.

[130] C.E. Condrat, D.C. Thompson, M.G. Barbu, O.L. Bugnar, A. Boboc, D. Cretoiu, N. Suciu, S.M. Cretoiu, S.C. Voinea, miRNAs as Biomarkers in Disease: Latest Findings Regarding Their Role in Diagnosis and Prognosis, Cells 9(2) (2020).

[131] Y. Liu, H. He, Z.X. Xiao, A. Ji, J. Ye, Q. Sun, Y. Cao, A systematic analysis of miRNA markers and classification algorithms for forensic body fluid identification, Brief Bioinform (2020).

[132] R. Rupaimoole, F.J. Slack, MicroRNA therapeutics: towards a new era for the management of cancer and other diseases, Nat Rev Drug Discov 16(3) (2017) 203-222.

[133] G. van Niel, G. D'Angelo, G. Raposo, Shedding light on the cell biology of extracellular vesicles, Nat Rev Mol Cell Biol 19(4) (2018) 213-228.

[134] K. O'Brien, K. Breyne, S. Ughetto, L.C. Laurent, X.O. Breakefield, RNA delivery by extracellular vesicles in mammalian cells and its applications, Nat Rev Mol Cell Biol 21(10) (2020) 585-606.

[135] A. Jeyaram, S.M. Jay, Preservation and Storage Stability of Extracellular Vesicles for Therapeutic Applications, AAPS J 20(1) (2017) 1.

[136] C. Lasser, V.S. Alikhani, K. Ekstrom, M. Eldh, P.T. Paredes, A. Bossios, M. Sjostrand, S. Gabrielsson, J. Lotvall, H. Valadi, Human saliva, plasma and breast milk exosomes contain RNA: uptake by macrophages, J Transl Med 9 (2011) 9.

[137] C. Yang, W.B. Guo, W.S. Zhang, J. Bian, J.K. Yang, T. Qi, C.Y. Wang, C.D. Liu, [Extraction and identification of semen-derived exosomes using PEG6000], Nan Fang Yi Ke Da Xue Xue Bao 36(11) (2016) 1531-1535.

[138] P. Peng, Y. Yan, S. Keng, Exosomes in the ascites of ovarian cancer patients: origin and effects on anti-tumor immunity, Oncol Rep 25(3) (2011) 749-62.

[139] E. Prieto-Fernandez, A.M. Aransay, F. Royo, E. Gonzalez, J.J. Lozano, B. Santos-Zorrozua, N. Macias-Camara, M. Gonzalez, R.P. Garay, J. Benito, A. Garcia-Orad, J.M. Falcon-Perez, A Comprehensive Study of Vesicular and Non-Vesicular miRNAs from a Volume of Cerebrospinal Fluid Compatible with Clinical Practice, Theranostics 9(16) (2019) 4567-4579.

[140] V. Mussack, G. Wittmann, M.W. Pfaffl, Comparing small urinary extracellular vesicle purification methods with a view to RNA sequencing-Enabling robust and non-invasive biomarker research, Biomol Detect Quantif 17 (2019) 100089.

[141] C. Théry, K.W. Witwer, E. Aikawa, M.J. Alcaraz, J.D. Anderson, R. Andriantsitohaina, A. Antoniou, T. Arab, F. Archer, G.K. Atkin-Smith, D.C. Ayre, J.-M. Bach, D. Bachurski, H. Baharvand, L. Balaj, S. Baldacchino, N.N. Bauer, A.A. Baxter, M. Bebawy, C. Beckham, A. Bedina Zavec, A. Benmoussa, A.C. Berardi, P. Bergese, E. Bielska, C. Blenkiron, S. Bobis-Wozowicz, E. Boilard, W. Boireau, A. Bongiovanni, F.E. Borràs, S. Bosch, C.M. Boulanger, X. Breakefield, A.M. Breglio, M.Á. Brennan, D.R. Brigstock, A. Brisson, M.L.D. Broekman, J.F. Bromberg, P. Bryl-Górecka, S. Buch, A.H. Buck, D. Burger, S. Busatto, D. Buschmann, B. Bussolati, E.I. Buzás, J.B. Byrd, G. Camussi, D.R.F. Carter, S. Caruso, L.W. Chamley, Y.-T. Chang, A.D. Chaudhuri, C. Chen, S. Chen, L. Cheng, A.R. Chin, A. Clayton, S.P. Clerici, A. Cocks, E. Cocucci, R.J. Coffey, A. Cordeiro-da-Silva, Y. Couch, F.A.W. Coumans, B. Coyle, R. Crescitelli, M.F. Criado, C. D'Souza-Schorey, S. Das, P. de Candia, E.F. De Santana, O. De Wever, H.A. del Portillo, T. Demaret, S. Deville, A. Devitt, B. Dhondt, D. Di Vizio, L.C. Dieterich, V. Dolo, A.P. Dominguez Rubio, M. Dominici, M.R. Dourado, T.A.P. Driedonks, F.V. Duarte, H.M. Duncan, R.M. Eichenberger, K. Ekström, S. El Andaloussi, C. Elie-Caille, U. Erdbrügger, J.M. Falcón-Pérez, F. Fatima, J.E. Fish, M. Flores-Bellver, A. Försönits, A. Frelet-Barrand, F. Fricke, G. Fuhrmann, S. Gabrielsson, A. Gámez-Valero, C. Gardiner, K. Gärtner, R. Gaudin, Y.S. Gho, B. Giebel, C. Gilbert, M. Gimona, I. Giusti, D.C.I. Goberdhan, A. Görgens, S.M. Gorski, D.W. Greening, J.C. Gross, A. Gualerzi, G.N. Gupta, D. Gustafson, A. Handberg, R.A. Haraszti, P. Harrison, H. Hegyesi, A. Hendrix, A.F. Hill, F.H. Hochberg, K.F. Hoffmann, B. Holder, H. Holthofer, B. Hosseinkhani, G. Hu, Y. Huang, V. Huber, S. Hunt, A.G.-E. Ibrahim, T. Ikezu, J.M. Inal, M. Isin, A. Ivanova, H.K. Jackson, S. Jacobsen, S.M. Jay, M. Jayachandran, G. Jenster, L. Jiang, S.M. Johnson, J.C. Jones, A. Jong, T. Jovanovic-Talisman, S. Jung, R. Kalluri, S.-i. Kano, S. Kaur, Y. Kawamura, E.T. Keller, D. Khamari, E. Khomyakova, A. Khvorova, P. Kierulf, K.P. Kim, T. Kislinger, M. Klingeborn, D.J. Klinke, M. Kornek, M.M. Kosanović, Á.F. Kovács, E.-M. Krämer-Albers, S. Krasemann, M. Krause, I.V. Kurochkin, G.D. Kusuma, S. Kuypers, S. Laitinen, S.M. Langevin, L.R. Languino, J. Lannigan, C. Lässer, L.C. Laurent, G. Lavieu, E. Lázaro-Ibáñez, S. Le Lay, M.-S. Lee, Y.X.F. Lee, D.S. Lemos, M. Lenassi, A. Leszczynska, I.T.S. Li, K. Liao, S.F. Libregts, E. Ligeti, R. Lim, S.K. Lim, A. Linē, K. Linnemannstöns, A. Llorente, C.A. Lombard, M.J. Lorenowicz, Á.M. Lörincz, J. Lötvall, J. Lovett, M.C. Lowry, X. Loyer, Q. Lu, B. Lukomska, T.R. Lunavat, S.L.N. Maas, H. Malhi, A. Marcilla, J. Mariani, J. Mariscal, E.S. Martens-Uzunova, L. Martin-Jaular, M.C. Martinez, V.R. Martins, M. Mathieu, S. Mathivanan, M. Maugeri, L.K. McGinnis, M.J. McVey, D.G. Meckes, K.L. Meehan, I. Mertens, V.R. Minciacchi, A. Möller, M. Møller Jørgensen, A. Morales-Kastresana, J. Morhayim, F. Mullier, M. Muraca, L. Musante, V. Mussack, D.C. Muth, K.H. Myburgh, T. Najrana, M. Nawaz, I. Nazarenko, P. Nejsum, C. Neri, T. Neri, R. Nieuwland, L. Nimrichter, J.P. Nolan, E.N.M. Nolte-'t Hoen, N.N. Hooten, L. O'Driscoll, T. O'Grady, A. O'Loghlen, T. Ochiya, M. Olivier, A. Ortiz, L.A. Ortiz, X. Osteikoetxea, O. Ostegaard, M. Ostrowski, J. Park, D.M. Pegtel, H. Peinado, F. Perut, M.W. Pfaffl, D.G. Phinney, B.C.H. Pieters, R.C. Pink, D.S. Pisetsky, E. Pogge von Strandmann, I. Polakovicova, I.K.H. Poon, B.H. Powell, I. Prada, L. Pulliam, P. Quesenberry, A. Radeghieri, R.L. Raffai, S. Raimondo, J. Rak, M.I. Ramirez, G. Raposo, M.S. Rayyan, N. Regev-Rudzki, F.L. Ricklefs, P.D. Robbins, D.D. Roberts, S.C. Rodrigues, E. Rohde, S. Rome, K.M.A. Rouschop, A. Rughetti, A.E. Russell, P. Saá, S. Sahoo, E. Salas-Huenuleo, C. Sánchez, J.A. Saugstad, M.J. Saul, R.M. Schiffelers, R. Schneider, T.H. Schøyen, A. Scott, E. Shahaj, S. Sharma, O. Shatnyeva, F. Shekari, G.V. Shelke, A.K. Shetty, K. Shiba, P.R.M. Siljander, A.M. Silva, A. Skowronek, O.L. Snyder, R.P. Soares, B.W. Sódar, C. Soekmadji, J. Sotillo, P.D. Stahl, W. Stoorvogel, S.L. Stott, E.F. Strasser, S. Swift, H. Tahara, M. Tewari, K. Timms, S. Tiwari, R. Tixeira, M. Tkach, W.S. Toh, R. Tomasini, A.C. Torrecilhas, J.P. Tosar, V. Toxavidis, L. Urbanelli, P. Vader, B.W.M. van Balkom, S.G. van der Grein, J. Van Deun, M.J.C. van Herwijnen, K.

Van Keuren-Jensen, G. van Niel, M.E. van Royen, A.J. van Wijnen, M.H. Vasconcelos, I.J. Vechetti, T.D. Veit, L.J. Vella, É. Velot, F.J. Verweij, B. Vestad, J.L. Viñas, T. Visnovitz, K.V. Vukman, J. Wahlgren, D.C. Watson, M.H.M. Wauben, A. Weaver, J.P. Webber, V. Weber, A.M. Wehman, D.J. Weiss, J.A. Welsh, S. Wendt, A.M. Wheelock, Z. Wiener, L. Witte, J. Wolfram, A. Xagorari, P. Xander, J. Xu, X. Yan, M. Yáñez-Mó, H. Yin, Y. Yuana, V. Zappulli, J. Zarubova, V. Žėkas, J.-y. Zhang, Z. Zhao, L. Zheng, A.R. Zheutlin, A.M. Zickler, P. Zimmermann, A.M. Zivkovic, D. Zocco, E.K. Zuba-Surma, Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines, Journal of Extracellular Vesicles 8(1) (2019) 1535750.

[142] J. Van Deun, P. Mestdagh, P. Agostinis, O. Akay, S. Anand, J. Anckaert, Z.A. Martinez, T. Baetens, E. Beghein, L. Bertier, G. Berx, J. Boere, S. Boukouris, M. Bremer, D. Buschmann, J.B. Byrd, C. Casert, L. Cheng, A. Cmoch, D. Daveloose, E. De Smedt, S. Demirsoy, V. Depoorter, B. Dhondt, T.A. Driedonks, A. Dudek, A. Elsharawy, I. Floris, A.D. Foers, K. Gartner, A.D. Garg, E. Geeurickx, J. Gettemans, F. Ghazavi, B. Giebel, T.G. Kormelink, G. Hancock, H. Helsmoortel, A.F. Hill, V. Hyenne, H. Kalra, D. Kim, J. Kowal, S. Kraemer, P. Leidinger, C. Leonelli, Y. Liang, L. Lippens, S. Liu, A. Lo Cicero, S. Martin, S. Mathivanan, P. Mathiyalagan, T. Matusek, G. Milani, M. Monguio-Tortajada, L.M. Mus, D.C. Muth, A. Nemeth, E.N. Nolte-'t Hoen, L. O'Driscoll, R. Palmulli, M.W. Pfaffl, B. Primdal-Bengtson, E. Romano, Q. Rousseau, S. Sahoo, N. Sampaio, M. Samuel, B. Scicluna, B. Soen, A. Steels, J.V. Swinnen, M. Takatalo, S. Thaminy, C. Thery, J. Tulkens, I. Van Audenhove, S. van der Grein, A. Van Goethem, M.J. van Herwijnen, G. Van Niel, N. Van Roy, A.R. Van Vliet, N. Vandamme, S. Vanhauwaert, G. Vergauwen, F. Verweij, A. Wallaert, M. Wauben, K.W. Witwer, M.I. Zonneveld, O. De Wever, J. Vandesompele, A. Hendrix, EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research, Nat Methods 14(3) (2017) 228-232.

[143] J. Lotvall, A.F. Hill, F. Hochberg, E.I. Buzas, D. Di Vizio, C. Gardiner, Y.S. Gho, I.V. Kurochkin, S. Mathivanan, P. Quesenberry, S. Sahoo, H. Tahara, M.H. Wauben, K.W. Witwer, C. Thery, Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles, J Extracell Vesicles 3 (2014) 26913.

[144] D. Buschmann, V. Mussack, J.B. Byrd, Separation, characterization, and standardization of extracellular vesicles for drug delivery applications, Adv Drug Deliv Rev 174 (2021) 348-368.

[145] R.M. Johnstone, M. Adam, J.R. Hammond, L. Orr, C. Turbide, Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes), J Biol Chem 262(19) (1987) 9412-20.

[146] T. Wollert, J.H. Hurley, Molecular mechanism of multivesicular body biogenesis by ESCRT complexes, Nature 464(7290) (2010) 864-9.

[147] C.E. Futter, A. Pearse, L.J. Hewlett, C.R. Hopkins, Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes, J Cell Biol 132(6) (1996) 1011-23.

[148] K. Trajkovic, C. Hsu, S. Chiantia, L. Rajendran, D. Wenzel, F. Wieland, P. Schwille, B. Brugger, M. Simons, Ceramide triggers budding of exosome vesicles into multivesicular endosomes, Science 319(5867) (2008) 1244-7.

[149] G. van Niel, S. Charrin, S. Simoes, M. Romao, L. Rochin, P. Saftig, M.S. Marks, E. Rubinstein, G. Raposo, The tetraspanin CD63 regulates ESCRT-independent and -dependent endosomal sorting during melanogenesis, Dev Cell 21(4) (2011) 708-21.

[150] M.F. Baietti, Z. Zhang, E. Mortier, A. Melchior, G. Degeest, A. Geeraerts, Y. Ivarsson, F. Depoortere, C. Coomans, E. Vermeiren, P. Zimmermann, G. David, Syndecan-syntenin-ALIX regulates the biogenesis of exosomes, Nat Cell Biol 14(7) (2012) 677-85.

[151] A. Chairoungdua, D.L. Smith, P. Pochard, M. Hull, M.J. Caplan, Exosome release of beta-catenin: a novel mechanism that antagonizes Wnt signaling, J Cell Biol 190(6) (2010) 1079-91.

[152] D. Perez-Hernandez, C. Gutierrez-Vazquez, I. Jorge, S. Lopez-Martin, A. Ursa, F. Sanchez-Madrid, J. Vazquez, M. Yanez-Mo, The intracellular interactome of tetraspanin-enriched microdomains reveals their function as sorting machineries toward exosomes, J Biol Chem 288(17) (2013) 11649-61.

[153] C.M. Fader, D.G. Sanchez, M.B. Mestre, M.I. Colombo, TI-VAMP/VAMP7 and VAMP3/cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways, Biochim Biophys Acta 1793(12) (2009) 1901-16.

[154] C. Hsu, Y. Morohashi, S. Yoshimura, N. Manrique-Hoyos, S. Jung, M.A. Lauterbach, M. Bakhti, M. Gronborg, W. Mobius, J. Rhee, F.A. Barr, M. Simons, Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C, J Cell Biol 189(2) (2010) 223-32.

[155] M. Ostrowski, N.B. Carmo, S. Krumeich, I. Fanget, G. Raposo, A. Savina, C.F. Moita, K. Schauer, A.N. Hume, R.P. Freitas, B. Goud, P. Benaroch, N. Hacohen, M. Fukuda, C. Desnos, M.C. Seabra, F. Darchen, S. Amigorena, L.F. Moita, C. Thery, Rab27a and Rab27b control different steps of the exosome secretion pathway, Nat Cell Biol 12(1) (2010) 19-30; sup pp 1-13.

[156] E. Cocucci, G. Racchetti, J. Meldolesi, Shedding microvesicles: artefacts no more, Trends Cell Biol 19(2) (2009) 43-51.

[157] F. Bianco, C. Perrotta, L. Novellino, M. Francolini, L. Riganti, E. Menna, L. Saglietti, E.H. Schuchman, R. Furlan, E. Clementi, M. Matteoli, C. Verderio, Acid sphingomyelinase activity triggers microparticle release from glial cells, EMBO J 28(8) (2009) 1043-54.

[158] K. Menck, C. Sonmezer, T.S. Worst, M. Schulz, G.H. Dihazi, F. Streit, G. Erdmann, S. Kling, M. Boutros, C. Binder, J.C. Gross, Neutral sphingomyelinases control extracellular vesicles budding from the plasma membrane, J Extracell Vesicles 6(1) (2017) 1378056.

[159] V. Muralidharan-Chari, J. Clancy, C. Plou, M. Romao, P. Chavrier, G. Raposo, C. D'Souza-Schorey, ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles, Curr Biol 19(22) (2009) 1875-85.

[160] P. Comfurius, J.M. Senden, R.H. Tilly, A.J. Schroit, E.M. Bevers, R.F. Zwaal, Loss of membrane phospholipid asymmetry in platelets and red cells may be associated with calcium-induced shedding of plasma membrane and inhibition of aminophospholipid translocase, Biochimica et Biophysica Acta (BBA)-Biomembranes 1026(2) (1990) 153-160.

[161] J.F. Nabhan, R. Hu, R.S. Oh, S.N. Cohen, Q. Lu, Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein, Proc Natl Acad Sci U S A 109(11) (2012) 4146-51.

[162] J.F. Kerr, A.H. Wyllie, A.R. Currie, Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics, Br J Cancer 26(4) (1972) 239-57.

[163] T. Ihara, T. Yamamoto, M. Sugamata, H. Okumura, Y. Ueno, The process of ultrastructural changes from nuclei to apoptotic body, Virchows Arch 433(5) (1998) 443-7.

[164] G.K. Atkin-Smith, R. Tixeira, S. Paone, S. Mathivanan, C. Collins, M. Liem, K.J. Goodall, K.S. Ravichandran, M.D. Hulett, I.K. Poon, A novel mechanism of generating extracellular vesicles during apoptosis via a beads-on-a-string membrane structure, Nat Commun 6 (2015) 7439.

[165] S. Caruso, G.K. Atkin-Smith, A.A. Baxter, R. Tixeira, L. Jiang, D.C. Ozkocak, J.P. Santavanond, M.D. Hulett, P. Lock, T.K. Phan, I.K.H. Poon, Defining the role of cytoskeletal components in the formation of apoptopodia and apoptotic bodies during apoptosis, Apoptosis 24(11-12) (2019) 862-877.

[166] B.K. Thakur, H. Zhang, A. Becker, I. Matei, Y. Huang, B. Costa-Silva, Y. Zheng, A. Hoshino, H. Brazier, J. Xiang, C. Williams, R. Rodriguez-Barrueco, J.M. Silva, W. Zhang, S. Hearn, O. Elemento, N. Paknejad, K. Manova-Todorova, K. Welte, J. Bromberg, H. Peinado, D. Lyden, Double-stranded DNA in exosomes: a novel biomarker in cancer detection, Cell Res 24(6) (2014) 766-9.

[167] C. Villarroya-Beltri, C. Gutierrez-Vazquez, F. Sanchez-Cabo, D. Perez-Hernandez, J. Vazquez, N. Martin-Cofreces, D.J. Martinez-Herrera, A. Pascual-Montano, M. Mittelbrunn, F. Sanchez-Madrid, Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs, Nat Commun 4 (2013) 2980.

[168] A.E. Russell, A. Sneider, K.W. Witwer, P. Bergese, S.N. Bhattacharyya, A. Cocks, E. Cocucci, U. Erdbrugger, J.M. Falcon-Perez, D.W. Freeman, T.M. Gallagher, S. Hu, Y. Huang, S.M. Jay, S.I. Kano, G. Lavieu, A. Leszczynska, A.M. Llorente, Q. Lu, V. Mahairaki, D.C. Muth, N. Noren Hooten, M. Ostrowski, I. Prada, S. Sahoo, T.H. Schoyen, L. Sheng, D. Tesch, G. Van Niel, R.E. Vandenbroucke, F.J. Verweij, A.V. Villar, M. Wauben, A.M. Wehman, H. Yin, D.R.F. Carter, P. Vader, Biological membranes in EV biogenesis, stability, uptake, and cargo transfer: an ISEV position paper arising from the ISEV membranes and EVs workshop, J Extracell Vesicles 8(1) (2019) 1684862.

[169] H. Wei, Q. Chen, L. Lin, C. Sha, T. Li, Y. Liu, X. Yin, Y. Xu, L. Chen, W. Gao, Y. Li, X. Zhu, Regulation of exosome production and cargo sorting, Int J Biol Sci 17(1) (2021) 163-177.

[170] S. Mathivanan, C.J. Fahner, G.E. Reid, R.J. Simpson, ExoCarta 2012: database of exosomal proteins, RNA and lipids, Nucleic Acids Res 40(Database issue) (2012) D1241-4.

[171] F. Fricke, J. Lee, M. Michalak, U. Warnken, I. Hausser, M. Suarez-Carmona, N. Halama, M. Schnolzer, J. Kopitz, J. Gebert, TGFBR2-dependent alterations of exosomal cargo and functions in DNA mismatch repair-deficient HCT116 colorectal cancer cells, Cell Commun Signal 15(1) (2017) 14.

[172] J. Kowal, G. Arras, M. Colombo, M. Jouve, J.P. Morath, B. Primdal-Bengtson, F. Dingli, D. Loew, M. Tkach, C. Thery, Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes, Proc Natl Acad Sci U S A 113(8) (2016) E968-77.

[173] B.J. Goldie, M.D. Dun, M. Lin, N.D. Smith, N.M. Verrills, C.V. Dayas, M.J. Cairns, Activity-associated miRNA are packaged in Map1b-enriched exosomes released from depolarized neurons, Nucleic Acids Res 42(14) (2014) 9195-208.

[174] T. Kogure, I.K. Yan, W.L. Lin, T. Patel, Extracellular Vesicle-Mediated Transfer of a Novel Long Noncoding RNA TUC339: A Mechanism of Intercellular Signaling in Human Hepatocellular Cancer, Genes Cancer 4(7-8) (2013) 261-72.

[175] T. Vagner, C. Spinelli, V.R. Minciacchi, L. Balaj, M. Zandian, A. Conley, A. Zijlstra, M.R. Freeman, F. Demichelis, S. De, E.M. Posadas, H. Tanaka, D. Di Vizio, Large extracellular vesicles carry most of the tumour DNA circulating in prostate cancer patient plasma, J Extracell Vesicles 7(1) (2018) 1505403.

[176] K. Laulagnier, C. Motta, S. Hamdi, S. Roy, F. Fauvelle, J.F. Pageaux, T. Kobayashi, J.P. Salles, B. Perret, C. Bonnerot, M. Record, Mast cell- and dendritic cell-derived exosomes display a specific lipid composition and an unusual membrane organization, Biochem J 380(Pt 1) (2004) 161-71.

[177] R.A. Haraszti, M.C. Didiot, E. Sapp, J. Leszyk, S.A. Shaffer, H.E. Rockwell, F. Gao, N.R. Narain, M. DiFiglia, M.A. Kiebish, N. Aronin, A. Khvorova, High-resolution proteomic and lipidomic analysis of exosomes and microvesicles from different cell sources, J Extracell Vesicles 5 (2016) 32570.

[178] A. Hoshino, B. Costa-Silva, T.L. Shen, G. Rodrigues, A. Hashimoto, M. Tesic Mark, H. Molina, S. Kohsaka, A. Di Giannatale, S. Ceder, S. Singh, C. Williams, N. Soplop, K. Uryu, L. Pharmer, T. King, L. Bojmar, A.E. Davies, Y. Ararso, T. Zhang, H. Zhang, J. Hernandez, J.M. Weiss, V.D. Dumont-Cole, K. Kramer, L.H. Wexler, A. Narendran, G.K. Schwartz, J.H. Healey, P. Sandstrom, K.J. Labori, E.H. Kure, P.M. Grandgenett, M.A. Hollingsworth, M. de Sousa, S. Kaur, M. Jain, K. Mallya, S.K. Batra, W.R. Jarnagin, M.S. Brady, O. Fodstad, V. Muller, K. Pantel, A.J. Minn, M.J. Bissell, B.A. Garcia, Y. Kang, V.K. Rajasekhar, C.M. Ghajar, I. Matei, H. Peinado, J. Bromberg, D. Lyden, Tumour exosome integrins determine organotropic metastasis, Nature 527(7578) (2015) 329-35.

[179] A. Montecalvo, A.T. Larregina, W.J. Shufesky, D.B. Stolz, M.L. Sullivan, J.M. Karlsson, C.J. Baty, G.A. Gibson, G. Erdos, Z. Wang, J. Milosevic, O.A. Tkacheva, S.J. Divito, R. Jordan, J. Lyons-Weiler, S.C. Watkins, A.E. Morelli, Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes, Blood 119(3) (2012) 756-66.

[180] K. Matsumoto, T. Morisaki, H. Kuroki, M. Kubo, H. Onishi, K. Nakamura, C. Nakahara, H. Kuga, E. Baba, M. Nakamura, K. Hirata, M. Tanaka, M. Katano, Exosomes secreted from monocyte-derived dendritic cells support in vitro naive CD4+ T cell survival through NF-(kappa)B activation, Cell Immunol 231(1-2) (2004) 20-9.

[181] T. Tian, Y.L. Zhu, Y.Y. Zhou, G.F. Liang, Y.Y. Wang, F.H. Hu, Z.D. Xiao, Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery, J Biol Chem 289(32) (2014) 22258-67.

[182] D. Feng, W.L. Zhao, Y.Y. Ye, X.C. Bai, R.Q. Liu, L.F. Chang, Q. Zhou, S.F. Sui, Cellular internalization of exosomes occurs through phagocytosis, Traffic 11(5) (2010) 675-87.

[183] K.J. Svensson, H.C. Christianson, A. Wittrup, E. Bourseau-Guilmain, E. Lindqvist, L.M. Svensson, M. Morgelin, M. Belting, Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1, J Biol Chem 288(24) (2013) 17713-24.

[184] B.S. Joshi, M.A. de Beer, B.N.G. Giepmans, I.S. Zuhorn, Endocytosis of Extracellular Vesicles and Release of Their Cargo from Endosomes, ACS Nano 14(4) (2020) 4444-4455.

[185] R.J. Berckmans, A. Sturk, L.M. van Tienen, M.C. Schaap, R. Nieuwland, Cell-derived vesicles exposing coagulant tissue factor in saliva, Blood 117(11) (2011) 3172-80.

[186] O. Gasser, J.A. Schifferli, Activated polymorphonuclear neutrophils disseminate anti-inflammatory microparticles by ectocytosis, Blood 104(8) (2004) 2543-8.

[187] H.C. O'Neill, B.J. Quah, Exosomes secreted by bacterially infected macrophages are proinflammatory, Sci Signal 1(6) (2008) pe8.

[188] J. Skog, T. Wurdinger, S. van Rijn, D.H. Meijer, L. Gainche, M. Sena-Esteves, W.T. Curry, Jr., B.S. Carter, A.M. Krichevsky, X.O. Breakefield, Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers, Nat Cell Biol 10(12) (2008) 1470-6.

[189] D.D. Taylor, C. Gercel-Taylor, MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer, Gynecol Oncol 110(1) (2008) 13-21.

[190] S. Li, J. Wei, C. Zhang, X. Li, W. Meng, X. Mo, Q. Zhang, Q. Liu, K. Ren, R. Du, H. Tian, J. Li, Cell-Derived Microparticles in Patients with Type 2 Diabetes Mellitus: a Systematic Review and Meta-Analysis, Cell Physiol Biochem 39(6) (2016) 2439-2450.

[191] A.E. Berezin, A.A. Kremzer, T.A. Berezina, Y.V. Martovitskaya, The pattern of circulating microparticles in patients with diabetes mellitus with asymptomatic atherosclerosis, Acta Clin Belg 71(1) (2016) 38-45.

[192] J. Shi, L. Kundrat, N. Pishesha, A. Bilate, C. Theile, T. Maruyama, S.K. Dougan, H.L. Ploegh, H.F. Lodish, Engineered red blood cells as carriers for systemic delivery of a wide array of functional probes, Proc Natl Acad Sci U S A 111(28) (2014) 10131-6.

[193] S.C. Voss, M. Jaganjac, A.M. Al-Thani, J.C. Grivel, C.M. Raynaud, H. Al-Jaber, A.S. Al-Menhali, Z.A. Merenkov, M. Alsayrafi, A. Latiff, C. Georgakopoulos, Analysis of RBC-microparticles in stored whole blood bags - a promising marker to detect blood doping in sports?, Drug Test Anal 9(11-12) (2017) 1794-1798.

[194] R.J. Almizraq, J.L. Holovati, J.P. Acker, Characteristics of Extracellular Vesicles in Red Blood Concentrates Change with Storage Time and Blood Manufacturing Method, Transfus Med Hemother 45(3) (2018) 185-193.

[195] M. de Almeida Fuzeta, N. Bernardes, F.D. Oliveira, A.C. Costa, A. Fernandes-Platzgummer, J.P. Farinha, C.A.V. Rodrigues, S. Jung, R.J. Tseng, W. Milligan, B. Lee, M. Castanho, D. Gaspar, J.M.S. Cabral, C.L. da Silva, Scalable Production of Human Mesenchymal Stromal Cell-Derived Extracellular

Vesicles Under Serum-/Xeno-Free Conditions in a Microcarrier-Based Bioreactor Culture System, Front Cell Dev Biol 8 (2020) 553444.

[196] T.J. Greenwalt, D.J. Bryan, U.J. Dumaswala, Erythrocyte membrane vesiculation and changes in membrane composition during storage in citrate-phosphate-dextrose-adenine-1, Vox Sang 47(4) (1984) 261-70.

[197] J.S. Wiley, K.E. McCulloch, D.S. Bowden, Increased calcium permeability of cold-stored erythrocytes, Blood 60(1) (1982) 92-8.

[198] A.B. Zimrin, J.R. Hess, Current issues relating to the transfusion of stored red blood cells, Vox Sang 96(2) (2009) 93-103.

[199] L. van Manen, A.L. Peters, P.M. van der Sluijs, R. Nieuwland, R. van Bruggen, N.P. Juffermans, Clearance and phenotype of extracellular vesicles after red blood cell transfusion in a human endotoxemia model, Transfus Apher Sci 58(4) (2019) 508-511.

[200] C. Roussel, A. Morel, M. Dussiot, M. Marin, M. Colard, A. Fricot-Monsinjon, A. Martinez, C. Chambrion, B. Henry, M. Casimir, G. Volle, M. Depond, S. Dokmak, F. Paye, A. Sauvanet, C. Le Van Kim, Y. Colin, S. Georgeault, P. Roingeard, S.L. Spitalnik, P.A. Ndour, O. Hermine, E.A. Hod, P.A. Buffet, P. Amireault, Rapid clearance of storage-induced microerythrocytes alters transfusion recovery, Blood 137(17) (2021) 2285-2298.

[201] Y. Cheng, X. Wang, J. Yang, X. Duan, Y. Yao, X. Shi, Z. Chen, Z. Fan, X. Liu, S. Qin, X. Tang, C. Zhang, A translational study of urine miRNAs in acute myocardial infarction, J Mol Cell Cardiol 53(5) (2012) 668-76.

[202] W.M. Usman, T.C. Pham, Y.Y. Kwok, L.T. Vu, V. Ma, B. Peng, Y.S. Chan, L. Wei, S.M. Chin, A. Azad, A.B. He, A.Y.H. Leung, M. Yang, N. Shyh-Chang, W.C. Cho, J. Shi, M.T.N. Le, Efficient RNA drug delivery using red blood cell extracellular vesicles, Nat Commun 9(1) (2018) 2359.

[203] Y. Kong, X. Tian, R. He, C. Li, H. Xu, L. Tian, Z. Liu, The accumulation of exosome-associated microRNA-1246 and microRNA-150-3p in human red blood cell suspensions, J Transl Med 19(1) (2021) 225.

[204] V. Mussack, G. Wittmann, M.W. Pfaffl, On the trail of blood doping-microRNA fingerprints to monitor autologous blood transfusions in vivo, Am J Hematol (2020).

[205] A. World Medical, World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects, JAMA 310(20) (2013) 2191-4.

[206] P.-E. Sottas, N. Robinson, S. Giraud, F. Taroni, M. Kamber, P. Mangin, M. Saugy, Statistical Classification of Abnormal Blood Profiles in Athletes, The International Journal of Biostatistics, 2006.

[207] P.E. Sottas, N. Baume, C. Saudan, C. Schweizer, M. Kamber, M. Saugy, Bayesian detection of abnormal values in longitudinal biomarkers with an application to T/E ratio, Biostatistics 8(2) (2007) 285-96.

[208] J.R. Delanghe, M.M. Speeckaert, Creatinine determination according to Jaffe-what does it stand for?, NDT Plus 4(2) (2011) 83-6.

[209] ISEV2018 abstract book, Journal of Extracellular Vesicles 7(sup1) (2018) 1461450.

[210] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin Chem 55(4) (2009) 611-22.

[211] V. Mussack, S. Hermann, D. Buschmann, B. Kirchner, M.W. Pfaffl, MIQE-Compliant Validation of MicroRNA Biomarker Signatures Established by Small RNA Sequencing, Methods Mol Biol 2065 (2020) 23-38.

[212] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, Genome Biol 3(7) (2002) RESEARCH0034.

[213] C.L. Andersen, J.L. Jensen, T.F. Orntoft, Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets, Cancer Res 64(15) (2004) 5245-50.

[214] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25(4) (2001) 402-8.

[215] R Core Team, R: A Language and Environment for Statistical Computing., R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/, 2018.

[216] T. Wilkin, A. Baoutina, N. Hamilton, Equine performance genes and the future of doping in horseracing, Drug Test Anal 9(9) (2017) 1456-1471.

[217] D. Boning, N. Maassen, A. Pries, The hematocrit paradox--how does blood doping really work?, Int J Sports Med 32(4) (2011) 242-6.

[218] C.M. Willinger, J. Rong, K. Tanriverdi, P.L. Courchesne, T. Huan, G.A. Wasserman, H. Lin, J. Dupuis, R. Joehanes, M.R. Jones, G. Chen, E.J. Benjamin, G.T. O'Connor, J.P. Mizgerd, J.E. Freedman, M.G. Larson, D. Levy, MicroRNA Signature of Cigarette Smoking and Evidence for a Putative Causal Role of MicroRNAs in Smoking-Related Inflammation and Target Organ Damage, Circ Cardiovasc Genet 10(5) (2017).

[219] L. Sun, F. Fan, R. Li, B. Niu, L. Zhu, S. Yu, S. Wang, C. Li, D. Wang, Different Erythrocyte MicroRNA Profiles in Low- and High-Altitude Individuals, Front Physiol 9 (2018) 1099.

[220] J. Mullen, L. Baekken, H. Bergstrom, L. Bjorkhem Bergman, M. Ericsson, L. Ekstrom, Fluctuations in hematological athlete biological passport biomarkers in relation to the menstrual cycle, Drug Test Anal (2020).

[221] R. Aikin, N. Baume, T. Equey, O. Rabin, Biomarkers of doping: uses, discovery and validation, Bioanalysis 12(11) (2020) 791-800.

[222] S.A. Solheim, J. Bejder, A. Breenfeldt Andersen, J. Morkeberg, N.B. Nordsborg, Autologous Blood Transfusion Enhances Exercise Performance-Strength of the Evidence and Physiological Mechanisms, Sports Med Open 5(1) (2019) 30.

[223] J.F. Brun, C. Bouchahda, D. Chaze, A.A. Benhaddad, J.P. Micallef, J. Mercier, The paradox of hematocrit in exercise physiology: which is the "normal" range from an hemorheologist's viewpoint?, Clin Hemorheol Microcirc 22(4) (2000) 287-303.

[224] T. Pottgiesser, W. Specker, M. Umhau, H.H. Dickhuth, K. Roecker, Y.O. Schumacher, Recovery of hemoglobin mass after blood donation, Transfusion 48(7) (2008) 1390-7.

[225] Bekanntmachung der Neufassung des Transfusionsgesetzes, in: F.M.o. Health (Ed.) BGBl. I S.2169, 2007.

[226] D. Marty, P. Nicholson, U. Haas, Report to the President of the Union Cycliste Internationale, Cycling Independent Reform Commission, Lausanne, 2005.

[227] J. Bejder, A. Breenfeldt Andersen, S.A. Solheim, M. Gybel-Brask, N.H. Secher, P.I. Johansson, N.B. Nordsborg, Time Trial Performance Is Sensitive to Low-Volume Autologous Blood Transfusion, Med Sci Sports Exerc 51(4) (2019) 692-700.

[228] D. Buschmann, A. Haberberger, B. Kirchner, M. Spornraft, I. Riedmaier, G. Schelling, M.W. Pfaffl, Toward reliable biomarker signatures in the age of liquid biopsies - how to standardize the small RNA-Seq workflow, Nucleic Acids Res 44(13) (2016) 5995-6018.

[229] H.D. VanGuilder, K.E. Vrana, W.M. Freeman, Twenty-five years of quantitative PCR for gene expression analysis, Biotechniques 44(5) (2008) 619-26.

[230] R.D. Gibbons, D. Hedeker, S. DuToit, Advances in analysis of longitudinal data, Annu Rev Clin Psychol 6 (2010) 79-107.

[231] C. Malm, I. Granlund, M. Hall, P. Lindén, M. Ericsson, J.I. Langridge, L.A. Gethings, C. Hughes, N. Khoo, DETECTION OF AUTOLOGOUS BLOOD DOPING, medRxiv (2020) 2020.07.04.20146217.

[232] J. Bejder, G.U. G, C. Cuparencu, F. Hall, M. Gybel-Brask, A. Breenfeldt Andersen, L.O. Dragsted, N.H. Secher, P.I. Johansson, N.B. Nordsborg, An Untargeted Urine Metabolomics Approach for Autologous Blood Transfusion Detection, Med Sci Sports Exerc 53(1) (2021) 236-243.

[233] VeloNews, Commentary: What German anti-doping probe may tell us about cycling, 2019. https://www.velonews.com/news/commentary-what-german-anti-doping-probe-may-tell-us-about-cycling/. (Accessed 08.10.2020.

[234] A. Marchand, I. Roulland, F. Semence, K. Schroder, V. Domergue, M. Audran, Detection of Hypoxia-Regulated MicroRNAs in Blood as Potential Biomarkers of HIF Stabilizer Molidustat, Microrna 8(3) (2019) 189-197.

[235] M. Kim, Y.S. Tan, W.C. Cheng, T.J. Kingsbury, S. Heimfeld, C.I. Civin, MIR144 and MIR451 regulate human erythropoiesis via RAB14, Br J Haematol 168(4) (2015) 583-97.

[236] S.P. Mittal, J. Mathai, A.P. Kulkarni, J.K. Pal, S. Chattopadhyay, miR-320a regulates erythroid differentiation through MAR binding protein SMAR1, Int J Biochem Cell Biol 45(11) (2013) 2519-29.

[237] M. Reithmair, D. Buschmann, M. Marte, B. Kirchner, D. Hagl, I. Kaufmann, M. Pfob, A. Chouker, O.K. Steinlein, M.W. Pfaffl, G. Schelling, Cellular and extracellular miRNAs are blood-compartment-specific diagnostic targets in sepsis, J Cell Mol Med (2017).

[238] M. Steinach, J. Lichti, M.A. Maggioni, M. Fahling, A fluid shift for endurance exercise-Why hydration matters, Acta Physiol (Oxf) 227(1) (2019) e13347.

[239] G.R. Adams, F.P. Zaldivar, D.M. Nance, E. Kodesh, S. Radom-Aizik, D.M. Cooper, Exercise and leukocyte interchange among central circulation, lung, spleen, and muscle, Brain Behav Immun 25(4) (2011) 658-66.

[240] Q. Zhou, C. Shi, Y. Lv, C. Zhao, Z. Jiao, T. Wang, Circulating microRNAs in Response to Exercise Training in Healthy Adults, Front Genet 11 (2020) 256.

[241] J.L. Barber, K.N. Zellars, K.G. Barringhaus, C. Bouchard, F.G. Spinale, M.A. Sarzynski, The Effects of Regular Exercise on Circulating Cardiovascular-related MicroRNAs, Sci Rep 9(1) (2019) 7527.

[242] M. Fernandez-Sanjurjo, N. Ubeda, B. Fernandez-Garcia, M. Del Valle, A. Ramirez de Molina, M.C. Crespo, R. Martin-Hernandez, P. Casas-Agustench, P. Martinez-Camblor, D. de Gonzalo-Calvo, S. Diez-Robles, A. Garcia-Gonzalez, A. Montero, F. Gonzalez-Gonzalez, M. Rabadan, A.E. Diaz-Martinez, M. Whitham, E. Iglesias-Gutierrez, A. Davalos, Exercise dose affects the circulating microRNA profile in response to acute endurance exercise in male amateur runners, Scand J Med Sci Sports 30(10) (2020) 1896-1907.

[243] J.P. Isbister, Physiology and pathophysiology of blood volume regulation, Transfus Sci 18(3) (1997) 409-23.

[244] A. Dunn, V. Lo, S. Donnelly, The role of the kidney in blood volume regulation: the kidney as a regulator of the hematocrit, Am J Med Sci 334(1) (2007) 65-71.

[245] K.C. Miranda, D.T. Bond, M. McKee, J. Skog, T.G. Paunescu, N. Da Silva, D. Brown, L.M. Russo, Nucleic acids within urinary exosomes/microvesicles are potential biomarkers for renal disease, Kidney Int 78(2) (2010) 191-9.

[246] H. Zhou, P.S. Yuen, T. Pisitkun, P.A. Gonzales, H. Yasuda, J.W. Dear, P. Gross, M.A. Knepper, R.A. Star, Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery, Kidney Int 69(8) (2006) 1471-6.

[247] U. Erdbrugger, C.J. Blijdorp, I.V. Bijnsdorp, F.E. Borras, D. Burger, B. Bussolati, J.B. Byrd, A. Clayton, J.W. Dear, J.M. Falcon-Perez, C. Grange, A.F. Hill, H. Holthofer, E.J. Hoorn, G. Jenster, C.R. Jimenez, K. Junker, J. Klein, M.A. Knepper, E.H. Koritzinsky, J.M. Luther, M. Lenassi, J. Leivo, I. Mertens, L. Musante, E. Oeyen, M. Puhka, M.E. van Royen, C. Sanchez, C. Soekmadji, V. Thongboonkerd, V. van Steijn, G. Verhaegh, J.P. Webber, K. Witwer, P.S.T. Yuen, L. Zheng, A. Llorente, E.S. Martens-Uzunova, Urinary extracellular vesicles: A position paper by the Urine Task Force of the International Society for Extracellular Vesicles, J Extracell Vesicles 10(7) (2021) e12093.

[248] B. Dhondt, J. Van Deun, S. Vermaerke, A. de Marco, N. Lumen, O. De Wever, A. Hendrix, Urinary Extracellular Vesicle Biomarkers in Urological Cancers: From Discovery Towards Clinical Implementation, Int J Biochem Cell Biol 2018).

[249] V. Mussack, G. Wittmann, M.W. Pfaffl, Autologous blood transfusions impact surface marker and microRNA profiles of urinary extracellular vesicles, unpublished manuscript (2021).

[250] T. Pisitkun, R.F. Shen, M.A. Knepper, Identification and proteomic profiling of exosomes in human urine, Proc Natl Acad Sci U S A 101(36) (2004) 13368-73.

[251] ISEV2020 Abstract Book, Journal of Extracellular Vesicles 9(sup1) (2020) 1784511.

[252] K.B. Fraser, A.B. Rawlins, R.G. Clark, R.N. Alcalay, D.G. Standaert, N. Liu, C. Parkinson's Disease Biomarker Program, A.B. West, Ser(P)-1292 LRRK2 in urinary exosomes is elevated in idiopathic Parkinson's disease, Mov Disord 31(10) (2016) 1543-1550.

[253] Y. Li, Y. Zhang, F. Qiu, Z. Qiu, Proteomic identification of exosomal LRG1: a potential urinary biomarker for detecting NSCLC, Electrophoresis 32(15) (2011) 1976-83.

[254] Z. Guo, Y. Zhang, L. Zou, D. Wang, C. Shao, Y. Wang, W. Sun, L. Zhang, A Proteomic Analysis of Individual and Gender Variations in Normal Human Urine and Cerebrospinal Fluid Using iTRAQ Quantification, PLoS One 10(7) (2015) e0133270.

[255] A.E. Turco, W. Lam, A.D. Rule, A. Denic, J.C. Lieske, V.M. Miller, J.J. Larson, W.K. Kremers, M. Jayachandran, Specific renal parenchymal-derived urinary extracellular vesicles identify age-associated structural changes in living donor kidneys, J Extracell Vesicles 5 (2016) 29642.

[256] P.M. Gunasekaran, J.M. Luther, J.B. Byrd, For what factors should we normalize urinary extracellular mRNA biomarkers?, Biomol Detect Quantif 17 (2019) 100090.

[257] C.J. Blijdorp, O.A.Z. Tutakhel, T.A. Hartjes, T.P.P. van den Bosch, M.H. van Heugten, J.P. Rigalli, R. Willemsen, U.M. Musterd-Bhaggoe, E.R. Barros, R. Carles-Fontana, C.A. Carvajal, O.J. Arntz, F.A.J. van de Loo, G. Jenster, M.C. Clahsen-van Groningen, C.A. Cuevas, D. Severs, R.A. Fenton, M.E. van Royen, J.G.J. Hoenderop, R.J.M. Bindels, E.J. Hoorn, Comparing Approaches to Normalize, Quantify, and Characterize Urinary Extracellular Vesicles, J Am Soc Nephrol 32(5) (2021) 1210-1226.

[258] C. Bongers, M. Alsady, T. Nijenhuis, A.D.M. Tulp, T.M.H. Eijsvogels, P.M.T. Deen, M.T.E. Hopman, Impact of acute versus prolonged exercise and dehydration on kidney function and injury, Physiol Rep 6(11) (2018) e13734.

[259] D. Tataruch-Weinert, L. Musante, O. Kretz, H. Holthofer, Urinary extracellular vesicles for RNA extraction: optimization of a protocol devoid of prokaryote contamination, J Extracell Vesicles 5 (2016) 30281.

[260] K. Koeppen, T.H. Hampton, M. Jarek, M. Scharfe, S.A. Gerber, D.W. Mielcarz, E.G. Demers, E.L. Dolben, J.H. Hammond, D.A. Hogan, B.A. Stanton, A Novel Mechanism of Host-Pathogen Interaction through sRNA in Bacterial Outer Membrane Vesicles, PLoS Pathog 12(6) (2016) e1005672.

[261] P. Fernandez-Llama, S. Khositseth, P.A. Gonzales, R.A. Star, T. Pisitkun, M.A. Knepper, Tamm-Horsfall protein and urinary exosome isolation, Kidney Int 77(8) (2010) 736-42.

[262] L. Cheng, X. Sun, B.J. Scicluna, B.M. Coleman, A.F. Hill, Characterization and deep sequencing analysis of exosomal and non-exosomal miRNA in human urine, Kidney Int 86(2) (2014) 433-44.

[263] D. Buschmann, B. Kirchner, S. Hermann, M. Marte, C. Wurmser, F. Brandes, S. Kotschote, M. Bonin, O.K. Steinlein, M.W. Pfaffl, G. Schelling, M. Reithmair, Evaluation of serum extracellular vesicle isolation methods for profiling miRNAs by next-generation sequencing, J Extracell Vesicles 7(1) (2018) 1481321.

[264] K. Barreiro, O.P. Dwivedi, G. Leparc, M. Rolser, D. Delic, C. Forsblom, P.H. Groop, L. Groop, T.B. Huber, M. Puhka, H. Holthofer, Comparison of urinary extracellular vesicle isolation methods for transcriptomic biomarker research in diabetic kidney disease, J Extracell Vesicles 10(2) (2020) e12038.

[265] F. Royo, C. Thery, J.M. Falcon-Perez, R. Nieuwland, K.W. Witwer, Methods for Separation and Characterization of Extracellular Vesicles: Results of a Worldwide Survey Performed by the ISEV Rigor and Standardization Subcommittee, Cells 9(9) (2020).

[266] Kiyotaka Shiba, Nobuyuki Sakamoto, Tomozumi Noda, S. Yamada, Method of Recovering Extracellular Vesicles and Container for Extracellular Vesicles, United States of America, 2020.

[267] K.W. Witwer, E.I. Buzas, L.T. Bemis, A. Bora, C. Lasser, J. Lotvall, E.N. Nolte-'t Hoen, M.G. Piper, S. Sivaraman, J. Skog, C. Thery, M.H. Wauben, F. Hochberg, Standardization of sample collection, isolation and analysis methods in extracellular vesicle research, J Extracell Vesicles 2 (2013).

[268] K.R. Yamamoto, B.M. Alberts, R. Benzinger, L. Lawhorne, G. Treiber, Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification, Virology 40(3) (1970) 734-44.

[269] R. Stranska, L. Gysbrechts, J. Wouters, P. Vermeersch, K. Bloch, D. Dierickx, G. Andrei, R. Snoeck, Comparison of membrane affinity-based method with size-exclusion chromatography for isolation of exosome-like vesicles from human plasma, J Transl Med 16(1) (2018) 1.

[270] Z. Andreu, M. Yanez-Mo, Tetraspanins in extracellular vesicle formation and function, Front Immunol 5 (2014) 442.

[271] F.G. Kugeratski, K. Hodge, S. Lilla, K.M. McAndrews, X. Zhou, R.F. Hwang, S. Zanivan, R. Kalluri, Quantitative proteomics identifies the core proteome of exosomes with syntenin-1 as the highest abundant protein and a putative universal biomarker, Nat Cell Biol 23(6) (2021) 631-641.

[272] W. Anderson, D. Kozak, V.A. Coleman, A.K. Jamting, M. Trau, A comparative study of submicron particle sizing platforms: accuracy, precision and resolution analysis of polydisperse particle size distributions, J Colloid Interface Sci 405 (2013) 322-30.

# List of scientific communications

## *Peer-reviewed original papers included in this thesis*

Veronika Mussack, Georg Wittmann, Michael W. Pfaffl

**Comparing small urinary extracellular vesicle purification methods with a view to RNA sequencing—Enabling robust and non-invasive biomarker research**

Biomolecular Detection and Quantification, DOI: 10.1016/j.bdq.2019.100089 (March 1st, 2019)

Veronika Mussack, Georg Wittmann, Michael W. Pfaffl

**On the trail of blood doping—microRNA fingerprints to monitor autologous blood transfusions *in vivo***

American Journal of Hematology, DOI: 10.1002/ajh.26078 (December 16th, 2020)

Dominik Buschmann, Veronika Mussack, James Brian Byrd

**Separation, characterization, and standardization of extracellular vesicles for drug delivery applications**

Advanced Drug Delivery Reviews, DOI: 10.1016/j.addr.2021.04.027 (May 5th, 2021)

## *Other peer-reviewed original papers*

Clotilde Théry, Kenneth W. Witwer *et al.* (including Veronika Mussack)

**Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines**

Journal of extracellular vesicles, DOI: 10.1080/20013078.2018.1535750 (November 23rd, 2018)

Fabia Fricke, Veronika Mussack, Dominik Buschmann, Ingrid Hausser, Michael W. Pfaffl, Jürgen Kopitz, Johannes Gebert

**TGFBR2-dependent alterations of microRNA profiles in extracellular vesicles and parental colorectal cancer cells**

International Journal of Oncology, DOI: 10.3892/ijo.2019.4859 (August 19th, 2019)

Pamela R. Matias-Garcia, Rory Wilson, Veronika Mussack, Eva Reischl, Melanie Waldenberger, Christian Gieger, Gabriele Anton, Annette Peters, Andrea Kuehn-Steven

**Impact of long-term storage and freeze-thawing on eight circulating microRNAs in plasma samples**

PLOS ONE, DOI: 10.1371/journal.pone.0227648 (January 14th,2020)

## *Book chapters*

Michael W. Pfaffl, Dominik Buschmann, Veronika Mussack, Benedikt Kirchner, Bajram Berisha

**Nucleic Acids | RNA Identification and Quantification via RT-qPCR**

Encyclopedia of Analytical Science (Third Edition), DOI: 10.1016/B978-0-12-409547-2.14496-8 (March 12th, 2019)

Dominik Buschmann, Benedikt Kirchner, Veronika Mussack, Michael W. Pfaffl

**Nucleic Acids | RNA Identification and Quantification via Next Generation Sequencing**

Encyclopedia of Analytical Science (Third Edition), DOI: 10.1016/B978-0-12-409547-2.14269-6 (March 12th, 2019)

Veronika Mussack, Stefanie Hermann, Dominik Buschmann, Benedikt Kirchner, Michael W. Pfaffl

**MIQE-compliant validation of microRNA biomarker signatures established by small RNA sequencing**

Quantitative Real-Time PCR. Methods in Molecular Biology, DOI: 10.1007/978-1-4939-9833-3_3 (October 2nd, 2019)

Marlene Reithmair, Anja Lindemann, Veronika Mussack, Michael W. Pfaffl

**Isolation and characterization of urinary extracellular vesicles for microRNA biomarker signature development with reference to MISEV compliance**

Extracellular Vesicles in Diagnosis and Therapy. Methods in Molecular Biology (accepted for publication)

## *Peer-reviewed poster presentations*

Veronika Mussack, Georg Wittmann, Michael W. Pfaffl

**Unveiling autologous blood doping: comparative analysis of different purification strategies for urinary extracellular vesicles pioneering miRNA biomarker research**

ISEV 2019 - Annual Meeting, Kyoto, Japan (April 24$^{th}$ – 28$^{th}$, 2019)

Fabia Fricke, Veronika Mussack, Dominik Buschmann, Michael W. Pfaffl, Jürgen Kopitz, Johannes Gebert

**Tumor driver TGFBR2-dependent microRNA profiles in colorectal cancer cells and their EVs**

ISEV 2019 - Annual Meeting, Kyoto, Japan (April 24$^{th}$ – 28$^{th}$, 2019)

Veronika Mussack, Michael Pfaffl

**Human urinary extracellular vesicles carry surface markers that are indicative of haematopoietic origin**

ISEV 2020 - Annual Meeting, online (July 20$^{th}$ – 22$^{nd}$, 2020)

Lin Li, Veronika Mussack, Elena Pepeldjiyska, Anne Hartz, Andreas Rank, Christoph Schmid, Erdem Oezkaya, Selda Ugur, Michael W. Pfaffl, Helga Schmetzer

**Role of exosomes as promotors or biomarkers to study activation of leukemia-derived dendritic cells (DCleu)-mediated antileukemic activation of adaptive and innate immune-reactive cells against AML-blast**

Immunotherapy of Cancer Conference - ITOC 2020, online (October 1$^{st}$, 2020)

Lin Li, Veronika Mussack, Elena Pepeldjiyska, Anne Hartz, Andreas Rank, Christoph Schmid, Erdem Oezkaya, Selda Ugur, Michael Pfaffl, Helga Schmetzer

**Role of exosomes as promotors or biomarkers to study activation of leukemia-derived dendritic cells (DCleu)-mediated antileukemic activation of adaptive and innate immune-reactive cells against AML-blast**

Bone Marrow Transplantation Conference, online (December 1st, 2020)

## *Other poster presentations*

Veronika Mussack, Anna Haberberger, Georg Wittmann, Michael W. Pfaffl

**Excursion: Detection of autologous blood doping on the miRNA level in a human study**

3rd HEZagrar PhD Symposium, Freising, Germany (April 25th, 2017)

Anna Haberberger, Veronika Mussack, Benedikt Kirchner, Irmgard Riedmaier, Christian Wichmann, Raymund Buhmann, Georg Wittmann, Reinhard Henschler, Michael W. Pfaffl

**Developing a potential biomarker set of miRNAs in packed RBCs to detect autologous blood doping**

GQ2017 Event – qPCR, dPCR & NGS Symposium, Freising, Germany (April 3rd – 7th, 2017)

Veronika Mussack, Georg Wittmann, Michael W. Pfaffl

**Urinary Extracellular Vesicles: Unveiling the Most Appropriate Purification Method with a View to RNA Sequencing and Biomarker Profiling**

GQ2019 Event – qPCR, dPCR & NGS Symposium, Freising, Germany (March 18th-22nd, 2019)

Veronika Mussack, Georg Wittmann, Michael W. Paffl

**Identifying appropriate urinary extracellular vesicle purifications for downstream transcriptomics**

GSEV Autumn Meeting 2019, Freising, Germany (November 28th-29th, 2019)

Veronika Mussack, Georg Wittmann, Michael W. Paffl

**Evaluation of differentially isolated small urinary EVs and its effect on downstream transcriptomics**

ISEV Workshop - Rigor and Standardization, Ghent, Belgium (December 2nd-3rd, 2019)

## *Oral presentations*

Veronika Mussack

**Urinary exosomes – an evaluation of different isolation strategies**

Mini-Symposium on Extracellular Vesicle Research, Erlangen, Germany (July 19th, 2017)

Veronika Mussack

**TGFBR2-dependent microRNA profiling of EVs and parental DNA mismatch repair deficient colorectal cancer cells**

GSEV Herbsttagung 2018, Marburg, Germany (November 11th, 2018)

Veronika Mussack

**WADA blood doping project – Basics, interim findings, and future perspectives**

Transfusionsmedizinisches Kolloquium, Munich, Germany (February 19th, 2019)

Veronika Mussack

**Human urinary extracellular vesicles carry surface markers that are indicative of hematopoietic origin**

ISEV 2020, online (July 21st, 2020)

Veronika Mussack

**Comparative analysis of differentially isolated urinary extracellular vesicles impacting downstream transcriptomics**

GSEV Jahressymposium 2020, Frankfurt a.M., Germany (March 5th, 2020)

Veronika Mussack

**Research Insights – microRNA fingerprints to monitor autologous blood doping transfusions *in vivo***

Partnership for Clean Competition – Research Insights, online (March 15th, 2021)

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