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„Exhaustive Exercise such as an Ironman Triathlon Alters MicroRNA Expression Pattern in Whole Blood“

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Abstract – English

MicroRNAs are ~ 22 nt short RNA stretches exhibiting fine-tuning properties on several cellular processes via either translational inhibition or mRNA degradation. The influence of sports, especially exhaustive exercise, on microRNA expression is just starting to emerge. Thus, the aim of this study was to determine a microRNA expression pattern upon exhaustive exercise. A microRNA expression profile of whole blood was determined from 15 male athletes before, immediately after the race as well as 1 day and 7 days after finishing the Ironman Austria triathlon. Using a PCR Array consisting of 84 observable microRNAs, 26 microRNAs were more than 2-fold down-regulated and 2 microRNAs more than 2-fold up-regulated directly after finishing the triathlon compared to pre-race conditions. Of note, miR-122 was more than 2-fold differentially regulated at all three time points post-race. The six most prominent miRNAs (miR-96, -15a, -126, -122, -223 and -23a) were further validated by RT-qPCR. MiR-15a and miR-126 could be positively verified and were found significantly decreased immediately after finishing the Ironman compared to pre-race levels. Generally, no sustained expression of microRNAs upon exhaustive exercise could be determined. It can be concluded, that the expression pattern of microRNAs is altered directly after exhaustive exercise and is not sustained over a longer period of time. Especially, miR-15a and miR-126 levels were altered immediately after the Ironman but did not show long-term regulation.

Abstract – Deutsch

MicroRNAs sind ~ 22 nt kurze RNA Stücke, welche fein-regulatorische Eigenschaften in verschiedenen zellulären Prozessen, entweder durch Inhibierung der Translation von Proteinen, oder Degradierung von mRNA, aufweisen. Der Einfluss von Sport, im Speziellen extreme Belastung auf die Expression von microRNAs konnte erst kürzlich gezeigt werden. Deshalb war es ein Ziel dieser Studie, ein Expressionmuster von microRNAs bei extremer Belastung zu bestimmen. Ein microRNA-Expressionsprofil aus Vollblut wurde von 15 männlichen Athleten vor, unmittelbar nach dem Wettkampf, ebenso wie 1 Tag und 7 Tage nach dem Ironman Austria Triathlon, bestimmt. 84 verschiedene microRNAs konnten unter Verwendung eines PCR Arrays detektiert werden. Unmittelbar nach dem Wettkampf wurden, im Vergleich zu Vorwettkampfwerten, 26 microRNAs mehr als 2-fach herunterreguliert und 2 microRNAs mehr als 2-fach hinaufreguliert vorgefunden. Eine microRNA (miR-122) wurde bei allen 3 Zeitpunkten nach dem Wettkampf mehr als 2-fach herunterreguliert detektiert. 6 Kandidaten-microRNAs (miR-96, -15a, -126, -122, -223 and -23a) wurden mittels RT-qPCR validiert. MiR-15a und miR-126 konnten positiv verifiziert werden. Verglichen mit Vorwettkampfwerten konnte für diese beiden microRNAs eine Verringerung der Expression unmittelbar nach dem Ironman Wettkampf bestimmt werden. Es konnte keine länger andauernde Regulation beobachtet werden. Aus den Ergebnissen dieser Studie folgt, dass sich das Expressionmuster von microRNAs auf Grund extremer Belastung verändert. Diese Veränderung ist jedoch nicht über einen längeren Zeitraum anhaltend. Im Speziellen ist die Expression der beiden microRNAs miR-15a und miR-126 direkt nach dem Ironman erniedrigt, zeigte jedoch keine langanhaltende Veränderung.

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1 Introduction

Sports can be defined as all forms of competitive or non-competitive physical activity with the aim to maintain and improve physical fitness, spirit and health. Moreover, moderate sports activity increases healthy life years as well as life expectancy partially by counteracting several diseases, e.g. sarcopenia and metabolic, cardiovascular or atherosclerotic diseases. However, the beneficial effects of sports are reduced upon high exercise intensity / training volume. Among others, one observed effect is an enhanced risk to acquire and infection upon exhaustive exercise. Nevertheless, from the perspective of elite athletes engaged in high-intensity trainings, it is of great importance to understand the mechanisms behind an increased susceptibility to acquire infections. Especially, infections such as coughs and colds, throat infections and middle ear infections, commonly referred to as upper respiratory tract infections (URTI) [22], are of major importance since they can dramatically reduce an athlete's performance. A J-shaped model has been suggested for the relationship between exercise intensity and URTI episodes. According to this model, moderate exercise is beneficial whereas participating in acute bouts of intense exercise and involvement in heavy schedules of training and competition increases the relative risk of URTI to above that of sedentary controls [17] (Figure 1). Whether or not an impaired immune response upon exhaustive load is associated or can even trigger this susceptibility for infection is not yet wholly clarified. It is thus an aim of this study to further improve the knowledge of URTI after exhaustive exercise.

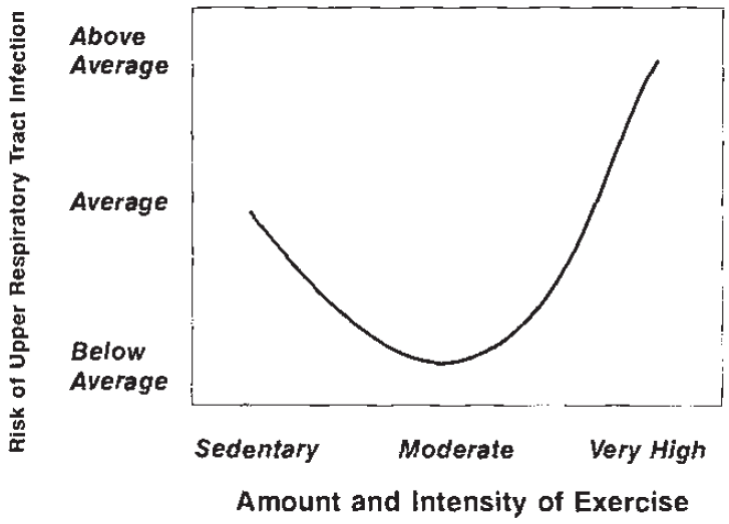

Figure 1. *The J-shaped Model of The Relationship Between Risk of Upper Respiratory Tract Infection (URTI) and Exercise Volume. [17]*

1.1 The Immune System

The human immune system is a multi-cellular system consisting of several cell types. It is capable to combat against viruses, parasites or bacteria and hence creates protection against foreign substances and organisms. A variety of different cell types of the immune system must be precisely orchestrated to recognize, attack and destroy foreign elements. The immune system splits into two major groups, the innate immunity and the adaptive immunity which work together synergistically. A primary defense layer is established by cells of the innate immune system, granulocytes (neutrophils, eosinophils, basophils), agranulocytes (monocytes/macrophages) and natural killer (NK) cells. These cells often initiate the activation of the secondary defense layer termed adaptive immune system. The adaptive immunity comprises of B and T cells with several sub-categories and different responsibilities for combating against certain pathogens. In general, a secondary immune response aids the innate immune systems defense mechanisms via specific adaption of its defense capabilities against specific pathogens. Moreover, it creates an important machinery to avoid overreaction against harmless antigens as well as to discriminate between components of ´self´ and ´non-self´. Thus, a precise temporal and spatial orchestration of both the innate as well as the adaptive immune system is crucial for a proper clearance of pathogens from a host since a false regulated immune response can do severe damage to the host. Whether or not an impaired immune response upon exhaustive load is associated with URTI is not yet wholly clarified. It is thus an aim of this study to further improve the knowledge of molecular parameters after exhaustive exercise.

1.2 Immune Function in Sport and Exercise

1.2.1 Innate Immunity

Leukocytosis is observed upon acute as well as upon exhaustive exercise [23-26] and is mainly due to release of neutrophils from the bone marrow [23, 26, 27], elevated monocyte counts [28] and elevated numbers of circulating dendritic cells [29, 30]. A second elevation of blood neutrophil numbers related to the intensity and duration of the exercise performed is detected after this primary elevation [25, 31]. Interestingly, an acute bout of exercise leads to increased activity of degranulation, phagocytosis and oxidative burst mediated by immune cells

whereas during recovery, bacterial stimulation reduces degranulation and oxidative burst activity [25, 31, 32].

In addition, monocyte numbers are elevated after acute exercise lasting for ~ 2h. The increase in monocyte numbers is most likely due to mobilization of marginated monocytes into the blood circulation and are destined to become mature tissue macrophages [28]. The circulating monocytes show reduced IL-6, IL1-a and TNF-α production which might be a consequence of down-regulated TLR expression [33-36]. MHC II is decreased in macrophages with ongoing exercise as well as their antigen presentation capacity [37-39]. Moreover, alveolar macrophages display reduced anti-viral capacity upon exhaustive exercise favoring susceptibility to Herpes simplex virus -1 infection [40, 41]. In contrast, moderate and intense acute exercise can have a positive effect on macrophages, e.g. enhanced phagocytosis [42], anti-tumour activity [43-45], reactive oxygen and nitrogen metabolism [44, 45] and chemotaxis [28, 46].

Lymphocytosis upon acute and exhaustive exercise is most exclusively a result of elevated cell numbers of NK cells in the blood stream [47-49]. A single bout of moderate or exhaustive exercise strongly enhances NK cell cytotoxicity [50, 51]. This effect is observed mainly due to increased NK cell numbers in the circulatory system [51]. A prolonged bout results in the repression of NK cytotoxicity which can last for several hours and might indicate a period of reduced ability to respond to an infection [52].

1.2.2 Adaptive Immunity

Increasing numbers of circulating cells in the blood stream immediately after exercise originates to a large extent from cells of the innate immune system. Nevertheless, a minor fraction of elevated cell numbers can be attributed to B and T cells. Similar to cells from the innate immune system, the intensity and duration is responsible for increased circulating B and T cell levels during and immediately after exercise [17, 53-55]. During the early stages of recovery, a drop below resting levels in B and T cell numbers is observed, before returning to baseline values eventually. During the resting state (> 24h after the last training session), lymphocyte numbers resemble those of non-athletes [56].

Of note, T cell populations are more affected than B cell populations [27, 57]. More exactly, the circulating T_H1 subpopulation is responsible for the observed drop in T cell levels whereas T_H2 levels remain almost steady [58, 59]. Whether the decline in type 1 T cell numbers results from enhanced apoptosis or redistribution to other compartments is unknown. Nevertheless, a reduced responsiveness of T cells to certain pathogens upon defective T cell functionality was

noticed to increase the incidence of viral infections [60] which might give a hint of enhanced susceptibility to viral re-activation following exhaustive exercise. B cell populations contribute less to the observed changes in cell counts. Thus, immunoglobulin (Ig) levels slightly increases or remain unchanged upon brief or prolonged exercise [61-63].

1.2.3 Cytokine Response upon Exercise

Upon exercise, the cytokine response is different than from infections (Figure 2). Production of TNF-α and IL-1β are typically increased during an infection whereas they are generally not elevated with exercise (reviewed in [64]). The first cytokine produced and secreted into the circulatory system is the pro-inflammatory molecule IL-6 and typically reaches its maximum level directly after exercise until it declines during the post-exercise period [65-67]. IL-6 is expressed in type I and II skeletal muscle fibers upon contraction [68] and released into the circulatory system during exercise [69, 70]. Down-stream signaling involves gp130Rβ/IL6-Rα homodimer, AMPK and PI3K and increases fat oxidation and glucose uptake [64, 71]. IL-6 can inhibit the production of TNF-α [72] and stimulates the expression of the anti-inflammatory cytokines IL-1ra and IL-10 [73-75]. Thus, exercise-induced IL-6 secretion establishes an anti-inflammatory environment via IL-10 and IL-1ra post-exercise.

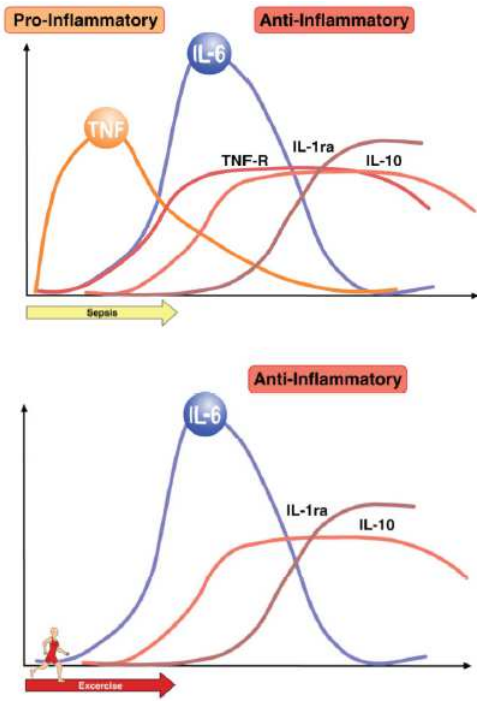

***Figure 2**. Expression Pattern of Circulating Cytokines. During sepsis (A), TNF-α is increased, followed by elevated IL-6 levels. In contrast, exercise-induced (B) IL-6 expression is not preceded by elevated TNF-α. [5]*

In contrast, strenuous physical exercise can elevate the production of the pro-inflammatory cytokines TNF-α and IL-1β which in-turn stimulate IL-6 production most likely by macrophages [75]. Elevated TNF-α levels after exhaustive exercise were observed in various studies [67, 73, 76-78] which might be a result of beginning inflammation upon muscular damage [77].

To what extent cytokine response upon strenuous exercise contributes to the susceptibility to acquire upper respiratory infections or to the development of upper respiratory symptoms (URS) remains unknown. By comparing cytokine levels in healthy and illness-prone athletes, Cox et al. (2007) demonstrated that upon exercise, IL-6 elevation is markedly higher in the latter group [79]. Moreover, IL-10 and IL-1ra levels were lower in the resting and post-exercise state in this group. This observation contributes to a scenario, where a dysregulated inflammatory response upon exercise favors the susceptibility to acquire URTI or develop URS.

1.2.4 Exercise and Infection Risk

Runners participating in a marathon or ultramarathon reported a 2 – 6 fold increase in upper respiratory infection symptoms in the following weeks [80, 81]. Remarkably, in a large cohort of elite athletes showing upper respiratory symptoms, no respiratory pathogen could be identified suggesting other mechanisms triggering these episodes of respiratory symptoms [82]. It is now questioned whether URTI are caused by an acquired infection per se or by other inflammatory stimuli [83, 84]. Since infections are not usually verified by pathological examinations, meaning that clinical investigations to examine the underlying causes of upper respiratory symptoms are rare [82, 83, 85], the concern must be raised whether URS are really caused by - newly acquired - infections or by an enhanced susceptibility to symptoms mimicking an infection as a result of immunosuppression / immunodysregulation after exhaustive exercise [82, 83].

Interestingly, elite athletes experience URTI symptoms at a similar rate as the general population does [82, 86, 87] but encounter episodes of URTI more frequently during or around competitions [81, 88-90] which endure ~ 1 - 3 days [84, 88]. Moreover, URS reports increase with increasing intensity of the training or competition load performed [81, 90-94]. Thus, the observation that URS last for a very short period of time and that they occur especially during or after intense loads of exercise suggests that the reported symptoms might be either due to viral re-activation rather than a primary infection or due to other exercise-induced dysregulated inflammatory responses [84, 88].

Further evidence supporting the ´viral re-activation´ hypothesis was reported in studies which included a clinical test to determine possible pathogens and noted that only ~ 5% of the episodes reported accounted for bacterial infections [84, 95-97]. The majority of symptoms were identified as viral infections. Nevertheless, these infections were responsible for only 30 – 40% of the upper respiratory symptoms reported [82, 97]. Additionally, the viral pathogens were identified as common respiratory pathogens which are associated with URTI in the general population [98]. Thus, URS observations might be, at least in part, a consequence of viral re-activation [84, 88] which would give an explanation for the short period of symptoms encountered.

Importantly, the health state of an athlete itself can contribute to an enhanced number of URS episodes [82, 84]. In general, the contribution of influencing parameters such as drying of the airways [99], asthma and allergic airway inflammation [83], psychological impacts of exercise on membrane integrity and immunity [100] and the migration of inflammatory cytokines, generated during the damage of muscles in eccentric exercise to the airways [101, 102] causing upper respiratory symptoms should be kept in mind although little or no direct evidence is yet available to support these issues.

To date, the most predictive factor favoring URTI prognosis is salivary immunoglobulin A (SIgA). Selective deficiency of SIgA [103] or low saliva flow rates [104] were related to a high incidence of URTI. Moreover, low levels of SIgA [105, 106] or even a transient fall [107] were associated with increased susceptibility to acquire upper respiratory tract infections and vice versa [108]. Saliva IgA levels decline during intensive periods of training [88, 105-107, 109-113]. Of note, some studies noticed an increased risk for infections [105-107, 110, 111] related to SIgA levels whereas other did not [109, 112, 114].

There is evidence that non-viral triggered inflammation can explain upper respiratory symptoms [115] meaning that aside viral-induced, a dysregulated inflammatory response upon exhaustive exercise mimics a URTI [79] and can serve as an explanation for the symptoms reported. A genetic variant leading to elevated IL-6 expression was demonstrated to correlate with an increased number of athletes reporting URS [116]. Additionally, IL-6 elevation after exercise was more pronounced in individuals prone to acquire upper respiratory illnesses compared to healthy participants [79]. Interestingly, athletes using anti-inflammatory sprays before a marathon race developed URS with the same frequency but less severe symptoms [91, 117]. Thus, proper regulation of the pro- and anti-inflammatory responses after exhaustive

exercise seems of great importance since a dysregulated / excessive inflammtory response can mimic an URTI.

Whether or not an impaired immune response upon exhaustive load is associated or even triggering a URTI episode is yet not wholly clarified. It is thus an aim of this study to further improve the knowledge of molecular parameters after exhaustive exercise.

Several fields in research (e.g. genomics, proteomics, metabolomics, and expression analysis) help to improve and elucidate the knowledge of molecular mechanisms which lead to upper respiratory symptoms observed upon exhaustive exercise. Recently, microRNAs were demonstrated differentially expressed upon exercise [118-120] and could be related to inflammatory processes [119, 120]. The ability of miRNAs to fine-tune the amount of various gene products at the translational level make them prone for further detailed studies in relation to exercise, immunodysregulation / immunosuppression, URS and even URTI development. Thus, an aim of this thesis was to gain an overview of the impact of extreme physiological exercise (Ironman Austria) on whole blood miRNA expression levels with a focus on parameters involved in the immune system. Moreover, a hypothetical outlook on downstream processes should be established. Thus, miRNAs involved during a phase of immunodysregulation / immunosuppression should be further elucidated by means of molecular biological approaches.

1.3 MicroRNAs

1.3.1 Biogenesis

1.3.1.1 Transcription

Canonical microRNAs are typically transcribed from intergenic or intronic sequences by the RNA polymerases II or III [121, 122] and are termed primary microRNAs (pri-miRNAs) (Figure 3, panel i and ii). RNA pol II is the more prominent transcription machinery for pri-miRNA generation since most observed transcripts include a cap structure and a poly-A tail tailored by this RNA polymerase similar to messenger RNAs (mRNAs) [123]. RNA pol III derived pri-miRNA transcript sequences are located in repetitive sequences or downstream of ALU elements [121, 124] whereas pri-miRNA sequences transcribed by RNA pol II originate from intronic sequences of messenger RNAs. Intron-derived pri-miRNAs are spliced from mRNA transcripts in a process that is neither interfering with mRNA splicing itself nor downstream mRNA processes [125]. Alternatively, microRNAs can originate from small introns of host genes (Figure 3, panel iii and iv). In this non-canonical pathway, pri-miRNAs are created via splicing and are independent of Drosha processing (reviewed in [126]).

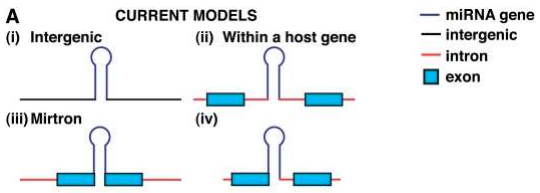

Figure 3. *MiRNA Gene Models. Canonical microRNAs can be transcribed from panel (i) intergenic or (panel ii) intronic sequences. Non-canonical miRNAs, termed mirtrons (panel iii and iv), originate from small introns of host genes. [9]*

1.3.1.2 Pri-miRNA Processing

Pri-miRNA transcripts are processed via a two-step cleavage mechanism. Initially, pri-miRNAs are cleaved inside the nucleus into ~ 70 nt long RNA stretches, termed premature-miRNAs (pre-miRNAs) [127] via a multiprotein complex, termed microprocessor [128]. Drosha, an RNAse class III enzyme, and DGCR8 are the main constituents of this machinery [128-130]. DGCR8 binds to the pri-miRNA and acts as a molecular ruler for the subsequent cleavage by Drosha [20]. Furthermore, DGCR8 stabilizes Drosha via protein-protein interactions [14]. A

more flexible basal ssRNA segment and to some extent a larger terminal loop may be beneficial for the correct binding and orientation of DGCR8 and thus for the whole processing procedure [20]. Interestingly, Drosha protein levels are regulated via an autoregulatory feedback loop. At high Drosha protein levels, Drosha mRNA destabilizes yielding to lower Drosha protein levels and decreased microprocessor activity [14] (Figure 4).

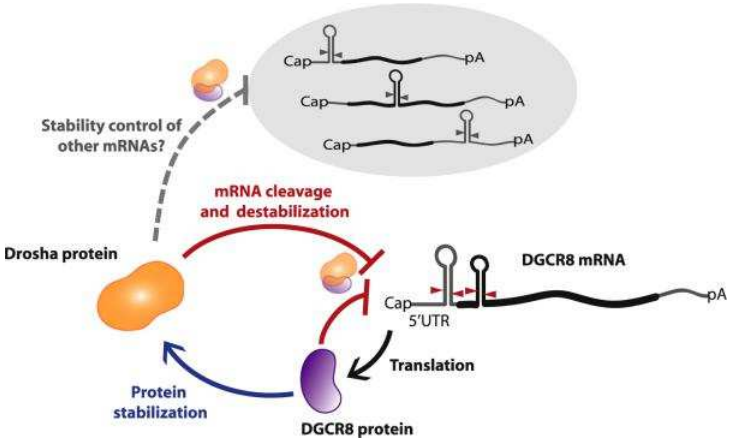

Figure 4. *Model for The Autoregulatory Feedback Loop between Drosha and DGCR8. Drosha protein destabilizes DGCR8 mRNA by cleaving the hairpins of the DGCR8 mRNA. Translated DGCR8 protein stabilizes Drosha protein levels via protein-protein interactions. [14]*

Drosha cleaves pri-miRNAs via its two distinct RNase III enzymes, RIIIDa and RIIIDb. These subunits form a dimer and act independently on either the 3´ (RIIIDa) or the 5´(RIIIDb) strand of the double stranded region of pri-miRNAs [18] (Figure 5).

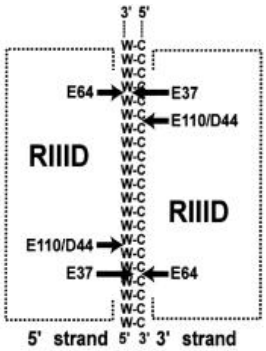

Figure 5. *A Model for The Cleavage Mechanism of RNase III. A dimer is formed by two RIIID units yielding into two processing centers. Each processing center is located in the double stranded region of a pri-miRNA and contains two catalytic sites that cleave two nearby phosphodiester bonds on the opposite RNA strands of dsRNA. W-C, Watson-Crick base pairs. [18]*

Single stranded RNA flanking sequences followed by a ~ 80 nt stem/loop structure are important factors for proper cleavage [131]. More exactly, the length of the stem (~ 30 nt) and the size of the terminal hairpin loop (> 10 nt) are crucial for this process. Drosha prefers larger

hairpins (> 10 nt) and measures about two helical turns (~ 22 nt) from the stem / loop junction and cuts the small RNA into ~ 22 nt long RNA stretches with a terminal loop and a single-stranded overhang [132] (Figure 6).

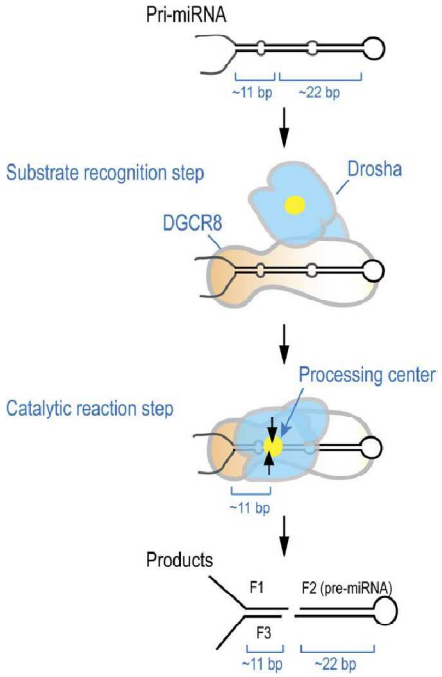

***Figure 6**. A Model for The Processing of Pri-miRNA by DGCR8 and Drosha. DGCR8 binds to the pri-miRNA and acts as a molecular ruler for the subsequent processing by Drosha. After target recognition, Dosha cleaves pri-miRNA in its double stranded region (yellow circle) at ~ 11 bp from the basal segments. [20]*

1.3.2 Nuclear Export

After transcription and pri-miRNA processing, a small RNA molecule consisting of ~ 22 nt long stem with a single-stranded overhang on the one and a loop region on the other end, termed premature-miRNA (pre-miRNA) is created. Pre-miRNAs are exported from the nucleus to the cytosplasm via binding to Exportin-5 (Exp-5) [133, 134]. Notably, Exp-5 only is able to facilitate pre-miRNA export, partially via binding sequence-independently to dsRNA but also via interactions with the single-stranded 3´-overhang but not with the loop region [135, 136]. 5´ overhangs were shown to block Exp-5 / pre-miRNA complex formation and are not exported into the cytoplasm via Exportin-5 suggesting another mechanism for the nuclear export of such RNA species [137].

RanGTP is essential for Exp-5 to bind to pre-miRNAs [134] and thereby greatly enhances this export mechanism [133]. More precisely, free Exp-5 is quite flexible and the 2 nt 3´ pre-miRNA stretch is hindered to insert into the binding pocket/groove (a tunnel comprised of four negatively charged amino acids) from Exp-5. Binding to RanGTP stabilizes Exportin-5. In turn,

pre-miRNA can bind to Exp-5:RanGTP via its 2 nt 3´ overhang forming a stable ternary complex (Figure 8 (A)). Further stabilization is not sequence specific and mainly achieved via hydrogen bonds and ionic interactions rather than base interactions [8, 16]. Additionally, the Exportin-5:Ran-GTP binary complex protects the stem of a pre-miRNA from degradation by nucleases [137]. Thus, RanGTP-stabilized Exp-5 strongly binds pre-miRNA at its 3´overhang whereas the stem contributes less (Figure 7).

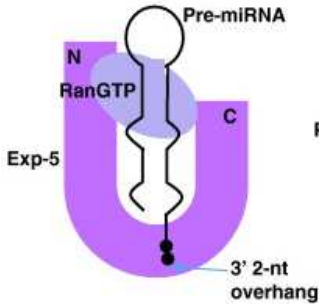

***Figure 7**. A Schematic Representation of The Exp-5:RanGTP:pre-miRNA Ternary Complex. [8]*

The ternary complex enters the cytoplasm where RanGTP hydrolyzes which in turn greatly reduces the binding affinity to the pre-miRNA and Exp-5. Hence, pre-miRNA is released into the cytoplasm (Figure 8 (B)). Of note, hydrolization and release of pre-miRNA may occur more or less simultaneously rather than as a serial event [16].

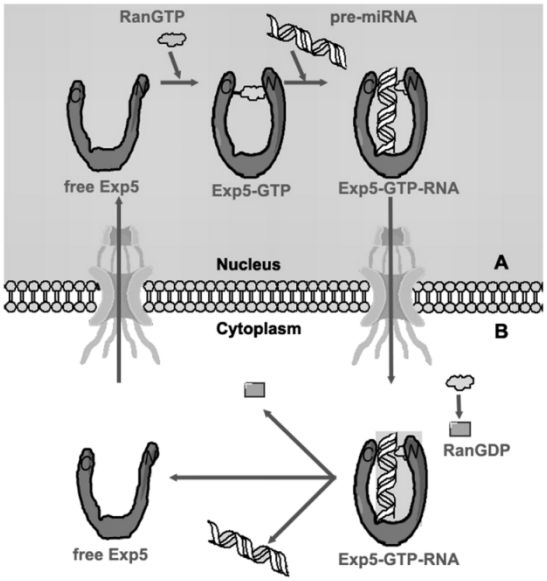

***Figure 8**. Schematic Representation of RanGTP-assisted Nuclear Export of Pre-miRNA. (A) Binding to RanGTP stabilizes Exportin-5. Subsequently, pre-miRNA can bind to this stabilized Exp-5:RanGTP binary complex via its 2nt 3´overhang forming a stable tertiary complex. The formed export complex is translocated through the nuclear pore complex (NPC) into the cytoplasm. (B) RanGTP from exported Exp-5:RanGTP:pre-miRNA complex is hydrolyzed and thus binding affinity to the pre-miRNA and Exp-5 is reduced. Subsequently, pre-miRNA is released into the cytoplasm. [16]*

1.3.3 Effects by Exported MiRNAs

1.3.3.1 RISC Loading Complex Formation

Prior pre-miRNA incorporation, Dicer and TRBP (HIV-1 TAR RNA binding protein) form a complex that is stabilized by the latter protein [138]. Subsequently, TRBP recruits Aronaute-2 (Ago2) to form a pre-assembled trimeric complex, termed miRNA loading complex (miRLC) or RLC (RISC loading complex) [139-142].

Dicer is important during miRNA biogenesis to produce 21 – 23 nt long ssRNA products [143, 144]. Unlike *D. melanogaster*, human Dicer cleaves its substrates without ATP consumption [145, 146]. The PAZ domain (Piwi Argonaut and Zwille domain) of Dicer interacts with the 3´overhang and TRBP (via a dsRBD (double strand RNA binding domain)) binds to the dsRNA region of a pre-miRNA [147, 148].

The processing center of Dicer is built-up of two subunits, RIIIa and RIIIb, which dimerize to form a catalytically active site and cleaves dsRNA in a two-step cleavage process [11, 148]. In this mechanism, pre-miRNA is cut first, released and rebound inversely for another round of cleavage producing ~ 23 nt long short RNAs with 2 nt 3´overhangs (Figure 9).

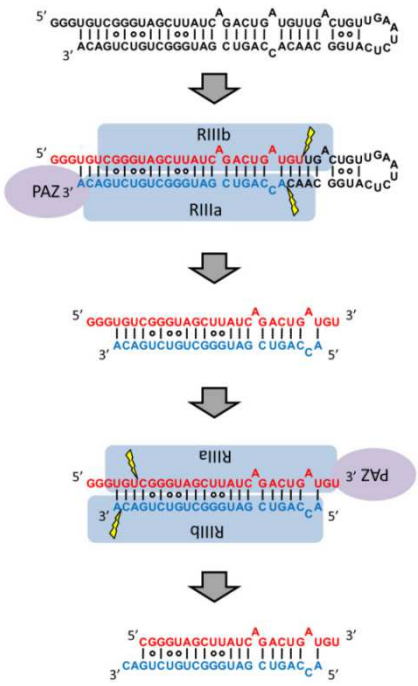

Figure 9. *Model of Two-step Processing of Pre-miRNA by Dicer Protein. In this mechanism, pre-miRNA is cut first, released and rebound inversely by the same or different Dicer molecule for another round of cleavage producing ~ 23 nt long short RNAs with 2 nt 3´overhangs. Lightning marks indicate the cleavage sites in the RNA. [11]*

Additionally, PACT (protein activator of PKR (PKR, protein kinase dsRNA dependent)) protein interacts with Dicers´ N-terminal helicase domain via its dsRBD [149]. Importantly, a RLC has the capability of dicing, selecting the guide-strand and slicing of pre-miRNAs [141]. Moreover, ATP is neither required for RLC assembly nor is it for the cleavage of pre-miRNAs or the unwinding of dsRNA species [140, 142-144, 150].

1.3.3.2 Dicer-dependent Pathways

Double-stranded pre-miRNA is incorporated in an asymmetrical manner into a pre-formed RLC and either Dicer activity only (Figure 9, left arm of the pathway) [140, 142, 151] or Ago2 and subsequent Dicer activity (Figure 9, right arm of the pathway) [19] mediate the cleavage of the passenger strand from a pre-miRNA. Both Ago2 and Dicer are involved as catalytically active enzymes in the latter pathway. In this pathway, Ago2 cleaves the pre-miRNA at its 3´overhang about 11-12 base pairs away creating a pre-miRNA intermediate, termed Ago2-cleaved pre-miRNA (ac-pre-miRNA). The unwinding process of the passenger strand from the pre-miRNA may be improved by this initial cleavage step. In a next step, ac-pre-miRNA is further cut into mature miRNA via Dicer [19] (Figure 10).

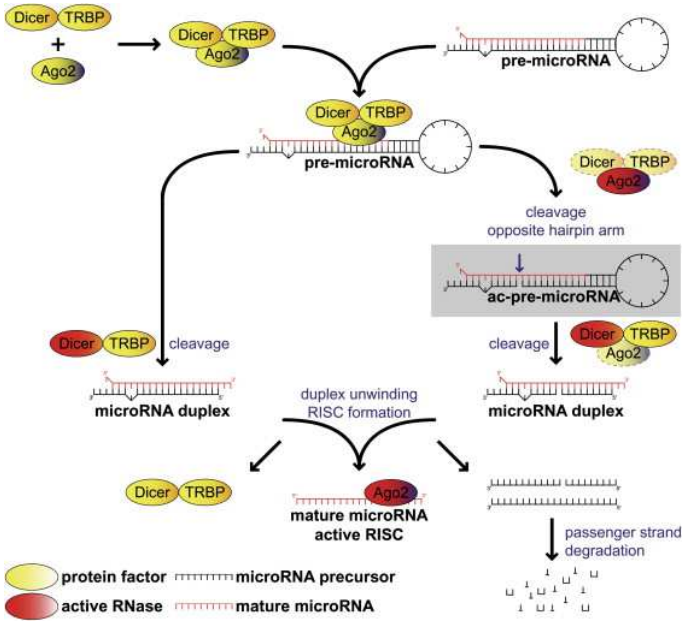

Figure 10. *Schematic Drawing of MicroRNA Processing. After nuclear export, the pre-miRNA incorporates into a preassembled trimeric complex, termed miRNA loading complex (miRLC) or RISC loading complex (RLC). Each miRLC/RLC consists of Dicer, TRBP and Ago2 protein. Either Dicer activity only (left arm of the pathway) or Ago2 and subsequent Dicer activity (right arm of the pathway) mediate the cleavage of the passenger strand from pre-miRNA. In the latter model, Ago2 cleavage creates a pre-miRNA intermediate, termed ac-pre-miRNA, which is further processed via Dicer into a miRNA duplex. After cleavage by Dicer, the resulting miRNA duplex is unwound, Dicer and TRBP dissociate, the passenger strand of the miRNA duplex is degraded, and the mature miRNA forms the RISC together with Ago2. [19]*

1.3.3.3 Dicer-independent Pathway:

Alternatively, a third, Dicer-independent pathway was identified recently. Instead of Dicer, Ago2 slicer activity facilitates the cleavage of pre-miRNAs into functional mature miRNAs indicating that the secondary structure of pre-miRNAs may be an important factor for the choice between the Dicer-dependent and -independent pathway [152].

1.3.3.4 Argonautes and RISC Formation:

Generally, miRNAs incorporate into all human Argonaute proteins (Ago1 – 4) without discrimination for its sequence and facilitate miRNA genesis. This might be established either through enhancing the production or by stabilizing mature miRNAs [19, 153]. The structures of human Ago1-4 are similar to those of fly Ago1 but not fly Ago2 [150]. It is speculated that translational inhibition is the major effect sustained by miRNAs rather than mRNA cleavage [153] since only Ago2 protein can actively cleave mRNAs via its Slicer activity [140, 150, 153, 154]. Hence, the role of Ago2 in miRNA biogenesis might be of major importance, unlike Ago1 or Ago3 [151, 154].

The crystal structure of Argonaute from *Pyrococcus furious* [13] (Figure 11 (A)) and recently from human Argonaute-2 [21] (Figure 11 (B)) give hint of how Argonaute proteins might interact with small RNAs. It is proposed, that the PAZ domain binds the 3´end of RNAs and the PIWI domain consists of an active site (RNase H domain). Overall, the spatial alignment of the respective domains establishes that mRNA and miRNA are bound to Ago in a way that the catalytic center of PIWI mediates mRNA cleavage [13, 21] (Figure 12).

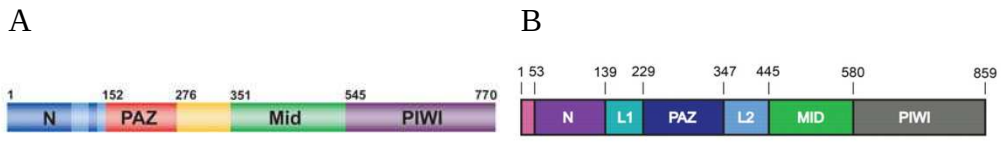

Figure 11. *Schematic Diagram of The Domain Borders of The Crystal Structure of (A) P. Furiosus [13] and (B) Human [21] Ago2 Protein.*

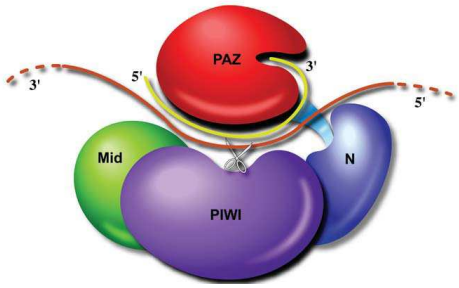

***Figure 12**. Drawing for MiRNA-guided Cleavage of MRNA by Argonaute. The miRNA (yellow) binds with its 3´ end in the PAZ cleft. The mRNA (brown) incorporates between the N-terminal (blue) and PAZ (red) domains. The active site in the PIWI (purple) domain (shown as scissors) cleaves the mRNA opposite the middle of the miRNA guide strand. [13]*

After processing of pre-miRNA to mature miRNA, the miRNA-loaded Ago2 dissociates from the loading complex and forms an active RISC (RNA-induced silencing complex) [141] facilitating cleavage of mRNA with full sequence complementarity to the guide strand of pre-miRNA [140]. ATP is not necessarily needed for the cleavage step but enhances RISC activity [140].

1.3.3.5 Targeting Messenger RNAs

There are 2 major groups describing how miRNAs can interact with a 3´ UTR sequence of mRNAs. The first, "5´ dominant sites", contains two sub-types; a "canonical type" (Figure 13, left) which shows binding at both the 5´ but also at the 3´ region and the other, called "seed type" (Figure 13, middle) that interacts mostly via its 5´ region without hardly any 3´ pairing support. In this class 7 – 8 base-pairs are sufficient for target recognition, pairing and translational repression. Additional binding sites enhance the functionality of pairing [10, 155]. The second major group of miRNAs, called "3´ compensatory sites" (Figure 13, right), interacts mainly via its 3´ region whereas 5´ base-pairing ability is reduced. 5 – 6mers are needed for proper binding functionality [10]. Interestingly, the "seed type"-group may be the largest group among the enormous amount of miRNAs [10, 155].

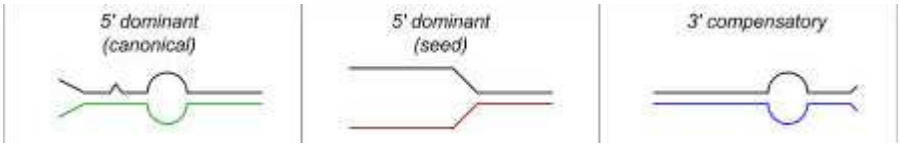

***Figure 13**. Illustration of The Three Classes of MiRNA Target Sites. Model of canonical (left), seed (middle) and 3' compensatory (right) target sites. The mode of pairing between target site (upper line) and miRNA (lower line, color) is illustrated. [10]*

1.3.3.6 Translational Repression or Target Cleavage

Partial sequence complementarities of a miRNA to the 3´UTR of an mRNA results in translational repression of this messenger RNA whereas total alignment leads to target cleavage. Notably, only the antisense strand is used for this mechanism [156, 157]. Additionally, translational repression or target cleavage can be mediated by the same miRNA on several mRNAs depending on the binding properties of the miRNA to its mRNA target [158]. Thus, a set of different miRNAs may show different inhibitory effects on a certain mRNA.

A vast majority of miRNAs were found associated with mRNAs during translation at polysomes. Importantly, miRNAs are bound to mRNAs and not to ribosomes per se [159]. Moreover, microRNAs seem to interfere with polypeptide growth [160] by blocking elongation of translation - rather than inhibition of initiation - and maintain subsequent ribosome drop-off from mRNA in eukaryotic cells [12] (Figure 14).

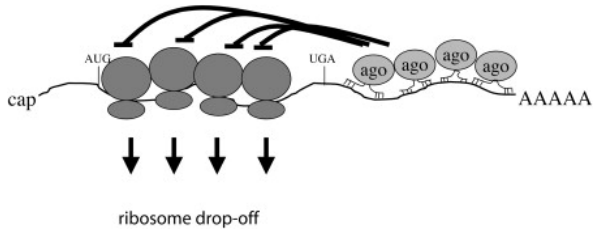

Figure 14*. Illustration of The Influence of MiRNAs on Ribosomes. MiRNAs incorporated into Argonaute proteins bind with mismatches to the 3' UTR of a target gene mRNA causing translating ribosomes to drop-off from the open reading frame of the mRNA. [12]*

MiRNAs promote translational repression of mRNAs via interaction of Argonaute proteins with the cap structure (m7G) of messenger RNAs. Furthermore, repressed mRNAs accumulate as processing bodies (P-bodies or GW-bodies) within the cytoplasm. Thus, processing body formation was suggested to be a consequence of translational inhibition of mRNAs via miRNAs [161]. More exactly, Argonaute proteins contain an m7GTP-cap binding domain which shows strong similarities with the elF4E-m7GTP-cap binding domain known to interact with the m7G-cap of mRNAs during translation. It is suggested that Argonautes can bind both the m7G-cap and miRNA simultaneously and thereby inhibit translation by competing with elF4e [15] (Figure 15). Interestingly, cap-independent translation events are also repressed via small RNAs [12]. Notably, mRNA isoforms carrying different 3´ and 5´UTR variants behave different to miRNA mediated translation inhibition. MiRNA composition on the 3´UTR on the one hand and 3´UTR binding-sites for miRNAs on the other are important factors for

maintaining the translation pattern. Thus, miRNAs can maintain protein levels due to translational inhibition [162, 163].

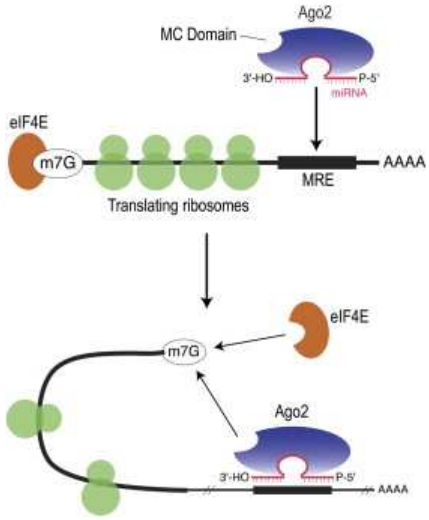

***Figure 15**. Proposed Mechanism of Translational Repression via MiRNAs. The MC domain of Ago2 can bind both the m7G-cap structure and microRNA simultaneously and thereby inhibit translation by competing with elF4e. [15]*

The main objective of miRNAs was proposed to deliver certain proteins to the right location during translational inhibition [164]. First, Argonaute proteins 1 and 2 were shown to be concentrated at certain cytoplasmic focis [165], the latter together with GW182 which is to some extent necessary for proper miRNA mediated mRNA translation inhibition and formation of P-bodies [166-168]. Ago1 and 2 may interact with Dcp1a and Dcp2 but not necessarily at P-body focis but also outside. The interaction of Argonautes with Dcp1a and 2 poses a link to mRNA decay inside P-bodies [168] which is another effect mediated by miRNAs.

Most factors required for 5´ - 3´ mRNA decay are detected in P-bodies; including deadenylation (hCcr4), decapping (hDcp1a, 1b, 2) and decapping activation (hLsm, rck/p54) [169]. Among other proteins, GW182 co-localizes with hLSm4, hDcp1 and hDcp2 [170, 171]. HDcp2 was identified to be the catalytic active enzyme that mediates mRNA decapping leading to m7GDP-intermediate [171] and m7GMP eventually [172] leaving a RNA-fragment with a 5´ terminal phosphate-group prone to degradation (e.g. via 5´-3´exonuclease Xm1). Interestingly, hDcp2 needs a long mRNA template as a substrate to fulfill its function [171]. Since mRNA decapping factors and mRNA breakdown intermediates are enriched in P-bodies it is evident that, among inhibition of translation, decapping and 5´ - 3´ exonucleolytic degradation takes place inside P-bodies [173].

Taken together, canonical miRNAs are transcribed via RNA polymerases II or III and spliced into primary transcripts, termed pri-miRNAs. A multi-protein complex, termed

microprocessor, cuts these small RNA molecules in a two-step cleavage mechanism, mediated by Drosha together with DGCR8, into ~ 70 nt long pre-mature miRNAs. Non-canonical pre-miRNAs are generated from small intronic miRNA (mirtron) sequences via splicesosome-mediated splicing followed by lariat-mediated debranching. Pre-miRNAs are exported into the cytoplasm in an Exportin-5- and Ran-GTP-dependent fashion. After exiting the nucleus, pre-miRNAs are incorporated into a RISC loading complex (RLC) where they are further cleaved either with or without the help of Dicer. Moreover, the guide strand of the resulting duplex is sliced out yielding mature miRNAs and together with Argonaute proteins leave the RLC and bind to complementary sequences in the 3´ UTR of mRNAs creating an active RISC. Translational repression or mRNA cleavage is established depending on either partial or full sequence complementarity of the miRNA to the 3´ UTR of an mRNA (Figure 16). RLC formation, RISC activation, targeting and inhibition/cleavage may occur inside the same cytoplasmic compartment, termed P-bodies or GW-bodies.

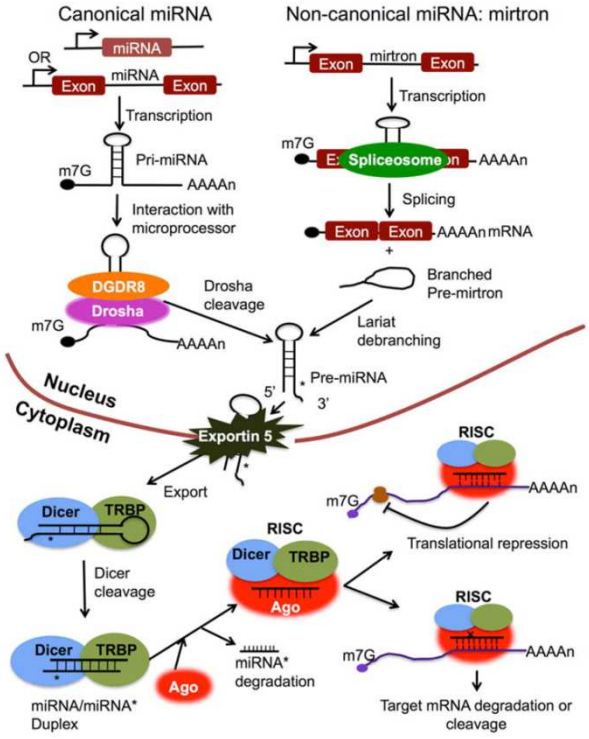

Figure 16*. MiRNA Biogenesis and Effects in Animal Cells. Pre-miRNA is either generated in a canonical pathway via Drosha and DGCR8 function, or in a non-canonical pathway via splicesosome and lariat debranching mechanisms. Following Exportin-5-mediated nuclear export, pre-miRNA is further processed into ~ 22 nt long mature miRNA via Dicer, TRBP and/or Argonaute protein resulting in an active RNA-induced silencing complex (RISC) eventually. RISC mediates either translational repression or target cleavage depending on binding properties of the miRNA to the mRNA. Translational repression is caused by partial sequence complementarities of the miRNA to the 3´UTR of an mRNA. Target cleavage is mediated via perfect sequence complementarity of the miRNA to the 3´UTR of an mRNA. [6]*

The potential of miRNAs to modulate the amount of a gene product at the post-transcriptional level establishes a new layer of regulation amidst the already known regulatory networks in eukaryotes. In contrast to several other regulatory mechanisms, miRNAs exhibit fine-tuning, rather than switching properties. During the recent years, miRNAs have emerged as important helpers in the innate and adaptive immunity. Regulatory mechanisms to various extents were demonstrated to influence the cellular state of immune cells.

1.4 MicroRNAs and The Immune System

The search term “miRNA AND immune system” on the NCBI / PubMed website (http://www.ncbi.nlm.nih.gov/pubmed/) listed 943 hits (Figure 17). Among others, miR-155, -223, -17-92 cluster, -146, -181 and 150 are described best and a short presentation of these microRNAs is given in the following section.

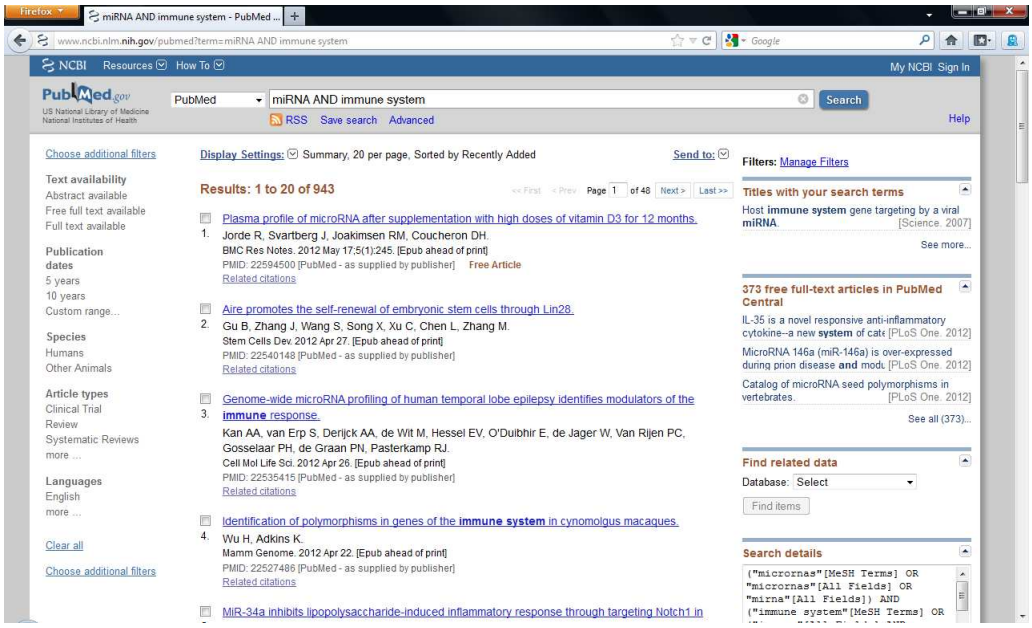

Figure 17*. Screenshot from The NCBI Homepage. The search term “miRNA AND immune system” listed 943 hits.*

A general perspective of microRNAs in the regulation of cell lineages from both the innate as well as the adaptive immune system is shown in Figure 18 (innate immune system) and Figure 19 (adaptive immune system). Evidently, microRNAs can fine-tune processes in the majority of cell types of the immune system.

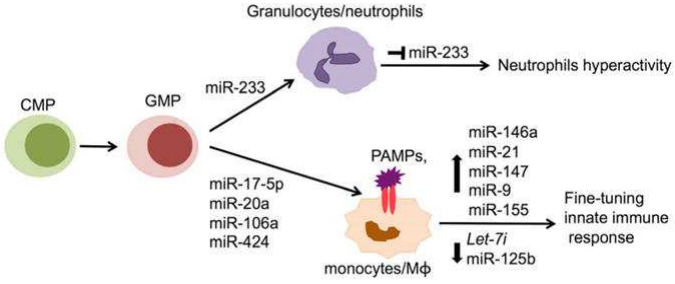

Figure 18*. Illustration of MiRNAs in The Regulation of Innate Immunity. MiRNAs that have been reported to be involved in the development and function of neutrophils and monocyte/macrophage (Mϕ). CMP, myeloid progenitor; GMP, granulocyte-monocyte progenitor; PAMPs, pathogen-associated molecular patterns. [6]*

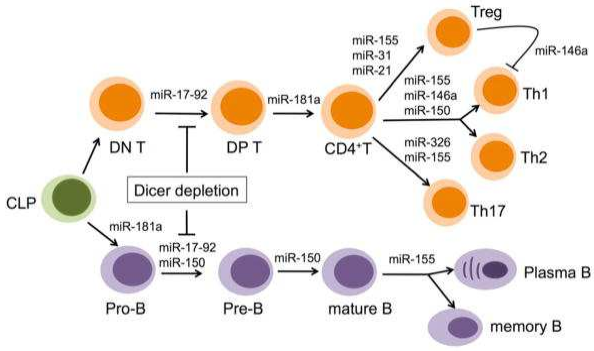

***Figure 19**. Illustration of MiRNAs in The Regulation of Adaptive Immunity. Various miRNAs that have been reported in the development and differentiation of T cells (upper part of the figure), and B cells (lower part of the figure) are shown. CLP, common lymphoid progenitor; DN, $CD4^-$ $CD8^-$ double negative; DP, $CD4^+CD8^+$ double positive; Treg, regulatory T cell. [6]*

1.4.1 Hsa-miR-155

So far, miR-155 is the best characterized miRNA during the immune response. It is located on chromosome 21q21 within a 13kb long sequence which consists of 3 exons, termed B cell integrated cluster (BIC) [174]. The transcript was proposed to act as a non-coding RNA [174, 175], later identified as the microRNA miR-155 [176]. BIC is expressed in the thymus, spleen, bone marrow and mobilized human peripheral blood stem cells [174, 177].

MiR-155 expression is induced in various tissues and cell types (e.g., Dendritic cells, macrophages, granulocytes) of the innate immunity upon poly I:C, INF-β, INF-γ, [178], LPS [3, 179-184], *H.pylori* [185, 186] or *F.tularensis* [187] and enhances TNF-α and IL-6 production [184, 187-189] or reduces IL-8 and GRO-α expression [185, 186]. Several direct targets of miR-155 were identified negatively regulated by miR-155 activation (e.g., FADD, IKKε, Ripk1 and SMAD2 [184, 186]; BACH1, ZIC3, HIVEP2, CEBPB, ZNF652, ARID2 and SMAD5 [190]; SHIP1 [3, 181, 187]; SOCS1 [179]; TAB2 [180]). The network of activators of miR-155 in several cell types and the huge amount of targets mentioned highlights the important role of miR-155 in the regulation of the innate immune response.

Upon encounter of macrophages to certain pathogens, signaling via MyD88 or TRIF [178] and PKC, NF-κB and AP1 activates miR-155 transcription [176, 186, 189, 191] which negatively regulates SHIP1 and activates Akt [181, 187]. Akt1 in turn further enhances miR-155 expression [179]. As a result, TNF-α and IL-6 production is positively influenced by miR-155 induction [187, 189].

The anti-inflammatory cytokine IL-10 counteracts LPS-induced miR-155 expression via STAT3-mediated inhibition of BIC/miR-155 transcription [3]. IL-10 stimulation leads to

increased SHIP1 protein levels (which was shown to limit TLR signaling [188]) due to reduced miR-155 levels [3] (Figure 20). As a consequence, TNF-α and IL-6 production is blocked [188].

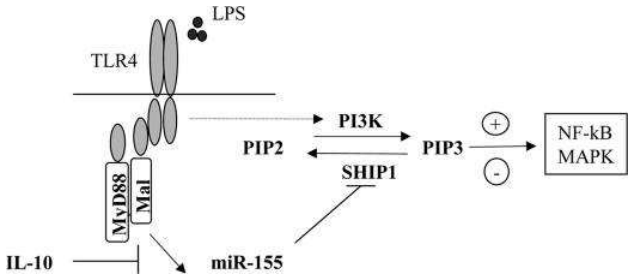

***Figure 20**. Schematic Presentation of a Possible Role for MiR-155. MicroRNA-155 is expressed in response to LPS and leads to decreased SHIP1 expression, thus allowing PI3K activation of NF-κB and MAPK to promote the pro-inflammatory response. However, IL-10 inhibits miR-155 expression and allows SHIP1 expression to recover and promote the conversion of PIP3 back to its inactive PIP2 state, switching off the pro-inflammatory response.* Mal*, MyD88-like adapter protein. [3]*

Additionally to the regulating properties of miR-155 observed in non lymphoid cells, a role for miR-155 in T and B cells was established recently where the loss of bic/miR155 resulted in impaired immune response and increased lung airway remodeling [192]. The humoral reponse to thymus-dependent and -independent antigens by microRNA-155 is required for a proper primary and secondary immune response in extrafollicular and germinal center B cells [193]. The expression of BIC to produce mature miRNA-155 is crucial for the proliferative activity of normal pre-B cells in spleen and bone marrow. Sustained overexpression of miR-155 leads to frank B cell malignancy eventually [194]. MiRNA-155 down-regulation and in turn up-regulation of one of its targets, PU.1, in wild type B cells results in fewer differentiated IgG1 producing cells [193]. The transcription factors IRF4 with BCL6, Pax5, IRF8, and PU.1 are co-expressed in the germinal center in B cells [195]. Upon PU.1 depletion, B cell development was totally blocked suggesting a strong necessity for PU.1 transcription factor in this cell type to initiate/maintain proper differentiation [196]. Thus, a role for miRNA-155 regulating PU.1 in antigen-driven B cell maturation and differentiation of IgG class-switched cells was outlined [193]. BIC/miR-155 induction in B cells was observed during the germinal center response and in primary spleen cells due to B cell receptor (BCR), CD40 or TLR activation via ERK and JNK but not p38 signaling pathways [190, 197]. Moreover, the activation of BCR leads to the induction of c-Fos, FosB and JunB. FosB and JunB in turn are recruited to the promoter region of BIC and can activate its transcription [190]. During class switch recombination (CSR), activation-induced cytidine deaminase (AID) is negatively regulated upon miR-155 activation in B lymphocytes [198, 199]. AID facilitates somatic hypermutations and class-switch

recombination by deaminating cytosine residues and introduction of U:G mismatches in DNA [200, 201]. As a consequence, miR-155 up-regulation maintains the activity of AID and thus has an effect on class switching recombination and somatic hypermutation in B lymphocytes during maturation [202]. AID regulation by microRNA-155 during CSR is important since up-regulated AID results in impaired affinity maturation and defective peripheral B cells eventually [199].

BIC/miR-155 expression was not detected in non-lymphoid tissues (lungs, kidney, brain, liver, and heart) as well as in naïve CD4+ T cells whereas its expression was induced early after activation [197, 203]. Following miR-155 depletion, the anti-inflammatory cytokines IL-4 and IL-10 are up-regulated whereas INF-γ is down-regulated in primary T cells [197]. A role for miR-155 helping to couple innate and adaptive immunity was demonstrated by O´Connell et al. in 2010. T cell development upon inflammatory conditions promotes the development of T_H17 and T_H1 cell subsets. LPS activated dendritic cells produced cytokines (IL-6, IL-23 p19, IL-12 and IL-23 p40, and TNF-α mRNA) necessary for the correct development of T cells, including T_H17 subpopulations, in the presence of miR-155. Loss of miR-155 resulted in defective inflammatory T cell development in vivo [204]. Loss of miR-155 results in reduced numbers of Tregs in thymus and spleen. MiR-155 is required for normal Treg development but is not necessary for proliferation and survival in the periphery [205]. Up-regulation of Foxp3 upon Treg activation is independent of miR-155 [205] whereas in the presence of IL-2, regulatory T cell populations require miR-155 expression to maintain the proliferative activity and thus cell numbers [206]. Interestingly, miR-155 expression is maintained by the transcription factor Foxp3 and sustained Foxp3 expression is required to keep miR-155 levels steady [206, 207].

1.4.2 Hsa-miR-223

The microRNA-223 was found exclusively expressed in the bone marrow but not in the thymus or spleen [208]. In contrast, expression of this miRNA was detected in bone marrow, spleen and lung tissues [1]. MiR-223 is involved in erythropoiesis [209] and granulopoiesis [2, 210, 211]. On the molecular level, PU.1 is an essential transcription factor (TF) to activate miR-223 production which is further enhanced in combination with C/EBPα [212]. Another transcription factor, NFI-A was demonstrated to activate transcription of miR-223 [210]. Following activation, miR-223 negatively regulates the TF E2F1 [210] and Mef2c [211] which is involved in granulocytic differentiation [213]. Thus, increasing levels of miR-223 promote granulocytic differentiation[210] (Figure 21, upper illustration). In contrast, E2F1 is an inhibitor of miR-223 and is repressed upon rising miR-223 levels. Repression of miR-223 by various

mechanisms leads to accumulated numbers of myeloid precursor cells [214] (Figure 21, lower illustration). Sustained miR-223 levels in granulocytes are necessary to maintain the cells proper state and avoid hypermature and hypersensitive granulocytic cells [211].

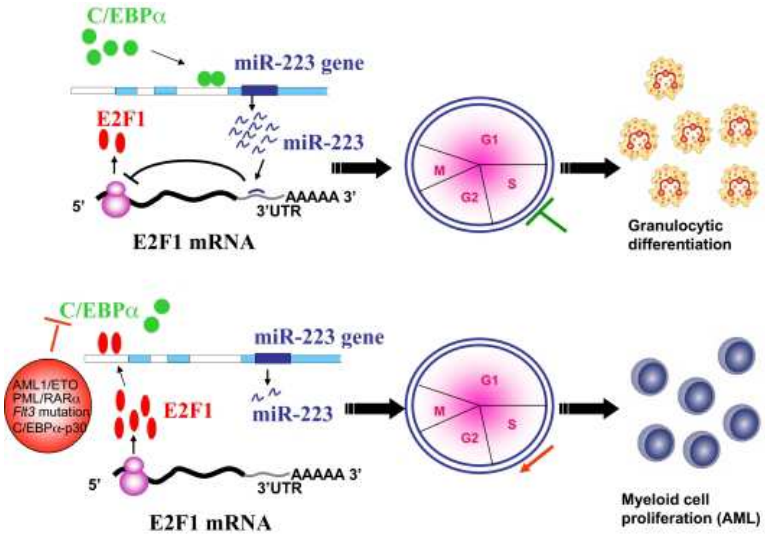

***Figure 21**. Schematic Representation of a Model for The Role of MiR-223 in Innate Immunity. MiR-223 expression promotes granulocytic cell fate via blocking E2F1. Upon various mechanisms inhibiting C/EBP-α and hence miR-223 expression, granulocyte differentiation is blocked and myeloid cell proliferation is promoted. [2]*

MiR-223 is also involved in the adaptive immune response. It is up-regulated in naïve and memory B cells where it negatively regulates LMO2 [215, 216], a germinal center (GC) specific transcription factor [217] and MYBL1 [216]. LMO2 expression was detected in erythroid and myeloid precursors, megakaryocytes, endothelial cells, spleen, various tumor cell lines but also in GC B cells [217]. Thus, downregulation of LMO2 by miR-223 might be crucial to promote the transition from naïve to GC and GC to memory B cells [215, 216].

1.4.3 Hsa-miR-17-92 Cluster

Upon M-CSF stimulation, microRNAs -17-5p, -20a and -106a are down-regulated in undifferentiated monocytic precursor cells. In turn, AML1 (=Runx1) protein, but not messenger RNA, is up-regulated and enhances M-CSFR transcription. Additionally, AML1 facilitates a negative feedback loop which inhibits the transcription of 15-5p-92 and 106a-92 clusters and thus further decreases miRNA -17-5p, -20a and -106a levels. As a result, monocytopoiesis is promoted [4] (Figure 22).

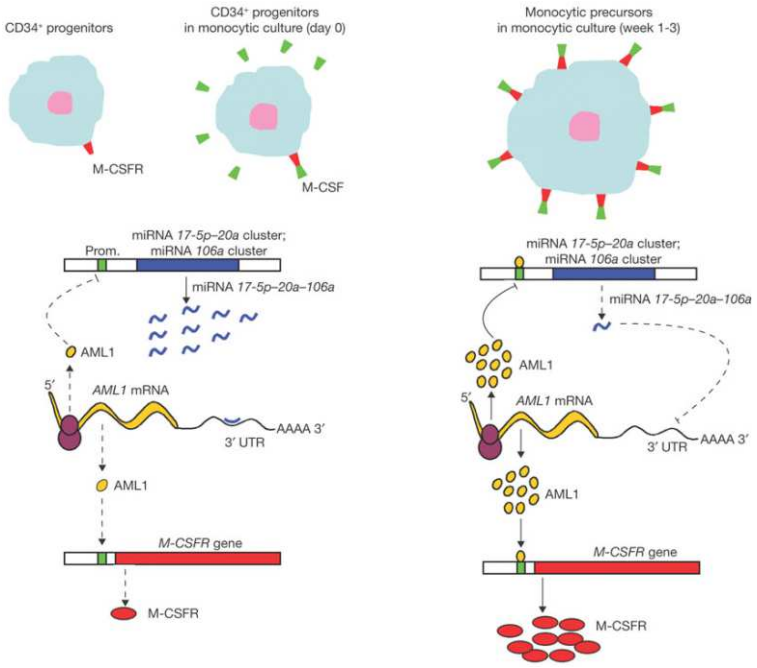

***Figure 22**. Schematic Illustration of MiR-17-5p, -20a and -106a in Innate Immunity. In CD34⁺ progenitor cells, miRNAs* 17-5p, 20a *and* 106a *inhibit* AML1 *mRNA translation and thus further enhance their expression. Low AML1 protein levels determine low M-CSFR expression. Stimulation of the monocytic culture with M-CSF down-regulates miRNA* 17-5p–20a–106a *and increases AML1 protein expression, which in turn inhibits miRNA* 17-5p–20a–106a *transcription and transactivates* M-CSFR*, thus promoting monocytopoiesis. [4]*

The genomic region C13orf25, located on the chromosome band 13q31-q32, hosts a cluster of miRNAs (miR-17, miR-18, miR-19a, miR-19b, miR-20, miR-91 and miR-92) [218] which is expressed in GC cells [216]. This miR-19-92 cluster negatively regulates protein levels of Pten and Bim by targeting its 3´UTR [219, 220]. Interestingly, Bim induces Myc in B cells [221]. Thus, miR-17-92 facilitates pro-B to pre-B transition via down-regulation of Bim protein [219]. Loss of miR-17-92 cluster results in neonatal lethality, defects in the development of the heart, lungs and B cells [219]. Conversely, overexpression of this miRNAs leads to increased lymphocytic proliferation, reduced activation-induced cell death and lymphoproliferative disease and autoimmunity eventually [220].

1.4.4 Hsa-miR-146

MiR-146 is broadly expressed in immune tissue in mice and is further induced in immune cells (myeloid and lymphoid cells) upon cell maturation and/or activation [222]. Activation of miR-146a/b via IL-1α, IL-1β or LPS [223-226] counteracts the production of certain pro-inflammatory cytokines (e.g.: IL-6, IL-8, TNF-α, RANTES) [7, 223, 225]. Expression of miR-146a upon LPS stimulation is up-regulated in precursor cells of the dendritic lineage [224] and in

macrophages [7, 222, 226]. LPS stimulation activates signaling via TLR4-NF-κB-E2F1, which in turn fully activates the transcription of the pro-inflammatory cytokine genes IL-6, TNF-α, CCL3, IL-1β and IL23A [222, 227]. Subsequently, miR-146a expression is induced. MiR-146a acts as a negative regulator of IRAK1 and TRAF6 via translational inhibition and degradation [222, 223, 226, 228]. Steady increasing miR-146a levels after inflammation activation result in decreasing IL-6, IL-8 and TNF-α production via repression of IRAK1 [7, 223] (Figure 23). The anti-inflammatory effect of miR-146 is crucial to prevent immune cells from hyper-responsivness and establishment of immune tolerance [7, 222].

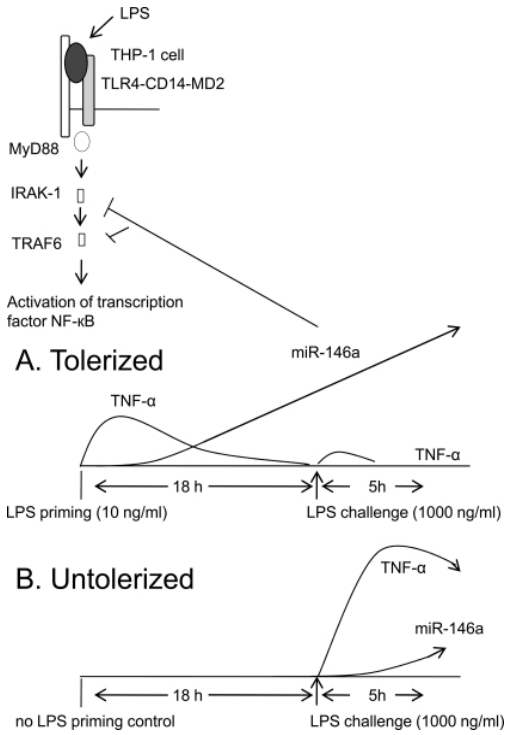

***Figure 23**. A Model of The Role of MiR-146a in LPS-TLR4-mediated Signal Transduction in Tolerized (A) Versus Untolerized (B) Cells. [7]*

The suppressive properties demonstrated in cells of the innate immunity can be allocated to regulatory T cells where miR-146 maintains immunological tolerance [229]. Additionally, miR-146 is expression is elevated in T_H1 and decreased in T_H2 lymphocytes compared to naïve T cell populations [1]. INF-γ activates miR-146a via LMP1 and NF-κB [230, 231]. Stat1, a key regulator for the promotion of T_H1 effector cell differentiation [232] is negatively regulated via miR-146a. Thus, upon activation of T_H1 cells, miR-146 expression counteracts differentiation into T_H1 effector cells [229].

1.4.5 Hsa-miR-181

Various tissues (e.g., thymus, brain, lung and bone marrow) and cell types (e.g., B-lymphoid cells of the bone marrow) in the hematopoietic lineage express miR-181. Hence, miR-181 was suggested to be a positive regulator for B cell differentiation [208]. Upon activation, B cells modulate proper AID function, at least in part, by miR-181b. MiR-181b expression decreases AID at both mRNA and protein levels. As a result, class switch recombination reaction is impaired. In sum, miR-181b is decreased upon B cell activation allowing AID to establish its function in a proper way [233]. Since miR-181 was highly expressed in thymus this miRNA might be of importance during T cell development [208]. The ability of miR-181a-1 to promote development of CD4 and CD8 double positive cells was demonstrated by Liu G et al. in 2008 [234]. Moreover, antigen sensitivity of mature T cells is regulated by miR-181a. During antigen activation, miR-181a modulates the signaling strength downstream of T cell receptor (TCR) by directly targeting of SHP-2, PTPN22, DUSP5 and DUSP6 [235].

1.4.6 Hsa-miR-150

During hematopoiesis, miR-150 promotes megakaryocyte differentiation by targeting the transcription factor MYB [236]. C-Myb fine-tuning is a crucial process in the formation of hematopoietic lineages. Whereas lower levels of c-Myb allows progenitor cells to expanse, suboptimal levels afterwards drives differentiation towards megakaryocytic and macrophage cell fate. In contrast, higher levels of c-Myb promote erythropoiesis and lymphopoiesis [237]. Hence, fine-tuning of c-Myb by miR-150 is a crucial step to initiate differentiation of hematopoietic cells into megakaryocytes [236]. Mature miR-150 is detected in the lymphocytic lineage in mature and resting T and B cells after the cells passed the double negative T cell or pro-B cell stages [1, 238, 239] (Figure 24). Downregulation in naïve T cells might be an effect initiated by TCR engagement which suggests that this miRNA either prevents naïve T cells from differentiation or helps during early stages in differentiation [1]. Immature activation of miR-150 in hematopoietic stem cells blocked the transition from the pro-B to pre-B cell state whereas development into CD4 and CD8 T cells, macrophages or granulocytes was not impaired [239]. C-Myb is a target for miR-150 [240]. Upon immature miR-150 induction, c-Myb protein levels decrease causing B cells to arrest into pro-B cell state [241]. As a consequence, proper temporal expression of miR-150 during lymphopoiesis is required for the development of terminally differentiated B cells.

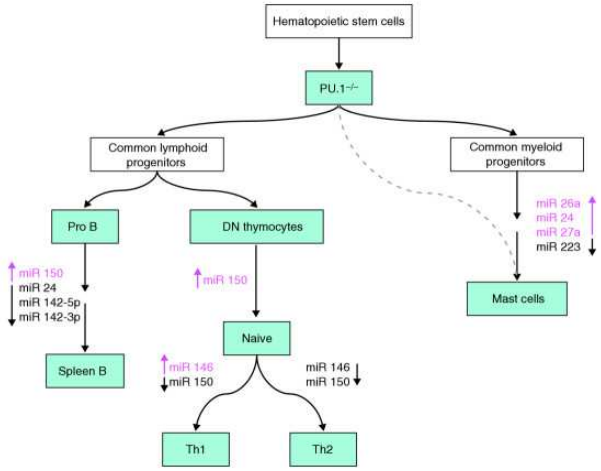

Figure 24*. MiRNA Expression and Lineage Commitment. Illustration of miR-150 in The Adaptive Immunity. Mature miR-150 is detected in the lymphocytic lineage in mature and resting T and B cells after the cells passed the double negative T cell or pro-B cell stages. DN, double-negative. [1]*

1.4.7 Further MiRNAs Involved in The Immune System

Several miRNAs were further identified to fine-tune the proper function of the immune system. IL-10 production upon LPS stimulation of human peripheral blood mononuclear cells (PBMCs) was induced by miR-21 in a MyD88-NF-κB-dependent manner. More exactly, miR-21 down-regulates PDCD4, a suppressor protein for the anti-inflammatory cytokines IL-4 and IL-10 [242, 243]. MiR-147 activation via the signaling cascade TLR4-MyD88-TRIF-NF-κB-STAT1α results in reduced IL-6 and TNF-α levels [244]. MiR-9 was identified as a fine-tuner of NFKB1 protein levels. Its activation upon LPS, TNF-α and IL-1β via TLR4, TLR2 and TLR7/8 was dependent on MyD88 and NF-κB [245]. LPS stimulation increases TNF-α levels partially via decreasing miR-125b, a negative regulator of TNF-α [184]. MiR-let-7i is a negative regulator of TLR4 and thereby decreases the immune response upon pathogen stimulation [246]. The transcription of miR-106a by Egr1 and Sp1 reduced IL-10 production [247]. The transcription factor PU.1 which activates miR-223 transcription [212] is to some extent involved in miR-424 expression in promyelocytic blasts [248]. Following activation, miR-424 induces monocytic differentiation by down-regulation of NFI-A, leading to activation of M-CSFR (monocytic lineage), but not G-CSFR (granulocytic lineage) [248].

The expression of Foxp3 in regulatory T cells is negatively regulated by miR-31 and positively influenced by miR-21 [249]. The repression of IRF4 and PRDM1/BLIMP1 in germinal center B lymphocytes is crucial to prevent the differentiation of these cells [250-252]. A microRNA, miR-125b is enriched in GC B cells and maintains the repression of IRF4 and

PRDM1/BLIMP1 [215]. The differentiation of progenitor T cells into IL-17 producing T_H17 T cells [253, 254] is positively influenced by miR-326 which targets Ets-1 [255], a negative regulator of T_H17 differentiation [256].

2 Aims of The Study

To date, the miRBase database (http://www.mirbase.org/index.shtml) contains 18,226 entries representing hairpin precursor miRNAs – expressing 21,643 mature miRNA products – in 168 different species. 1,527 from these 21,643 mature miRNAs account for homo sapiens. Since miRNAs can modulate immune function, the question must be raised to which extent they contribute to the effects observed after exhaustive exercise. It is of interest, whether specific differentially expressed miRNAs can improve the understanding of processes leading to upper respiratory symptoms. Thus, the aim of this thesis was to gain an overview concerning the impact of extreme physiological exercise (Ironman Austria triathlon) on the expression pattern of microRNAs. More exactly, miRNAs derived from cells homing in the blood stream are of certain interest. Immediate changes in expression levels directly after finshing are of certain interest as well as long term alterations. Moreover, a hypothetical outlook on downstream processes with a focus on immunological parameters should be established. This study should help to understand the involvement of miRNAs upon exhaustive exercise and furthermore elucidate the phase of immunodysregulation / immunosuppression (open window theory) by means of molecular-biological approaches.

3 Methods

3.1 Study Design

The study was conducted in accordance with the Austrian Federal Law, the Declaration of Helsinki (in the revised version of Edinburgh 2000) as well as the ICH-GCP guidelines. The study design was hence developed under particular scientific and ethical care. The study was concipated as a one group analysis conducting a pre-test and three follow-up tests which means a testing point before and three more testing points after (< 20 min, 1 day and 7 days after finishing the race) the competition. Male triathletes aged between 18 – 60 years and having a fixed starting place in the Ironman Austria were set up as inclusion criteria. In contrast, an infection less than one week before the basal test, known autoimmune diseases or known diseases of the immune system or medical intake modulating the immune system (e.g. non-steroidal antirheumatica) were set up as exclusion criteria. Any volunteer participating for the study was excluded from the study when at least on exclusion criterion was applied. Upon an approved ethical comitee vote (Ek.Nr. 454/2010) potential study participants which met the inclusion criteria were invited to a short briefing and were informed about the requirements, aims and procedures of the study. All participants were informed about the attending medical scientist and were provided with the phone number to clarify specific questions. After delivering a signed informed consent, a basal test was conducted and first blood drawing was scheduled.

3.2 Subjects

18 male athletes volunteered to participate in the study. All participants were fully informed about the rationale for the study and of all experimental procedures undertaken. All subjects provided written consent to participate in the study. Subjects were enrolled after having fulfilled all inclusion criteria and presenting none of the exclusion criteria. Subjects completed a comprehensive anthropometric measurement and provided a trainings diary (kind and duration of training as well as training load) before the start of the study. The training diary was used to

estimate the physical condition and the expected finishing time. 2 subjects did not finish (DNF) and 1 did not provide a blood sample 7 days post-race.

3.3 Blood Collection

Blood collection was established using the procedure described in PAXgene® Blood miRNA Kit Handbook (May 2009) (PreAnalytiX, Hombrechtikon, Switzerland). In short, after a small amount of whole blood was drawn into a discard tube, 2.5 mL whole blood was collected from 15 athletes, who participated in the "Ironman Austria", into PAXgene™ Blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) using a BD Vacutainer Safety-LOK Blood Collection Set (BD Vacutainer, New Jersey, USA). Blood was drawn before the competition ("PRE"), within 20 min after finishing ("POST"), 1 day ("1d POST") and 7 days later ("7d POST"). The tubes were inverted immediately 10 times to mix the whole blood with the RNA-stabilizing reagent and stored at -20°C.

3.4 Isolation of Total RNA, Including Small RNAs

Total RNA, including small RNAs, was isolated from whole blood with PAXgene® Blood miRNA Kit (PreAnalytiX, Hombrechtikon, Switzerland) using the automation procedure on QIAcube (QIAGEN, Hilden, Germany) according to the manufacturers protocol. For short, whole blood collected in PAXgene® Blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) were thawed, equilibrated to room temperature and inverted 10 times. The samples were incubated 12 hours to improve RNA yield. Afterwards, samples were centrifuged at 4,165 x g for 10 min and RT. The pellet was then washed once with 4 mL RNase free water and thoroughly resuspended in 350µl Buffer BM1. The sample was loaded onto the QIAcube together with the reagents from the PAXgene® Blood miRNA Kit according to the customers´ instructions and the protocol "*RNA_PAXgeneBloodmiRNA_Blood_PAXgeneBloodmiRNAPartAandB_V1.qpf*" obtained from the QIAGEN homepage (http://www.qiagen.com/qiacube/standard/search.aspx) was run. Afterwards, the samples containing the isolated total RNA were stored at -80°C.

After the automated protocol was fully finished, the quantity and purity of the samples were determined using NanoDrop ND-1000 (Thermo Fisher Scientific, Wilmington, USA). Briefly, 2 µl of each sample was loaded onto NanoDrop ND-1000 and A_{260} and A_{280} values were measured. The quantity and $A_{260/A280}$ ratio were determined for each sample.

Additionally, the integrity (RNA integrated number (RIN)) of the samples was categorized with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany) according to the manufacturer.

3.5 MiRNA PCR Array

3.5.1 Pooling and Reverse Transcription

Total RNA from five individuals (IMI 6, 7, 8, 10, 17) which showed accurate purity ($A_{260/280}$ between 1.9 – 2.1) and quality (RIN > 6.0) were pooled in a time point dependent manner (PRE, POST, 1d POST, 7d POST). Samples were diluted to 18.96 ng / µl in a total volume of 25 µl. Mature miRNA from 12 µl pooled total RNA (227.5 ng total RNA, yielding 0.94 ng cDNA from each sample per well) was reverse transcribed into cDNA using the miScript™ II RT Kit (QIAGEN, Hilden, Germany) and miScript™ HiSpec Buffer according to the manufacturer's instructions. Briefly, each reaction batch (20 µl) - containing 4 µl 5x miScript™ HiSpec Buffer, 2 µl 10x miScript™ Nucleics Mix, 2 µl miScript™ Reverse Transcriptase Mix and 12 µl template RNA - was incubated for 60 min and 37°C on a Thermocycler (Techne, New Jersey, USA). Reverse Transcriptase was inactivated at 95°C and 5 min. Samples were placed on ice and diluted with 200 µl RNase-free water immediately afterwards. CDNA was stored as two 110 µl aliquots at -20°C until further analysis.

3.5.2 PCR Array

Human miFinder miScript™ miRNA PCR Array (QIAGEN, Hilden, Germany) analysis was used for expression profiling of the pooled samples according to the customers protocol (miScript™ miRNA PCR Array Handbook, October 2011). A schematic illustration for this PCR Array is shown in Figure 25. Two replicates for each time point were performed. For each

replicate, from a master mix containing 1,375 µl 2x QuantiTect PCR SYBR Green MasterMix (QIAGEN, Hilden, Germany), 275 µl 10x miScript™ Universal Primer (included in PCR SYBR Green MasterMix), 1,000 µl RNase free water and 100 µl diluted cDNA, 25 µl were used for each well on the 96 well PCR array plate yielding 0.94 ng reverse transcribed mature RNA in each well. PCR was initiated at 95°C and 15 min, 40 cycles - 94°C and 15 sec; 55°C and 45 sec; 70°C and 30 sec - and a dissociation curve analysis was performed on an ABI 7500 real-time cycler (ABI, California, USA). "Automatic baseline" and a C_t threshold of 0.02 were fixed across all PCR Arrays performed for data consistency. The results were exported as a MS Excel 2007 spread sheet for further analysis.

Array Layout

hsa-miR-142-5p A01	hsa-miR-9 A02	hsa-miR-150 A03	hsa-miR-27b A04	hsa-miR-101 A05	hsa-let-7d A06	hsa-miR-103a A07	hsa-miR-16 A08	hsa-miR-26a A09	hsa-miR-32 A10	hsa-miR-26b A11	hsa-let-7g A12
hsa-miR-30c B01	hsa-miR-96 B02	hsa-miR-185 B03	hsa-miR-142-3p B04	hsa-miR-24 B05	hsa-miR-155 B06	hsa-miR-146a B07	hsa-miR-425 B08	hsa-miR-181b B09	hsa-miR-302b B10	hsa-miR-30b B11	hsa-miR-21 B12
hsa-miR-30e C01	hsa-miR-200c C02	hsa-miR-15b C03	hsa-miR-223 C04	hsa-miR-194 C05	hsa-miR-210 C06	hsa-miR-15a C07	hsa-miR-181a C08	hsa-miR-125b C09	hsa-miR-99a C10	hsa-miR-28-5p C11	hsa-miR-320a C12
hsa-miR-125a-5p D01	hsa-miR-29b D02	hsa-miR-29a D03	hsa-miR-141 D04	hsa-miR-19a D05	hsa-miR-18a D06	hsa-miR-374a D07	hsa-miR-423-5p D08	hsa-let-7a D09	hsa-miR-124 D10	hsa-miR-92a D11	hsa-miR-23a D12
hsa-miR-25 E01	hsa-let-7e E02	hsa-miR-376c E03	hsa-miR-126 E04	hsa-miR-144 E05	hsa-miR-424 E06	hsa-miR-30a E07	hsa-miR-23b E08	hsa-miR-151-5p E09	hsa-miR-195 E10	hsa-miR-143 E11	hsa-miR-30d E12
hsa-miR-191 F01	hsa-let-7i F02	hsa-miR-302a F03	hsa-miR-222 F04	hsa-let-7b F05	hsa-miR-19b F06	hsa-miR-17 F07	hsa-miR-93 F08	hsa-miR-186 F09	hsa-miR-196b F10	hsa-miR-27a F11	hsa-miR-22 F12
hsa-miR-130a G01	hsa-let-7c G02	hsa-miR-29c G03	hsa-miR-140-3p G04	hsa-miR-128 G05	hsa-let-7f G06	hsa-miR-122 G07	hsa-miR-20a G08	hsa-miR-106b G09	hsa-miR-7 G10	hsa-miR-100 G11	hsa-miR-302c G12
cel-miR-39 H01	cel-miR-39 H02	SNORD61 H03	SNORD68 H04	SNORD72 H05	SNORD95 H06	SNORD96A H07	RNU6-2 H08	miRTC H09	miRTC H10	PPC H11	PPC H12

***Figure 25**. MiScript Human MiFinder PCR Array Layout. 84 different miRNAs can be detected using this Array. Sample preparation control (cel-miR-39) (not used for this study). House-keeping genes (SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, RNU6-2). miRTC, miRNA reverse transcription control. PPC, positive PCR control.*

3.5.3 Data Analysis

Dissociation curve was analyzed (mature miRNAs T_m = 74.5 – 76.0 °C, Control "PPC" T_m = 17.0 – 19.0 °C) and a default Excel-based miScript™ PCR Array Data Analysis Template for 96-well miScript miRNA PCR Array plates was downloaded from the homepage of SA Biosciences (http://www.sabiosciences.com/mirnaArrayDataAnalysis.php). According to the instructions for the analysis provided in this Excel sheet, C_t values were copy-pasted in the respective cells. The three time points (POST, 1d POST, 7d POST) were compared individually to the control (PRE) in three separate spread sheets and fold-changes were calculated using the $\Delta\Delta C_t$ method. Hsa-miR-30b and SNORD95 were used as "house-keeping" genes. Samples with a fold regulation more or less than 2 where preselected as potential candidates. From this

candidates, the six most prominent miRNAs (hsa-miR-223, -23a, -96, -15a, -126, and -122) were further validated by RT-qPCR.

3.6 Evaluation of Individual Samples

3.6.1 Reverse Transcription

Only the samples where all time points were available (IMI 1 – 11, 13, 14, 17 and 18) were used for reverse transcription. Prior to reverse transcription, the samples were diluted to 10.49 ng / µl in a total volume of 27.6 µl. 12 µl (125.88 ngtotal RNA) was reverse transcribed into cDNA using the miScript™ II RT Kit (QIAGEN, Hilden, Germany) and miScript™ HiFlex Buffer according to the manufacturer's instructions. Briefly, each reaction batch (20 µl) - containing 4 µl 5x miScript™ HiFlex Buffer, 2 µl 10x miScript™ Nucleics Mix, 2 µl miScript™ Reverse Transcriptase Mix and 12 µl template RNA - was incubated for 60 min and 37°C on a Thermocycler (Techne, New Jersey, USA). Reverse Transcriptase was inactivated at 95°C and 5 min. The reaction batches were immediately placed on ice and 3 µl from each sample was diluted with 15.88 µl RNase-free water to yield a concentration of 1 ng / µl. The cDNA stock and the dilutions were stored at -20°C until further analysis.

3.6.2 Standard Curve Generation

Standards for absolute quantification were established using miScript™ miRNA Mimics (miR-223, -23a, -96, -15a, -126 and -122) (QIAGEN, Hilden, Germany) (Table 2). The miRNA mimcs preparations and standard curve dilutions are described in the miScript™ PCR System Handbook, Appendix C (QIAGEN, Hilden, Germany). In short, bacterial carrier RNA (ABI, California, USA) was diluted to 10 ng / µl and 1 ng / µl. MiRNA Mimics were briefly centrifuged, diluted in 250 µl RNase-free water, vortexed and centrifuged again yielding a 20 µM dilution (= 10^{12} copies / µl) for each mimic. 1 µl from the dilutedmimic was further diluted in 1203 µl RNase-free water to obtain a concentration of 10^{10} copies / µl. The miScript™ II RT Kit (QIAGEN, Hilden, Germany) was used to reverse transcribe 5 µl mimic (5 x 10^{10} copies / µl) and 5 µl bacterial carrier RNA (5 x 10 ng / µl) foreach mimic. 5 µl of cDNA was diluted in 120 µl bacterial carrier RNA (ABI, California, USA) (120 x 1 ng / µl) yielding in 10^{8} copies / µl.

Serial dilutions were prepared according to Table 1. 2 µl from each of the dilutions $1x10^3$ - $1x10^7$ copies / µl were used to create a standard curve for absolute quantification.

Table 1. *Dilution Series for The Generation of a Standard Curve from Individual MiRNA Mimics.*

Dilution tube	Volume	Volume (Carrier RNA)	Copies / µl
1	125 µl diluted cDNA		1×10^8
2	5 µl from tube 1	45 µl	1×10^7
3	5 µl from tube 2	45 µl	1×10^6
4	5 µl from tube 3	45 µl	1×10^5
5	5 µl from tube 4	45 µl	1×10^4
6	5 µl from tube 5	45 µl	1×10^3
7	5 µl from tube 6	45 µl	1×10^2

3.6.3 RT-qPCR

MiScript™ Primer Assays (QIAGEN, Hilden, Germany) along with miScript™ MiRNA Mimics (QIAGEN, Hilden, Germany) (Table 2) were used to create a standard curve for each miRNA to validate and absolute quantificate the microRNAs miR-223, -23a, -96, -15a, -126, and -122. MiScript™ Primer Assays (QIAGEN, Hilden, Germany) were diluted in 550 µl TE, pH 8.0 (Sigma-Aldrich, Missouri, USA) and miScript™ MiRNA Mimics (QIAGEN, Hilden, Germany) in 250 µl RNase-free H2O. RT-qPCR runs were performed in 25 µl reaction volumes according to the customers´ instructions. In brief, 1 ng cDNA from each individual and 2 µl from each of the respective standard dilutions (10^3 – 10^7 copies) were taken for individual reactions. Each reaction was performed as duplets. On an ABI 7500 real-time cycler (ABI, California, USA) PCRs were initiated at 95°C and 15 min followed by 40 cycles with 94°C and 15 sec, 55°C and 45 sec and 70°C and 30 sec followed by a dissociation curve analysis directly afterwards. "Automatic baseline" and a "C_t threshold" of 0.02 were fixed across all PCR runs for data consistency. The results were exported as a MS Excel 2007 spread sheet for further analysis.

Table 2. *Primer and Mimic Sequences Used for RT-qPCR Runs and Generation of Individual Standard Curves.*

MiScript Primer Assay	Primer sequence
hsa-miR-223	5' UGUCAGUUUGUCAAAUACCCCA
hsa-miR-23a	5' AUCACAUUGCCAGGGAUUUCC
hsa-miR-96	5' UUUGGCACUAGCACAUUUUUGCU
hsa-miR-15a	5' UAGCAGCACAUAAUGGUUUGUG
hsa-miR-126	5' UCGUACCGUGAGUAAUAAUGCG
hsa-miR-122	5' UGGAGUGUGACAAUGGUGUUUG

MiScript miRNA Mimic	Mimic sequence
hsa-miR-223	5' UGUCAGUUUGUCAAAUACCCCA
hsa-miR-23a	5' AUCACAUUGCCAGGGAUUUCC
hsa-miR-96	5' UUUGGCACUAGCACAUUUUUGCU
hsa-miR-15a	5' UAGCAGCACAUAAUGGUUUGUG
hsa-miR-126	5' UCGUACCGUGAGUAAUAAUGCG
hsa-miR-122	5' UGGAGUGUGACAAUGGUGUUUG

3.7 Statistical Analysis

Data were analyzed per-protocol meaning that only those data were included in the statistical analyses where all time points were available (n=15, 83.3%). A descriptive statistical analysis was performed for the general data gathered from the athletes. All measured data (concentrations from NanoDrop ND-1000 and 2100 Bioanalyzer; RIN values from 2100 Bioanalyzer; absolute quantities of individual samples from RT-qPCR) were tested for normal distribution using Kolomogorov-Smirnov (total samples) and Shapiro-Wilk (separated for time points) test. P-values above 0.05 were considered as normally distributed. A Pearson product-moment correlation was applied to determine a relationship between the concentrations measured with NanoDrop ND-1000 and those obtained from the 2100 Bioanalyzer. A p-value below 0.05 was considered as significant for the correlation tests. A repeated measures ANOVA was further used to determine effects of time point in the individual measurements (concentrations from NanoDrop ND-1000 and 2100 Bioanalyzer; RIN values from 2100 Bioanalyzer; absolute quantities of individual samples from RT-qPCR). To test the data for homogeneity of variance and covariance a Mauchly´s test was conducted. If Mauchly´s sphericity test was not violated ($p > 0.05$), sphericity was assumed, otherwise ($p < 0.05$), a Greenhouse-Geisser correction was applied. If an effect of time was observed a Bonferroni post-hoc test was used and a p-value below 0.05 was considered as significant. Additionally, a Pearson product-moment correlation was applied to determine the relationship between the mean fold change values calculated from the mean absolute quantities of the PCR Array and the RT-qPCR. A p-value below 0.05 was considered as significant for the correlation tests. An unpaired t-test was used to test for an influence of low RIN values on the RT-qPCR outcome. Absolute quantities obtained from RT-qPCR were divided into groups (“All samples” group represents the absolute quantities from all samples, “PCR Array samples” group includes only samples used for

the PCR Array and the “Remaining samples” group which includes all samples except those used for the PCR Array) and differences were determined for each time point.

To understand why these specific statistical tests were used, a short description of each statistical test used in this study is given in the following section:

In this study, data (concentrations from NanoDrop ND-1000 and 2100 Bioanalyzer; RIN values from 2100 Bioanalyzer; absolute quantities of individual samples from RT-qPCR) is analyzed using a repeated measures ANOVA (analysis of variance). Data can be analyzed using a repeated measures ANOVA for two types of study design. Studies that investigate either (1) changes in mean scores over three or more time points, or (2) differences in mean scores under three or more different conditions. Given, that in this study whole blood from the same subjects was analyzed at four separate time points and according to (1), a repeated measures ANOVA could be applied in this case and hence test the data for an effect of time point on measured parameters (absolute miRNA quantities in this study). Repeated measures ANOVA is the equivalent of the one-way ANOVA but for related, not independent groups and is the extension of the dependent t-test. It has the further advantage that it can remove the variability due to the individual differences between subjects since the same subjects are used in each group.

A repeated measures ANOVA is also referred to as a within-subjects ANOVA or ANOVA for correlated samples. All these names imply the nature of the repeated measures ANOVA that of a test to detect any overall differences between related means. The null hypothesis (H_0) states that the means are equal:

$$H_0: \mu_1 = \mu_2 = \mu_3 = \ldots = \mu_k$$

where μ = population mean and k = number of related groups. The alternative hypothesis (H_A) states that the related population means are not equal (at least one mean is different to another mean):

$$H_A: \textit{at least two means are significantly different}$$

Similar to other ANOVA tests, each level of the independent variable needs to be approximately normally distributed since normal data is an underlying assumption in parametric testing. Thus, to run a repeated measures ANOVA, data must be checked for normality (e.g. Kolomogorov-Smirnov (total samples) or Shapiro-Wilk (separated for time points) test). If normality is violated a non-parametric test must be applied.

When data is normally distributed, a repeated measures ANOVA can be applied. Another thing to keep in mind is the fact that ANOVAs with repeated measures (within-subject factors) are particularly susceptible to the violation of the assumption of sphericity. Sphericity is the condition where the variances of the differences between all combinations of related groups (levels) are equal. Violation of sphericity is when the variances of the differences between all combinations of related groups are not equal. Sphericity can be likened to homogeneity of variances in a between-subjects ANOVA. The violation of sphericity is serious for the repeated measures ANOVA, with violation causing the test to become too liberal (i.e. an increase in the Type I error rate). Determining whether sphericity has been violated is, therefore, very important. If violations of sphericity do occur then corrections have been developed to produce a more valid critical *F*-value (i.e. reduce the increase in Type I error rate). This is achieved by estimating the degree to which sphericity has been violated and applying a correction factor to the degrees of freedom of the *F*-distribution.

Taken together, a repeated measures ANOVA can only be applied to analyze changes in mean scores over three or more time points with the prerequisite that data is normally distributed. After checking data for sphericity, a *F*-statistic test is used to determine statistical significant overall differences between related means. The degrees of freedom for time (df_{time}) and error (df_{error}) are determined as well as the effect size (η^2) and must be presented in the results. Anyway, such a test includes no information of where the differences between groups lie and if it is statistically significant. To determine where these specific differences occur, a *post-hoc* test (e.g. Bonferroni, Dunnett, Student Newman-Keuls or Tukey test) must be applied.

A Pearson moment correlation analysis was used to determine a relationship between the concentrations measured with NanoDrop ND-1000 and those obtained from the 2100 Bioanalyzer as well as between the mean fold change values calculated from the mean absolute quantities of the PCR Array and the RT-qPCR. In a normal distributed data set the Pearson product-moment correlation coefficient (or Pearson correlation coefficient for short) is a measure of the strength of a linear association between two variables and is denoted by *r*. Basically, a Pearson product-moment correlation attempts to draw a line of best fit through the data of two variables, and the Pearson correlation coefficient, *r*, indicates how far away all these data points are to this line of best fit. The Pearson correlation coefficient, *r*, can take a range of values from +1 to -1. A value of 0 indicates that there is no association between the two variables. A value greater than 0 indicates a positive association, that is, as the value of one variable increases so

does the value of the other variable. A value less than 0 indicates a negative association, that is, as the value of one variable increases the value of the other variable decreases.

An unpaired t-test was used to test for an influence of low RIN values on the RT-qPCR outcome. Absolute quantities obtained from RT-qPCR were divided into groups ("All samples" group represents the absolute quantities from all samples, "PCR Array samples" group includes only samples used for the PCR Array and the "Remaining samples" group which includes all samples except those used for the PCR Array) and differences were determined for each time point. The unpaired t-test (independent t-test), also called the two sample t-test or student's t-test is an inferential statistical test that determines whether there is a statistically significant difference between the means in two unrelated groups. Unrelated groups, also called unpaired groups or independent groups, are groups in which the cases in each group are different. Using an unpaired t-test requires that the dependent variable is approximately normally distributed within each group. The unpaired t-test assumes equality of the variances of the two groups that are measured. This assumption is determined using Levene's Test of Equality of Variances, If the variances are unequal then this can affect the Type I error rate.

The null hypothesis for the unpaired t-test is that the population means from the two unrelated groups are equal:

$$H_0: u_1 = u_2$$

In most cases, we are looking to see if we can show that we can reject the null hypothesis and accept the alternative hypothesis, which is that the population means are not equal:

$$H_A: u_1 \neq u_2$$

4 Material List

PAXgene® Blood miRNA Kit

Cat. Nr. 763134
PreAnalytiX GmbH
Feldbachstrasse
CH – 8634 Hombrechtikon, Switzerland

Agilent RNA 6000 Nano Kit

Cat. Nr. 5067-1511
Agilent Technologies, Inc.
Hewlett-Packard-Straße 8
76337 Waldbronn, Germany

RNaseZAP®

Cat. Nr. 9780
AMBION, INC
2130 WOODWARD ST.
AUSTIN, TX, 78744, USA

MiScript™ II RT Kit

Cat. Nr. 218160 or 218161
QIAGEN GmbH
QIAGEN Straße 1
D-40472 Hilden, Germany

Human miFinder miScript™ miRNA PCR Array

Cat. Nr. MIHS-001ZA-12
QIAGEN GmbH
QIAGEN Straße 1
D-40472 Hilden, Germany

QuantiTect® PCR SYBR® Green MasterMix

Cat. Nr. 218073 or 218075
QIAGEN GmbH
QIAGEN Straße 1
D-40472 Hilden, Germany

MiScript™ miRNA Mimics

hsa-miR-223 (Cat. Nr. MSY0000280)
hsa-miR-23a (Cat. Nr. MSY0000078)
hsa-miR-96 (Cat. Nr. MSY0000095)

hsa-miR-15a (Cat. Nr. MSY0000068)
hsa-miR-126 (Cat. Nr. MSY0000445)
hsa-miR-122 (Cat. Nr. MSY0000421)
QIAGEN GmbH
QIAGEN Straße 1
D-40472 Hilden, Germany

10x miScript™ Primer Assays

hsa-miR-223 (Cat. Nr. MS00003871)
hsa-miR-23a (Cat. Nr. MS00031633)
hsa-miR-96 (Cat. Nr. MSY0000078)
hsa-miR-15a (Cat. Nr. MS00003178)
hsa-miR-126 (Cat. Nr. MS00003430)
hsa-miR-122 (Cat. Nr. MS00003416)
QIAGEN GmbH
QIAGEN Straße 1
D-40472 Hilden, Germany

TRIS EDTA Buffer Solution pH 8.0

Cat. Nr. 93283
Sigma-Aldrich Corporate
3050 Spruce St.
St. Louis, MO 63103, USA

***E. coli* Total RNA (200µg)**

Cat. Nr. AM7940
Applied Biosystems
850 Lincoln Centre Drive
Foster City, CA 94404, USA

7500 Real-Time PCR System with Dell™ Tower (7500 System SDS Software 1.3.1.22)

Cat. Nr. 4351105
Applied Biosystems
850 Lincoln Centre Drive
Foster City, CA 94404, USA

MicroAmp® Optical 96-Well Reaction Plate

Cat. Nr. 4306737
Applied Biosystems
850 Lincoln Centre Drive
Foster City, CA 94404, USA

TC-512 Techne® Thermal Cycler

Cat. Nr. FTC51H2D
Barloworld Scientific US Ltd
Techne Inc
3 Terri Lane Suite 10
Burlington N.J. 08016, USA

PAXgene™ Blood RNA tubes

Cat. Nr. 762165
PreAnalytiX GmbH
Feldbachstrasse
CH – 8634 Hombrechtikon, Switzerland

BD Vacutainer Safety-LOK Blood Collection Set

Cat. Nr. 367281
BD Diagnostics
Preanalytical Systems
1 Becton Drive
Franklin Lakes, NJ 07417, USA

QIAcube

Cat. Nr. 9001293
QIAGEN GmbH
QIAGEN Straße 1
D-40472 Hilden, Germany

NanoDrop® ND-1000 Spectrophotometer (V3.6.0.)

Device not available any more.
Thermo Fisher Scientific
3411 Silverside Road
Bancroft Building, Suite 100
Wilmington, DE 19810, USA

Agilent 2100 Bioanalyzer

Cat. Nr. G2939AA
Agilent Technologies, Inc.
Hewlett-Packard-Straße 8
76337 Waldbronn, Germany

HERAEUS Fresco 17 Centrifuge

Cat. Nr. 75002420
Thermo Fisher Scientific
3411 Silverside Road

Bancroft Building, Suite 100
Wilmington, DE 19810, USA

HERAEUS Zentrifugen Rotina 420R

Cat. Nr. 4706-50
Hettich AG
Seestrasse 204a
CH-8806 Bäch

5 Results

5.1 Individuals

From 18 male athletes who volunteered to participate, 15 subjects completed the study, 2 subjects did not finish (DNF) the race and 1 did not provide a blood sample 7 days post-race. The general anthropometric data as well as the results from the questionnaire regarding training loads are shown as mean ± SD in Table 3.

***Table 3**. Anthropometric, Training Diary and Ironman Results.*

Anthropometric measurements	**Value**	**N**
Age [yr]	39.8 ± 10.1	15
Body mass [kg]	77.8 ± 8.7	15
Height [cm]	182.1 ± 6.2	15
BMI [kg/m²]	23.4 ± 1.7	15
Training / Race history	**Value**	**N**
Engaged in aerobic exercise [yr]	8.2 ± 6.2	15
Engaged as active triathlete [yr]	3.5 ± 3.0	14
Finished short-distance triathlons	7.7 ± 7.7	12
Finished Ironmans	1.7 ± 0.8	7
Personal best short-distance triathlon [h]	2.3 ± 0.4	9
Personal best Ironman [h]	11.5 ± 1.0	7
Training load before the Ironman	**Value**	**N**
April		
Total [h]	43.6 ± 22.9	15
Swimming [h]	6.5 ± 4.7	15
Cycling [h]	23.6 ± 16.1	15
Running [h]	13.5 ± 7.2	15
May		
Total [h]	36.7 ± 18.2	15
Swimming [h]	6.2 ± 3.9	15
Cycling [h]	17.9 ± 11.9	15

Running [h]	12.4 ± 6.0	15
June		
Total [h]	38.1 ± 19.4	15
Swimming [h]	4.8 ± 3.4	15
Cycling [h]	22.7 ± 15.3	15
Running [h]	10.5 ± 4.9	15
Total training load per week		
Total [h / week]	9.6 ± 5.1	15
Swimming [h / week]	1.5 ± 0.9	15
Cycling [h / week]	5.4 ± 3.3	15
Running [h / week]	3.2 ± 1.6	15
Performance in the Ironman triathlon	**Value**	**N**
Expected		
Total race [h]	11.6 ± 0.7	15
3.8-km swimming [h]	1.4 ± 0.3	15
180-km cycling [h]	5.7 ± 0.4	15
42.2-km running [h]	4.2 ± 0.3	15
Achieved		
Total race [h]	11.8 ± 1.2	15
3.8-km swimming [h]	1.4 ± 0.3	15
180-km cycling [h]	5.8 ± 0.5	15
42.2-km running [h]	4.4 ± 0.5	15

Values are expressed as means ± SD. BMI, body mass index.

5.2 RNA Isolation

5.2.1 NanoDrop ND-1000 – Quantity and Purity

After RNA isolation from whole blood, absorbance at 260 and 280 nm was measured with NanoDrop ND-1000. Total RNA concentration and the purity ($A_{260/280}$ ratio) from the isolated RNA are shown in Table 4. A repeated measures ANOVA was used to test the effect of time point on concentrations. Mauchly´s sphericity test was not violated ($p = 0.170$) and thus sphericity was assumed. There was a significant difference for total RNA concentrations over time for NanoDrop ND-1000 measurements ($F_{3,39} = 2.949$; $p = 0.045$; $\eta^2 = 0.185$). A Bonferroni post-hoc analysis did not confirm this test, although a trend ($p = 0.062$) towards altered concentrations between the time points PRE and 1d POST was reported for NanoDrop ND-1000

measurements. $A_{260/280}$ ratio means were between 2.0 and 2.1 for each time point (1.9 – 2.1 are recommended according to the "PAXgene Blood miRNA Kit" handbook, May 2009).

***Table 4**. Averages for Total RNA Concentrations, $A_{260/280}$ Ratio from Isolated Samples for Each Time Point from NanoDrop ND-1000 measurements.*

Time point	c (NanoDrop ND-1000) [ng/µl]	$A_{260/280}$
PRE	54.71 ± 14.80	2.07 ± 0.06
POST	97.04 ± 55.99	2.01 ± 0.07
1d POST	87.04 ± 37.74	2.08 ± 0.06
7d POST	70.55 ± 37.74	2.09 ± 0.06

5.2.2 Agilent 2100 Bioanalyzer – RNA Integrity

Before PCR Array expression analysis, total RNA integrity must be determined via Agilent 2100 Bioanalyzer. Total RNA concentrations and RIN values were calculated from an electropherogram obtained from this analysis (Table 5). A repeated measures ANOVA was used to test the effect of time point on concentrations. Mauchly´s sphericity test was not violated ($p = 0.243$) and thus sphericity was assumed. No significant effect of time point on total RNA concentrations from Bioanalyzer measurements ($F_{3,39} = 2.048$; $p = 0.123$; $\eta^2 = 0.136$) was reported.

A repeated measures ANOVA was used to test the effect of time point on RIN values. Mauchly´s sphericity test was violated ($p = 0.000$) and a Greenhouse-Geisser correction was applied. No significant effect of time point on RIN values from Bioanalyzer measurements ($F_{1.018,5.088} = 1.911$; $p = 0.225$; $\eta^2 = 0.276$) was reported. RIN value criteria (RIN > 6) were not met for 11 out of 60 samples which could not be used for the PCR Array. Interestingly, 9 of the 11 samples which did not meet the criteria were obtained from time point POST. Taken together, only RIN values from individuals IMI 6, 7, 8, 10 and 17 met the RIN value criteria at all four time points and could be further used for pooling in the PCR Array experiment. In contrast, absolute quantities were determined for all 60 samples RT-qPCR regardless of the RIN value.

***Table 5**. Averages for Total RNA Concentrations, Average RIN Values and Number of Valid Samples for Each Time Point from 2100 Bioanalyzer Measurements.*

Time point	c (Bioanalyzer 2100) [ng/µl]	Average (RIN)	# (valid samples)
PRE	52.00 ± 15.29	8.31 ± 0.26	15
POST	82.93 ± 51.53	7.30 ± 1.53	6

1d POST	81.73 ± 42.36	7.99 ± 0.41	13
7d POST	72.53 ± 38.97	8.11 ± 0.31	15

As mentioned, 9 samples which did not meet the RIN value criteria for the PCR Array were obtained directly after the competition (POST) and showed slightly degraded total RNA. Since RNA fragments from ongoing RNA degradation is masking especially the small RNA fraction (~ 25 – 30 sec) (Figure 26, black rectangle), a RNA-integrated number (RIN) could not be calculated from those samples. In comparison, a RIN value of 8.8 was calculated for the sample IMI 1-1 (Figure 27) and thus posed the most intact sample obtained among all samples. The difference of the eluted RNA fraction during 25 – 30 sec, where normally small RNAs elute, is easily noticeable (black rectangles in Figure 26 and 27).

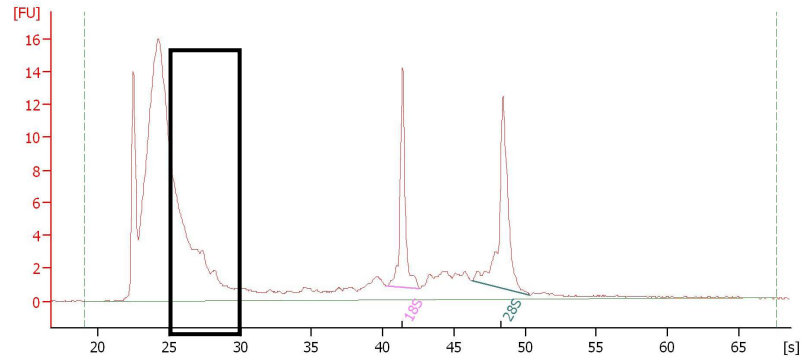

***Figure 26**. Partially Degraded Total RNA Represented in a 2100 Bioanalyzer Electropherogramm. This electropherogram from the sample IMI 2-2 shows partially degraded RNA masking the fraction where typically small RNAs elute (~ 25 – 30 sec, black rectangle). A RIN value could not be calculated from this sample.*

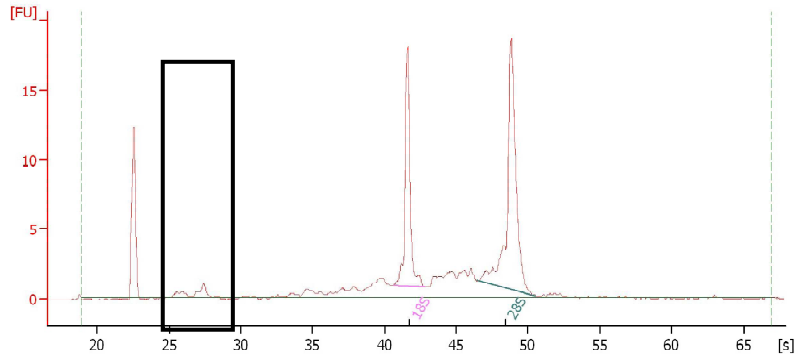

***Figure 27**. Intact Total RNA Represented in a 2100 Bioanalyzer Electropherogramm . This electropherogram from the sample IMI 1-1 and a RIN of 8.80 is of very good quality and represents the highest RIN obtained among all other samples. Of notice is the small RNA fraction eluting at ~ 25 – 30 sec (black rectangle). RIN value from this samples was 8.8.*

5.2.3 Comparison of NanoDrop ND-1000 and Agilent 2100 Bioanalyzer Concentrations

The concentrations obtained from the NanoDrop ND-1000 and 2100 Bioanalyzer measurements were then compared to show the reproducibility of those results. A Pearson product-moment correlation was applied to determine the relationship between the concentrations measured with NanoDrop ND-1000 and those obtained from the 2100 Bioanalyzer. There was a strong, positive correlation between the measured concentrations, which was statistically significant (r = 0.956, n = 59, p = .000) (Figure 28). As a result, both methods provide comparable concentration values.

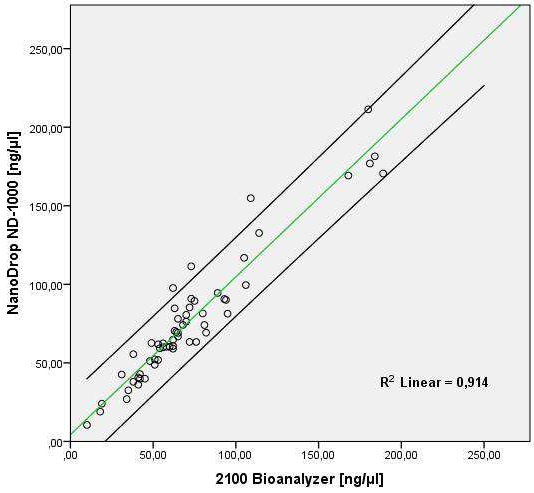

***Figure 28**. Correlation between The Concentrations Obtained from NanoDrop ND-1000 and 2100 Bioanalyzer Measurements. A linear regression curve (green) together with a 95% confidence interval was inserted.*

5.3 Human MiFinder MiScript™ MiRNA PCR Array

SNORD95 and hsa-miR-30b were used as house-keeping genes for the $\Delta\Delta C_t$ method to calculate fold changes for each miRNA after the competition in relation to pre-race conditions. Among 84 observed miRNAs, 26 microRNAs were more than 2-fold down- and 2 microRNAs more than 2-fold up-regulated directly after finishing the triathlon (POST / PRE) (Table 6, highlighted in green and orange). From those miRNAs, only miR-122 was more than 2-fold differentially regulated at all three post-race time points (POST / PRE, 1d POST / PRE and 7d POST / PRE) when compared to pre-race levels. Mature miR-122 levels were more than 2-fold down-regulated directly after the race and remained below pre-race levels even 1 and 7 days after finishing the competition.

***Table 6**. MiRNA PCR Array Fold Regulation. MiRNAs with a fold-change < 2 in at least one time point are shown in orange, miRNAs with a fold change > 2 in at least one time point are shown in green.*

	POST /PRE	1d POST /PRE	7d POST /PRE		POST /PRE	1d POST /PRE	7d POST /PRE
Down-regulated				**Down-regulated**			
hsa-miR-96	-7.44	-1.69	-1.23	hsa-let-7c	-2.43	-1.66	-1.50
hsa-miR-15a	-3.53	-1.29	-1.04	hsa-miR-17	-2.37	-1.66	-1.53
hsa-miR-126	-3.43	-1.74	-1.70	hsa-miR-32	-2.35	-1.44	-1.32
hsa-miR-144	-3.36	-1.37	-1.31	hsa-miR-210	-2.33	-1.35	1.10
hsa-miR-101	-3.33	-1.42	-1.21	hsa-let-7e	-2.32	-1.64	-1.59
hsa-miR-16	-2.84	-1.56	-1.49	hsa-let-7b	-2.29	-1.68	-1.39
hsa-let-7f	-2.69	-1.74	-1.92	hsa-miR-93	-2.26	-1.62	-1.50
hsa-miR-195	-2.67	-1.48	-1.51	hsa-let-7a	-2.19	-1.70	-1.63
hsa-miR-20a	-2.65	-1.64	-1.47	hsa-let-7i	-2.14	-1.62	-1.53
hsa-let-7g	-2.62	-1.60	-1.85	hsa-miR-376c	-2.12	-1.30	1.01
hsa-miR-22	-2.59	-1.23	-1.05	hsa-miR-18a	-2.08	-1.53	-1.40
hsa-miR-122	-2.50	-2.09	-2.27				
hsa-miR-142-5p	-2.49	1.06	1.23	**Up-regulated**			
hsa-miR-106b	-2.46	-1.58	-1.29	hsa-miR-23a	2.51	-1.07	-1.17
hsa-miR-103a	-2.45	-1.57	-1.28	hsa-miR-223	2.70	1.00	-1.02

The most peculiar microRNAs, thus, the three most down- (hsa-miR-96, -15a and -126) and two most up-regulated (hsa-miR-23a and -223) miRNAs as well as hsa-miR-122 (down-regulated at all time points post-race) were chosen for further validation using RT-qPCR.

5.4 RT-qPCR

5.4.1 Absolute Quantification

Six candidate miRNAs were validated with Real-time quantitative PCR (RT-qPCR). Absolute quantities were determined by individual standard curves generated for each miRNA (Table 7).

***Table 7**. Absolute Quantities Obtained from RT-qPCRs.*

[Copies /ng RNA]	PRE	POST	1d POST	7d POST
hsa-miR-96	$(3.6 \pm 1.2) \times 10^4$	$(3.2 \pm 1.6) \times 10^4$	$(3.3 \pm 2.0) \times 10^4$	$(2.7 \pm 0.8) \times 10^4$
hsa-miR-15a	$(3.2 \pm 1.5) \times 10^6$	$(1.6 \pm 1.2) \times 10^6$	$(2.3 \pm 1.2) \times 10^6$	$(2.1 \pm 1.0) \times 10^6$
hsa-miR-126	$(2.4 \pm 1.6) \times 10^6$	$(1.0 \pm 1.2) \times 10^6$	$(1.8 \pm 1.5) \times 10^6$	$(1.6 \pm 1.1) \times 10^6$
hsa-miR-122	$(5.0 \pm 3.4) \times 10^3$	$(2.0 \pm 1.2) \times 10^3$	$(5.8 \pm 7.7) \times 10^3$	$(5.0 \pm 3.3) \times 10^3$
hsa-miR-23a	$(1.8 \pm 0.6) \times 10^7$	$(2.3 \pm 1.9) \times 10^7$	$(1.5 \pm 0.2) \times 10^7$	$(1.2 \pm 0.3) \times 10^7$
hsa-miR-223	$(6.6 \pm 0.0) \times 10^7$	$(11.6 \pm 10.4) \times 10^7$	$(7.1 \pm 2.5) \times 10^7$	$(6.0 \pm 2.1) \times 10^7$

5.4.2 Repeated Measures ANOVA

To test whether the miRNA expression changes over time, a repeated measures ANOVA and a post-hoc Bonferroni test were used. Mauchly's Test of Sphericity indicated that the assumption of sphericity had been violated for all miRNAs, except for miR-15a, where sphericity could be assumed ($p = 0.080$). Therefore, a Greenhouse-Geisser correction was used for these miRNAs. There was a significant effect of time point on absolute quantities of miR-15a ($F_{3,42} = 5.956$; $p = 0.002$; $\eta^2 = 0.298$) and miR-126 ($F_{1.574,22.041} = 4.980$; $p = 0.023$; $\eta^2 = 0.262$). A tendency was reported for miR-23a ($F_{1.194,16.711} = 3.115$; $p = 0.090$; $\eta^2 = 0.182$) and -223 ($F_{1.108,15.511} = 3.358$; $p = 0.083$; $\eta^2 = 0.193$). Time point had no effect on absolute quantities for miR-96 ($F_{1.944,27.220} = 1.473$; $p = 0.247$; $\eta^2 = 0.095$) and -122 ($F_{1.492,20.892} = 2.403$; $p = 0.126$; $\eta^2 = 0.147$). MiR-15a ($p = 0.024$) and miR-126 ($p = 0.047$) levels showed a significant change between the time points PRE and POST. Between POST and 7d POST, miR-126 was significantly altered ($p = 0.013$).

5.4.3 Hsa-miR-15a

According to the PCR Array results listed in Table 6, miR-15a was -3.53 fold decreased directly after (POST / PRE = -3.53), still slightly decreased 1d after (1d POST / PRE = -1.29) and was detected at baseline levels 7d after (7d POST / PRE = -1.04) finishing the competition compared to pre-race values. The results obtained from RT-qPCR confirmed that microRNA levels of miR-15a significantly decreased immediately post-race (POST / PRE = -1.95, p = 0.024) and were not significantly different to pre-race values 1d (p = 0.127) and 7d (p = 0.115) after finishing compared to pre-race conditions. Of note, the decrease observed from RT-qPCR data (-1.95) is to a lesser extent than from observed from the PCR Array data (-3.53) and is very likely an effect from sample pooling. An overall time-course for miR-15a is presented in Figure 29. Figure 30 shows an overview of miR-15a expression changes from each individual (Figure 30 (A)) as well as with certain respect to individuals used for PCR Array (Figure 30 (B)).

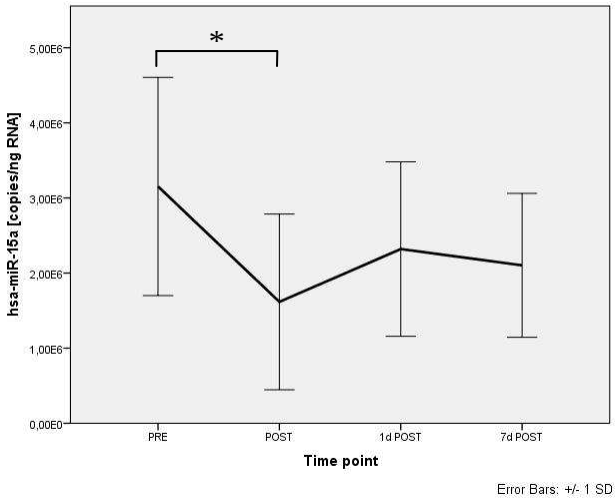

***Figure 29**. Time-course for Hsa-miR-15a from RT-qPCR Results. MiR-15a levels were significantly decreased directly after completion of the Ironman. Error bars indicate ± 1 standard deviation. N = 15.*

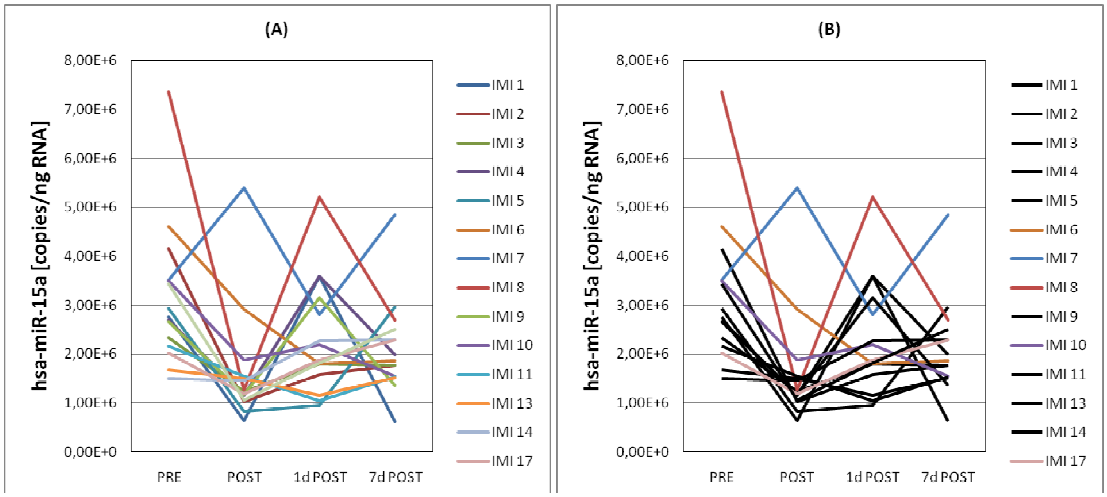

***Figure 30**. Time-course for Individual Subjects for MiR-15a. A time-course from the RT-qPCR results is shown for each subject. (A) Every subject is depicted in an individual color whereas in (B) only time-courses from subjects used for PCR Array are colored.*

5.4.4 Hsa-miR-126

According to the PCR Array results listed in Table 6, miR-126 was -3.43 fold decreased directly after (POST / PRE = -3.43) and remained slightly decreased 1d after (1d POST / PRE = -1.74) and 7d after (7d POST / PRE = -1.70) finishing the competition compared to pre-race values. The results obtained from RT-qPCR confirmed that microRNA levels of miR-126 significantly decreased immediately post-race (POST / PRE = -2.37, p = 0.047) and were not different to pre-race values 1d (p = 0.217) and 7d (p = 0.381) after finishing compared to pre-race conditions. Additionally, miR-126 levels were significantly 1.60 fold up-regulated 7d after compared to immediately post-rave levels (7d POST / POST = 1.60, p = 0.013). Of note, the decrease observed from RT-qPCR data directly post-race (-2.37) is to a lesser extent than from observed from the PCR Array data (-3.43) and is very likely an effect from sample pooling. An overall time-course for miR-126 is presented in Figure 31. Figure 32 shows an overview of miR-126 expression changes from each individual (Figure 32 (A)) as well as with certain respect to individuals used for PCR Array (Figure 32 (B)).

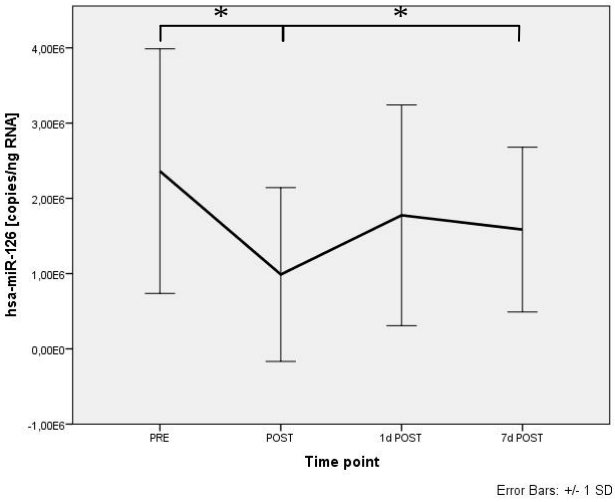

Figure 31. *Time-course for Hsa-miR-126 from RT-qPCR Results. MiR-126 levels were significantly decreased directly after completion of the Ironman. Expression levels were detected significantly lower POST compared to 7d POST. Error bars indicate ±1 standard deviation. N = 15.*

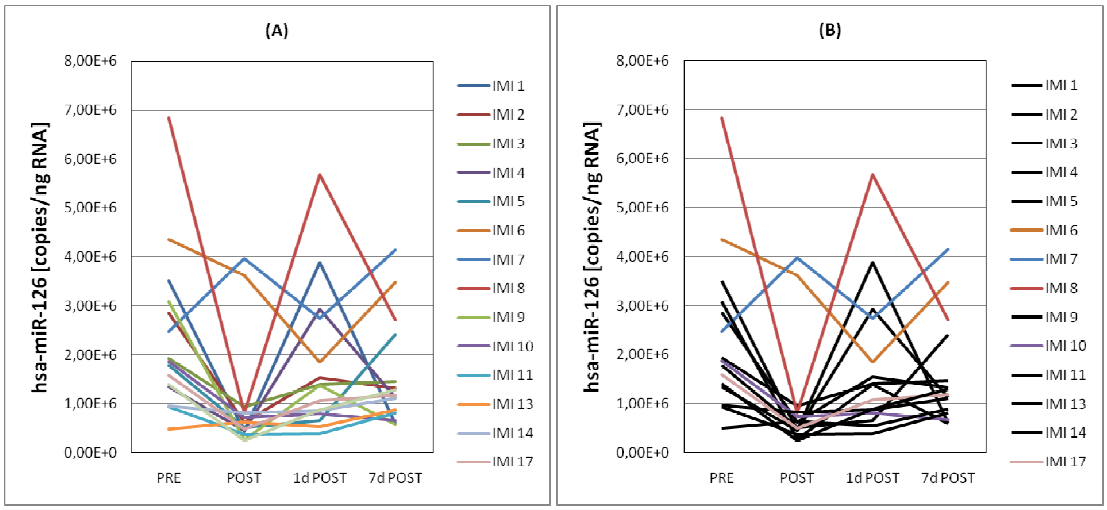

Figure 32. *Time-course for Individual Subjects for MiR-126. A time-course from the RT-qPCR results is shown for each subject. (A) Every subject is depicted in an individual color whereas in (B) only time-courses from subjects used for PCR Array are colored.*

5.4.5 Hsa-miR-96

The PCR Array results are listed in Table 6. Compared to pre-race values, miR-96 was markedly down-regulated directly after (POST / PRE = -7.44) and remained slightly down-regulated 1 day after (1d POST / PRE = -1.69) and 7 days after (7d POST / PRE = -1.23) finishing the competition. These results were not confirmed by RT-qPCR since no effect of absolute quantities over time was reported ($F_{1.944,27.220}$ = 1.473; p = 0.247; η^2 = 0.095). A time-course for miR-96 is presented in Figure 31. An overall time-course for miR-96 is presented in

Figure 33. Figure 34 shows an overview of miR-96 expression changes from each individual (Figure 34 (A)) as well as with certain respect to individuals used for PCR Array (Figure 34 (B)).

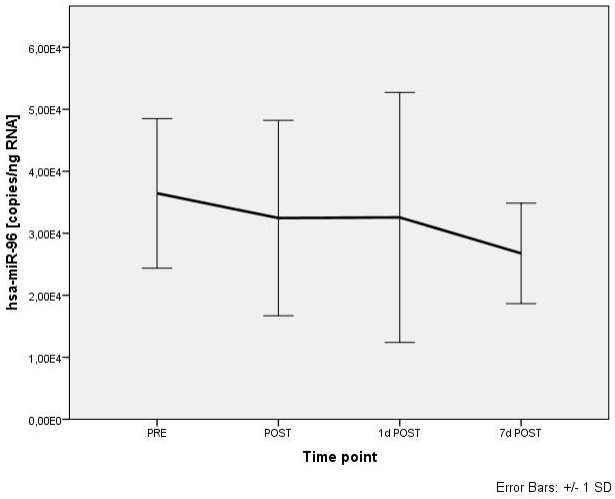

***Figure 33**. Time-course for Hsa-miR-96 from RT-qPCR Results. MiR-96 levels remained unchanged throughout all time points. Error bars indicate ±1 standard deviation. N = 15.*

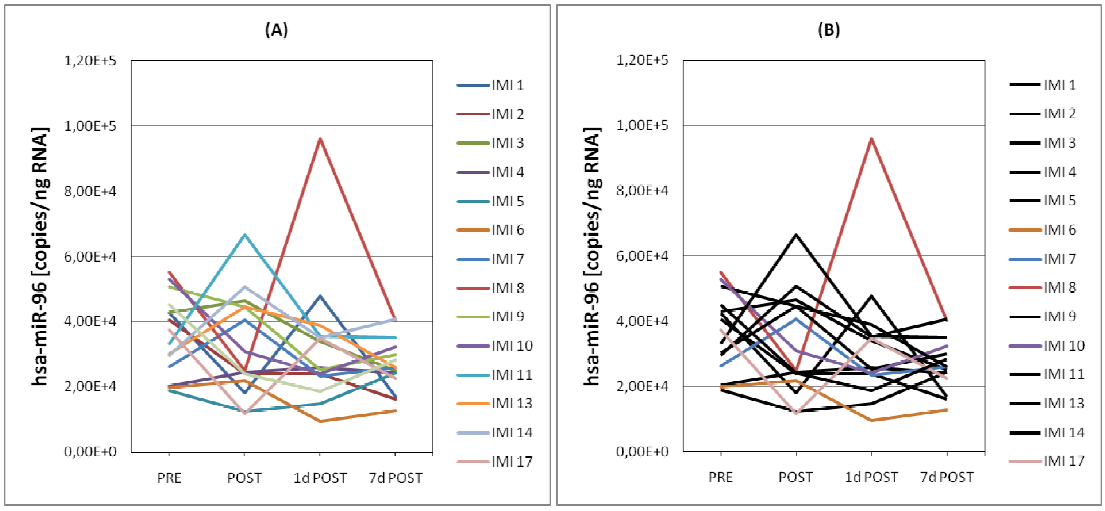

***Figure 34**. Time-course for Individual Subjects for MiR-96. A time-course from the RT-qPCR results is shown for each subject. (A) Every subject is depicted in an individual color whereas in (B) only time-courses from subjects used for PCR Array are colored.*

5.4.6 Hsa-miR-23a

According to the PCR Array results listed in Table 6, miR-23a was up-regulated directly after (POST / PRE = 2.51) and returned to baseline levels 1d after (1d POST / PRE = -1.07) and 7d after (7d POST / PRE = -1.17) finishing the competition compared to pre-race values. The results from the PCR Array were not confirmed by RT-qPCR were only a tendency ($F_{1.194,16.711}$ = 3.115; p = 0.090; η^2 = 0.182) for an effect of time point on miR-23a levels was reported. An

overall time-course for miR-23a is presented in Figure 35. Figure 36 shows an overview of miR-23a expression changes from each individual (Figure 36 (A)) as well as with certain respect to individuals used for PCR Array (Figure 36 (B)).

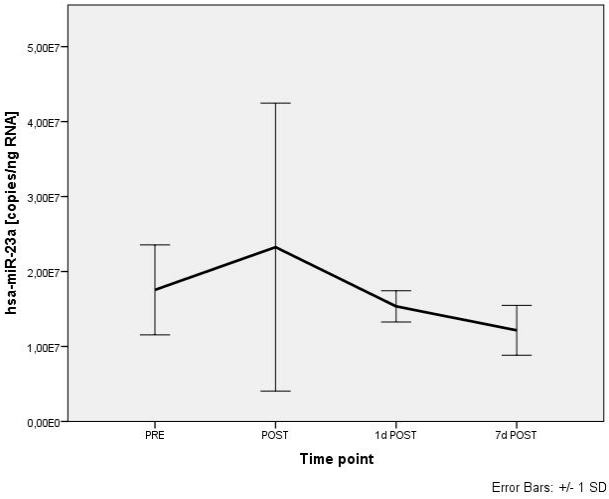

***Figure 35**. Time-course for Hsa-miR-23a from RT-qPCR Results. MiR-23a levels remained unchanged throughout all time points. Error bars indicate ±1 standard deviation. N = 15.*

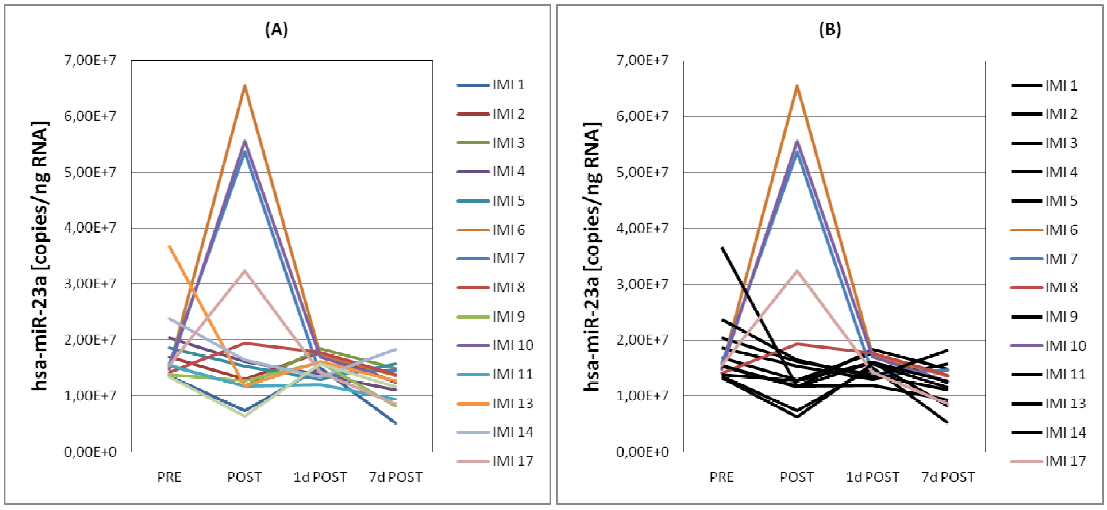

***Figure 36**. Time-course for Individual Subjects for MiR-23a. A time-course from the RT-qPCR results is shown for each subject. (A) Every subject is depicted in an individual color whereas in (B) only time-courses from subjects used for PCR Array are colored.*

5.4.7 Hsa-miR-223

According to the PCR Array results listed in Table 6, miR-23a was up-regulated directly after (POST / PRE = 2.70) and returned to baseline levels 1d after (1d POST / PRE = 1.00) and 7d after (7d POST / PRE = -1.02) finishing the competition compared to pre-race values. RT-

qPCR results reported only a tendency ($F_{1.108,15.511} = 3.358$; $p = 0.083$; $\eta^2 = 0.193$) for an effect of time point on miR-223 levels and thus did not confirm the results from the PCR Array. An overall time-course for miR-223 is presented in Figure 37. Figure 38 shows an overview of miR-223 expression changes from each individual (Figure 38 (A)) as well as with certain respect to individuals used for PCR Array (Figure 38 (B)).

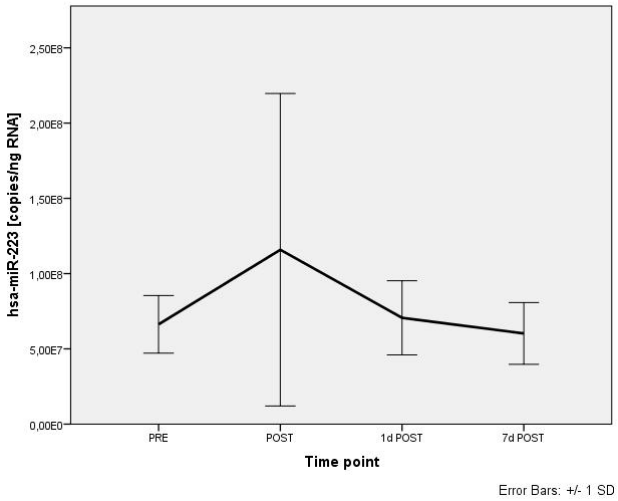

***Figure 37**. Time-course for Hsa-miR-223 from RT-qPCR Results. MiR-223 levels remained unchanged throughout all time points. Error bars indicate ±1 standard deviation. N = 15.*

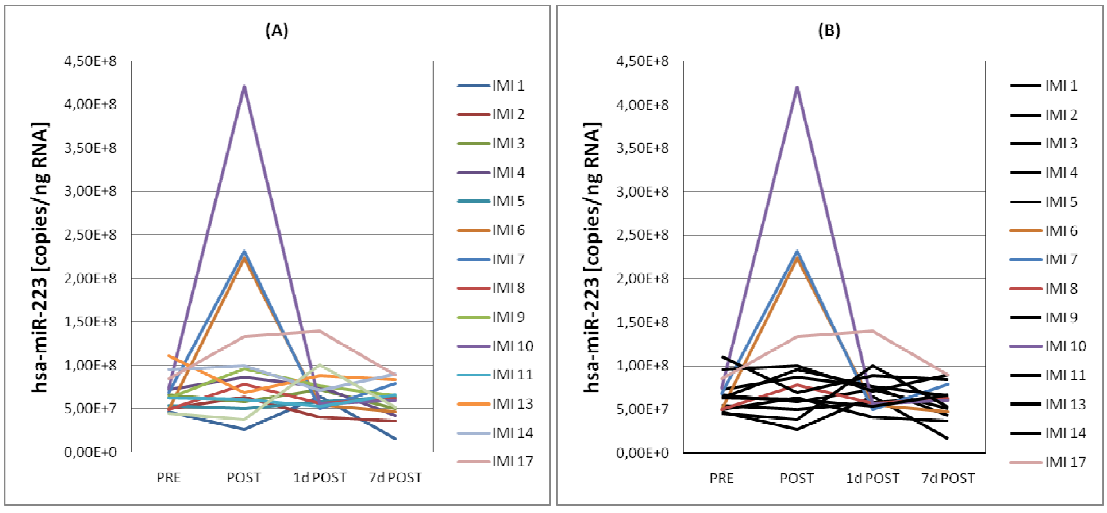

***Figure 38**. Time-course for Individual Subjects for MiR-223. A time-course from the RT-qPCR results is shown for each subject. (A) Every subject is depicted in an individual color whereas in (B) only time-courses from subjects used for PCR Array are colored.*

5.4.8 Hsa-miR-122

The PCR Array results listed in Table 6 shows that miR-122 was down-regulated directly after (POST / PRE = -2.50) and remained down-regulated even 1 day (1d POST / PRE = -2.09) and 7 days (7d POST / PRE = -2.27) after finishing the competition compared to pre-race values. These results were not confirmed since no effect of absolute quantities over time point was reported from the RT-qPCR results ($F_{1.492,20.892}$= 2.403; p = 0.126; η^2 = 0.147). An overall time-course for miR-122 is presented in Figure 39. Figure 40 shows an overview of miR-122 expression changes from each individual (Figure 40 (A)) as well as with certain respect to individuals used for PCR Array (Figure 40 (B)).

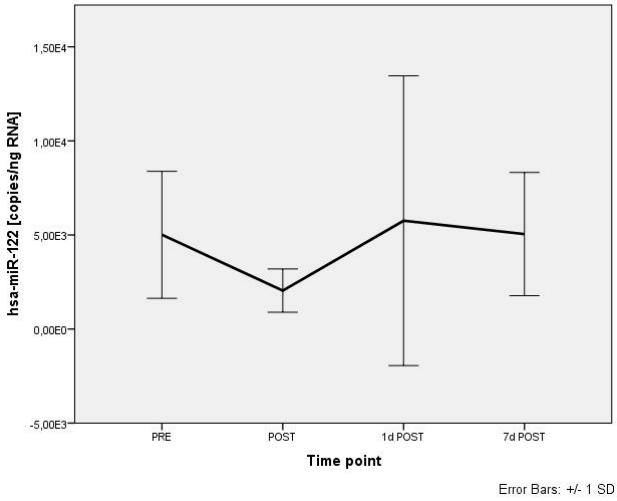

***Figure 39**. Time-course for Hsa-miR-122 from RT-qPCR Results. MiR-122 levels remained unchanged throughout all time points. Error bars indicate ± 1 standard deviation. N = 15.*

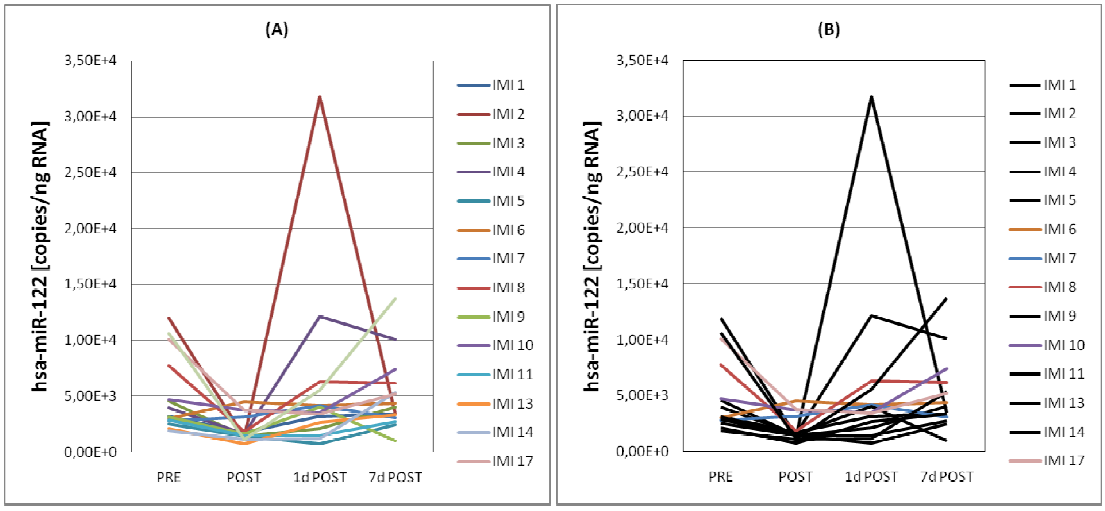

***Figure 40**. Time-course for Individual Subjects for MiR-122. A time-course from the RT-qPCR results is shown for each subject. (A) Every subject is depicted in an individual color whereas in (B) only time-courses from subjects used for PCR Array are colored.*

5.5 Influence of RIN Values on MiRNA Quantities

A Pearson product-moment correlation was applied to determine the relationship between the mean fold change values calculated from the mean absolute quantities of the PCR Array and the RT-qPCR. There was a positive correlation between the obtained mean fold changes from the PCR Array and the RT-qPCR which was statistically significant (r = 0.574; n = 18; p = 0.013) (Figure 35). Thus, both methods provide comparable results.

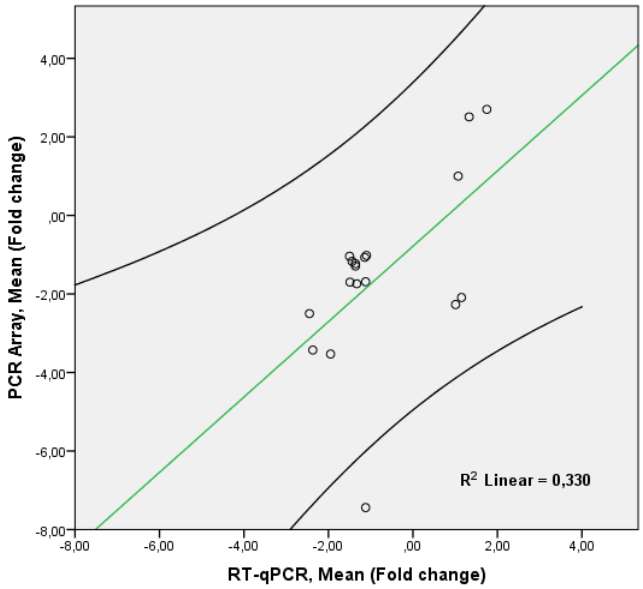

Figure 41. *Correlation between The Mean Fold Changes Obtained from PCR Array and RT-qPCR Measurements. A linear regression curve (green) together with a 95% confidence interval was inserted.*

However, it remains of interest, whether samples with RIN value < 6.0 can influence the overall results obtained from RT-qPCR. Therefore, the results from the RT-qPCR were divided into certain groups within each time point and compared to each other with an independent t-test. Thus, three different absolute quantity groups were set up for each time point; "All samples" group represents the absolute quantities from all samples, "PCR Array samples" group includes only samples used for the PCR Array with RIN values for all time points above 6.0 and the "Remaining samples" group which includes all samples except those used for the "PCR Array samples" group (Figure 36).

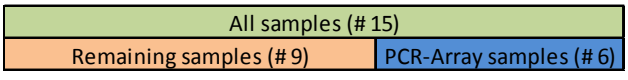

Figure 42. *Groups Created for Each Time Point to Determine a Possible Influence of Low RIN Values. Note that for time point POST "PCR-Array samples" consists of samples with RIN values > 6.0 whereas "Remaining samples consists of samples with RIN values < 6.0.*

MiR-122 and -23a showed significant differences between the groups “All samples” and “PCR Array samples” (miR-122 ($t(18) = -2.315$, $p = 0.030$); miR-23a ($t(18) = -2.231$, $p = 0.039$)), between “All samples” and “Remaining samples” (miR-122 ($t(16.433) = 2.189$, $p = 0.043$); miR-23a ($t(15.278) = 2.172$, $p = 0.046$)) as well as between “PCR Array samples” and “Remaining samples” (miR-122 ($t(4.323) = 4.475$, $p = 0.009$); miR-23a ($t(4.129) = 3.893$, $p = 0.017$)) for the time point POST. Of note, the miR-223 groups showed tendencies ($p < 0.10$ between all three groups) to differ from each other; “All samples” and “PCR Array samples” ($t(18) = -1.716$, $p = 0.091$); “All samples” and “Remaining samples” ($t(16.173) = 1.826$, $p = 0.086$); “PCR Array samples” and “Remaining samples” ($t(4.136) = 2.594$, $p = 0.058$). No significant differences between the groups were reported for miR-15a, -96 and -126. Taken together, significant differences between the means of the individual quantity groups (“All samples”, “PCR Array samples” and “Remaining samples”) were reported for miR-122 and -23a solely at the time point POST. No statistically significant differences were noticed for the other three time points (PRE, 1d POST, 7d POST) for any other miRNA. This indicates that the majority of samples with low RIN values observed - which were taken at the second time point (POST) - might influence the RT-qPCR results. However, only 2 out of 6 tested microRNAs exhibited this pattern. Thus, low RIN values might only to some extent influence RT-qPCR results. Moreover, the RT-qPCR results with significantly differing averages (miR-122 and -23a) must be treated and interpreted with caution, since insufficient sample quality might influence or bias those results.

6 Discussion

To date, a role for miRNAs in sports as well as in the context of URTI / URS development is unclear. Thus, an aim of this study was to gain insight into which miRNAs are differentially regulated upon exhaustive exercise and to what extent. In this study, 15 individuals took part in the Ironman Klagenfurt Triathlon in 2010. Whole blood samples were taken before, immediately after (< 20 min) as well as 1 day and 7 days post-race. Total RNA including small RNAs was isolated and further analyzed using a Bioanalyzer 2100. Total RNA samples with a RIN more than 6 were pooled in a time-dependent manner and the expression pattern of 84 miRNAs was determined with a miRNA PCR Array. 28 miRNAs were found more or less than 2-fold regulated directly after finishing the race compared to pre-race conditions. The most differentially regulated miRNAs (miR-96 (-7.44), -15a (-3.53), -126 (-3.43), -223 (+2.70), -23a (+2.51)) as well as miR-122 - which remained decreased at all time points post-race compared to pre-race values (-2.50 ; -2.09 ; -2.27) - were further validated using RT-qPCR. The PCR Array results obtained for miR-96, -223, -23a as well as -122 could not be confirmed from the RT-qPCR results although a tendency for up-regulated miR-223 and -23a levels post-race was noticed. Decreased mir-15a (-1.95, $p = 0.024$) and -126 (-2.37, $p = 0.047$) levels post-race compared to pre-race conditions were detected from RT-qPCR results and thus confirmed the observations from the PCR Array.

It remains of interest, whether the unconfirmed miRNAs (miR-96, -223, -23a and -122) would nevertheless have a potential role in mechanisms of cells of the immune system. MiR-96 could not be attributed to certain immunological processes so far. This microRNA was shown to negatively regulate FOXO1 [257] and FOXO3 [258] resulting in metastasis of certain tumors. Moreover, platelete reactivity could be attributed to miR-96 action [259] as well as progressive hearing loss [260]. Thus, miR-96 can either be unknown in sports exercise or simply be a false positive result obtained from the PCR Array data. In contrast, miR-223 modulates several mechanisms in the immune system such as interleukin production in peripheral blood leukocytes [261], modulation of the non-canonical NF-κB pathway during macrophage differentiation [262], modulation of CD4+ naïve lymphocytes of rheumatoid arthritis patients [263], modulation of memory B cells [215] as well as regulation of granulocyte development [210] and function [211]. Since a tendency for miR-223 regulation after the Ironman was detected by RT-qPCR

measurements, it remains of interest whether significant changes in miR-223 levels would be observed in a larger study cohort. Unlike the obvious important role for miR-223 in immune function, miR-23a could not be attributed to such a variety of mechanisms. However, an important function was shown by Zhu et al. in 2010 [264] where a negative regulation of interleukin-6 receptor by miR-23a was demonstrated. Given, that its ligand, IL-6, is up-regulated upon exhaustive exercise [65-67], a modulation of IL6R by miR-23a [264] might have an impact on one of the most important signaling pathways of the immune system. Like miR-223, a study with larger cohorts would clarify whether there is really a significant difference of miR-23a upon exhaustive exercise. The last microRNA which could not be confirmed by RT-qPCR is resembled by miR-122. Interestingly, this miRNA was reported down-regulated at all time-points post-race compared to pre-race values by PCR Array analysis. To date, miR-122 was reported to be involved in hepatocellular carcinomas [265] where it directly targets cyclin G1 [266] and Bcl-w [267]. Furthermore, miR-122 enhances hepatitis C virus replication [268] via stimulating hepatitis C virus RNA translation [269]. A link between sports and miR-122 is not yet established and may be a task for future studies.

Unlike miR-96, -223, -23a and -122, microRNAs miR-15a and miR-126 levels showed significant altered expression levels after the Ironman. How and to what extent miR-15a and miR-126 might contribute to a dysregulated immunological response upon exhaustive exercise is unclear. Mir-15a was shown to form an autoregulatory feedback loop with c-Myb creating an important function for this miRNA in normal hematopoietic lineage [270]. Forced miR-15a expression halted K562 myeloid leukemia cells as well as human CD34+ cells in G1 phase and blocked erythroid and myeloid colony formation in the latter. Thus, miR-15a expression can be activated by c-myb in hematopoietic cells and regulate the cell cycle as well as differentiation [270]. C-myb protein, which expression can be induced via IL-2 in T-lymphocytes, is important to protect T-cells from programmed cell death [271]. Given that miR-15a was found decreased in this study, it is of interest whether c-myb levels were elevated and T-cell apoptosis decreased. It can be speculated whether miR-15a contributes to dysregulated T-cell populations observed upon exhaustive exercise [58, 59].

Recently, uncoupling protein 2 (UCP-2) was determined as as target for miR-15a and UCP-2 protein levels are negatively regulated by this miRNA [272]. Overexpression of miR-15a decreased UCP-2 levels which in-turn resulted in intracellular accumulation of insulin in MIN6 cells. UCP-1, UCP-2 and UCP-3 are constituents of the inner mitochondrial membrane [273]. Trenker et al. (2007) [274] demonstrated the importance for UCP-2 and UCP-3 in Ca^{2+} uptake

into mitochondria. Various roles for uncoupling proteins are described, e.g. after endurance exercise, elevated UCP gene expression [275, 276] and protein levels [277] were observed. No effect on UCP-2 was detected upon acute endurance exercise in human volunteers whereas UCP-3 gene expression was increased in untrained subjects [278]. UCP-2 is involved in controlling the generation of reactive oxygen species (ROS) [279] by mitochondria [280]. Overexpression of UCP-2 in endothelial cells reduced ROS generation [281]. A function for UCP-2 in the heart was presented by Teshima et al. in 2003 where overexpression of UCP-2 protected cardiomyocytes against oxidative stress by H_2O_2 [282]. Interestingly, UCP-2 expression in monocytes is typically low and is elevated upon H_2O_2 [283]. As a consequence, elevated UCP-2 levels decrease expression of adhesion molecules, e.g. β2 integrin, CCR2, ICAM-1 and VCAM-1 [283, 284] and may reduce the ability of monocytes to adhere to endothelial cell layers. Taken together, decreased miR-15a levels upon exhaustive exercise may further elevate UCP-2 protein levels in monocytes to counteract cellular stress. Moreover, adherence to endothelial cells by monocytes may decrease by a reduction of adhesion molecules (e.g. β2 integrin, CCR2, ICAM-1 and VCAM-1) [283, 284]. This in-turn may lead to elevated circulating monocytes observed upon exhaustive exercise lasting for ~ 2h and is most likely due to mobilization of marginated monocytes into the blood circulation destined to become mature tissue macrophages [28]. The circulating monocytes show reduced IL-6, IL1-α and TNF-α production which might be a consequence of down-regulated TLR expression [33-36]. In contrast to monocytes, UCP-2 is expressed in human mast cells and can down-regulate degranulation upon stimulation. Additionally, UCP-2 can negatively regulate histamine production and IL-6 secretion [285]. Of certain interest, LPS-stimulated macrophages showed reduced UCP-2 levels [286] and UCP-$2^{-/-}$ mice were immune to infection with *Toxoplasma gondii* as well as *Listeria monocytogenes* caused by dramatically elevated ROS levels [280, 287] suggesting an important role for UCP-2 in the immune system.

Taken together, differentially UCP-2 expression is observed in various cell types of the innate immune system and can be regulated by miR-15a. To what extent miR-15a contributes to this regulatory feedback remains unknown as well as if a dysregulation of UCP-2 can lead to upper respiratory symptoms observed after extreme physiological exercise. It is of interest, whether antagomirs for miR-15a would help to reduce upper respiratory symptoms observed after exhaustive exercise. Our observation would suggest increased UCP-2 protein levels and hence reduced ROS activity in cells of the innate immune response. Given that microRNAs exhibit fine-tuning properties, miR-15a regulation of UCP-2 protein levels may sustain a

regulatory pattern to inhibit hyperreactivity induced by reactive oxygen species. Moreover, monocytopoiesis observed upon exhaustive exercise might be a consequence of miR-15a/UCP-2/adhesion molecule regulation. Since reduced IL-6 release was observed from circulating monocytes upon exhaustive exercise [33-36], a regulation for IL-6 release that involves UCP-2 similar to mast cells [285] may exist in monocytes.

Similar to miR-15a, miR-126 was down-regulated as well directly after finishing the race (-2.37, $p = 0.047$) compared to pre-race values. No further changes were reported for this microRNA. MiR-126 is transcribed from chromosome 9q34.3 from an intronic sequence between Exons 7 and 8 of the epidermal growth factor-like 7 (Egfl 7) gene [288] and prominently expressed in endothelial cells from unbilical vein, aorta skin and brain [288] as well as from heart, lung and other vascularized tissues [289-291]. Ets-1 and Ets-2 were validated as activators of the miR-126/Egfl 7 gene [288].

The proinflammatory molecules Angiotensin II and TNF-α can induce Ets-1 expression in vascular smooth muscle cells (VSMC) and macrophages via ERK1/2 [292]. Ets-1 induces monocytes chemoattractant protein-1 (MCP-1), VCAM-1 [293] and miR-126 [288]. Taken together, TNF-α is released into the circulatory system after exhaustive exercise [67, 73, 76-78] which might be a result of beginning inflammation upon muscular damage [77] and can induce Ets-1 expression in VSMCs and macrophages. Ets-1 in-turn activates transcription of MCP-1 and VCAM-1. However, Ets-1 induces miR-126 transcription as well and miR-126 was shown to negative regulate VCAM-1 translation [289]. Given, that elevated miR-126 levels were not observed directly after finishing the triathlon but the other way around, other simuli might dominate over TNF-α/ERK1/2/Ets-1 mediated miR-126 expression. Similar to miR-15a [272, 283, 284], decreased miR-126 levels might lead to reduced adherence of monocytes/macrophages and hence might contribute to monocytopoiesis observed upon exhaustive exercise [28].

MiR-126 can be down-regulated upon ER (endoplasmic reticulum) stress and was shown to negatively regulate TOM1 [294]. Overexpression of TOM1 can suppress activation of NF-κB and AP-1 induced by IL-1β or TNF-α [295]. In addition, negative signaling to NF-κB by TOM1 can be mediated upon LPS stimulation via TLR4 as well [294]. TOM1 knockdown exhibited a significant elevation of NF-κB mediated IL-8 secretion in response to LPS or IL-1β [294]. IL-8 is a member of the CXC chemokine family and can facilitate neutrophil attraction [296-299]. Diminished IL-8 secretion caused delayed neutrophils chemotaxis in cystic fibrosis lung tissues [300]. Thus, LPS, IL-1β or TNF-α can elevate IL-8 secretion via activation of NF-κB amplifying

neutrophil chemotaxis eventually [294, 295, 301]. NF-κB can be suppressed by TOM1 which is in-turn negatively regulated by miR-126 [294] (Figure 37).

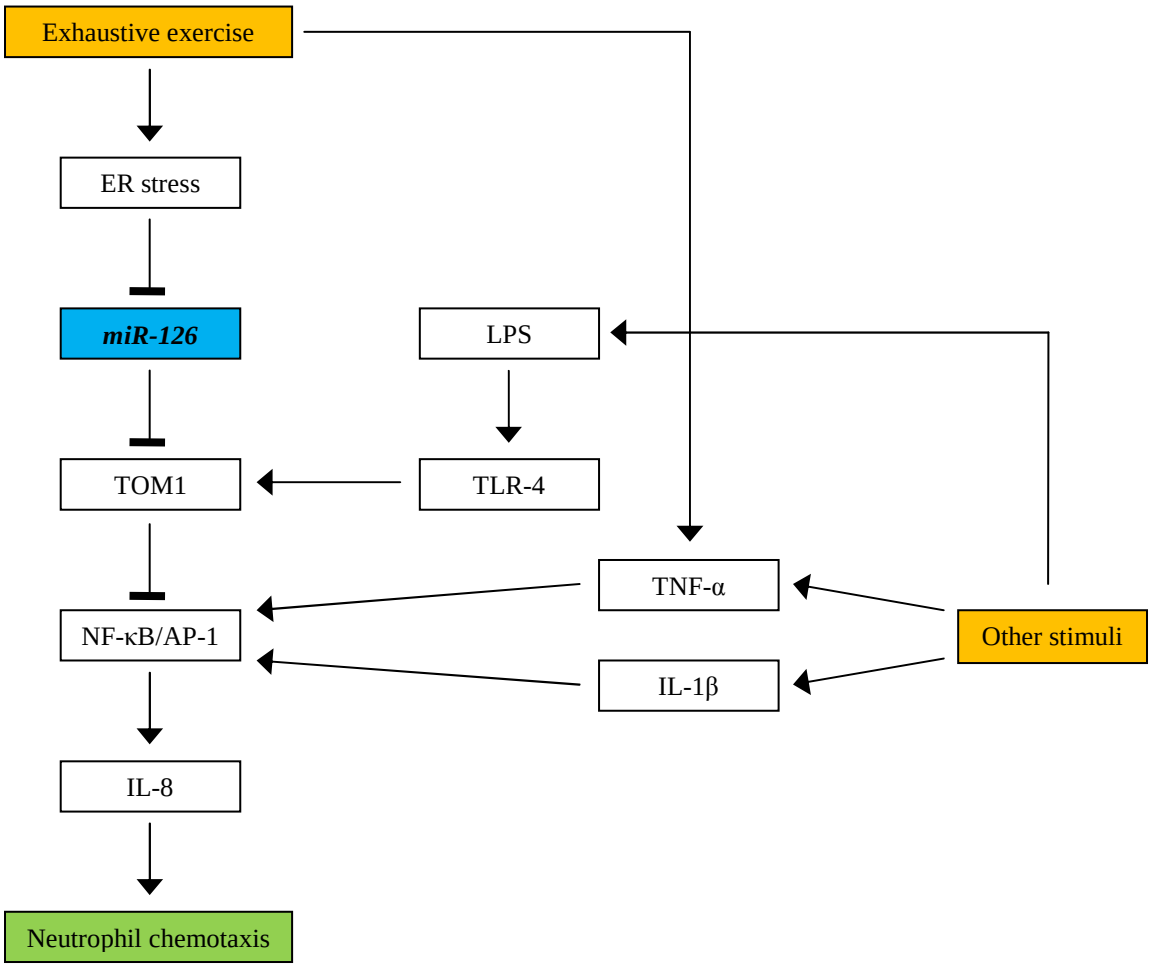

***Figure 43**. Schematic Drawing of a Hypothetical Pathway Regulating Neutrophil Chemotaxis Involving MiR-126. Upon exhaustive exercise, ER (endoplasmic reticulum) stress blocks miR-126 expression. Subsequently, TOM1, a direct target of miR-126, is up-regulated and counteracts TNF-α or IL-1 induced NF-κB/AP-1 signaling. In-turn, IL-8 and hence neutrophil chemotaxis is reduced. LPS signaling via TLR-4 enhances miR-126 action by further activating TOM1 expression. Ectopic stimuli a colored orange, miR-126 is colored blue and effects are colored green.*

Our results showed reduced miR-126 levels directly after finishing the race compared to pre-race conditions which would suggest higher TOM1 and lower NF-κB levels respectively. Moreover, it remains of interest whether this would lead to decreased IL-8 levels and thus result in reduced neutrophil chemotaxis to the lung. Whether the increased number of circulating neutrophilic cells upon acute exercise [25, 31] is a result of reduced chemotaxis to lung tissue by this cell type remains unknown. In other words, are neutrophilic cells inhibited to invade lung tissue because of decreased IL-8 chemoattractant mediated by reduced miR-126 levels? Is this a

reason for the increased number of circulating neutrophils observed upon exhaustive exercise because of the reduced attractance to invade certain tissues mediated by miR-126 and IL-8? Furthermore, to what extent neutrophils contribute to URS remains unknown.

In a recent report, Zhao et al. [302] showed that miR-126 specifically targets DNA methyltranferase 1 (Dnmt1). Moreover, overexpression of miR-126/Egfl 7 gene resulted in reduced Egfl 7 promoter methylation. In CD4+ T cells overexpressing miR-126, demethylation of CD11a and CD70 gene loci was noticed resulting in elevated CD11a and CD70 protein levels. As a consequence, T cell and B cell activity was increased. CD11a is an important adhesion molecule of the integrin family and can enhance the adherence of T lymphocytes to other immune cells. CD70 is transiently expressed on the surface of activated T and B cells stimulating the synthesis of IgG in B cells [303]. In sum, miR-126 is involved in T cell and B cell surface molecule expression. It remains a question whether decreased miR-126 levels down-regulate CD11a and CD70 and thus fine-tune T cell and B cell activity.

A strong effect for miR-126 regulation was demonstrated by Mattes et al. in 2009 [304]. They showed that BALB/c mice exposed to house dust mite (HDM) increased miR-126 expression in the airways. MiR-126 induction was mediated via TLR4 and MyD88. By specifically inhibiting miR-126, airway hyperresponsiveness and mucus hypersecretion as well as eosinophils recruitment/accumulation in the airways and lung tissue was suppressed. Interestingly, these processes are normally mediated via IL-5 and IL-13 which are typically produced from T_H2 T cells [305-309]. Moreover, CD4 T cells (predominantly T_H2 cells in their study) as well as T_H2 promoting dendritic cells were not recruited to the airways. Thus, the effect of miR-126 on T_H2 cells might be of certain interest since the observed upper respiratory symptoms upon exhaustive exercise may be mediated in part by this microRNA.

7 Limitations

Despite the results presented here, some limitations must be addressed. The expression profile in this study is a result of whole blood expression. Given, that whole blood constitutes of several cell types which were not separated in this study it cannot be addressed which cell type contributes mostly to the expression pattern observed. Rapid RNA degradation of whole blood samples is an important factor influencing the outcome of several methods. Unfortunately, no methodology for cell separation and simultanous RNA stabilization is yet established. Thus, RNA-stabilizing tubes were used in this study prohibiting premature RNA degradation and thus enhance storage conditions and improve study results to the disadvantage of no separated cell populations.

Furthermore, no hematocrit value and thus no plasma volume were determined. Plasma volume is increased after an Ironman and is dependent on fluid intake during the competition [310]. A concentration is determined by two factors, the amount and the volume. Given, that the plasma volume is altered after an Ironman race, certain measured parameters might be influenced by changed plasma volumes. Hypothetically, increasing plasma volume after an Ironman [310] would alter the overall microRNA levels observed. Decreasing miRNA levels might be even more pronounced in a hypothetical steady plasma volume environment after an Ironman. Increasing miRNA levels on the other hand might be to some extent masked upon increasing plasma levels.

In addition, low RIN values might have an influence on the overall results which is an important issue to keep in mind. RIN values < 6.0 or N/A were almost exclusively observed in samples acquired directly after finishing the competition. Why these samples showed such a pattern remains unknown. It is of importance to pinpoint that those samples seemed to have altered the RT-qPCR results as can be observed in the graphs for each miRNA (Figures 29 – 34). Hence, all RT-qPCR results must be interpreted with caution. Nevertheless, miR-15a and miR-126 are most likely reliable despite this problem since a very conservative statistical analysis was applied. More significant changes on time and for each miRNA were obtained from a normal t-test (not shown) but we decided in the favor of the more conservative RM-ANOVA and a Bonferroni post-hoc analysis since the results might be influenced by low RIN values.

8 Conclusions and Future Prospects

Compared to pre-race levels, miR-15a and miR-126 were down-regulated in male athletes directly after finishing an Ironmen competition. Both microRNAs could be attributed to certain processes of the innate as well as the adaptive immune system. Whether and to what extent these microRNAs might contribute to symptoms observed upon exhaustive exercise (URTI, URS) remains unknown and should be further addressed in future experiments possibly by using miR-15a or miR-126 antagomir inhibitors.

In future studies, miRNA expression patterns from separated cell populations of the circulating system would further improve the understanding of microRNAs in the regulation of certain mechanisms in both the innate as well as the adaptive immune system. Understanding the mechanisms in certain cell types upon exhaustive exercise is crucial to improve the regeneration process of athletes. In addition, the contribution of varying plasma volumes upon exhaustive exercise should be investigated in more detail. It must be questioned, to what extent expression levels can be influenced by varying plasma levels would help to improve the accuracy of future studies. Moreover, pathway analysis should be applied to determine the influence of miR-15a or miR-126 by verifying direct interactions of these miRNAs with possible candidate gene mRNAs. The results would help to improve the knowledge of the cellular processes mentioned and their consequences on immune cell signaling. Moreover, it is yet unknown whether there is a correlation between the physical condition of an athlete and certain miRNA expression. In general, does physical fitness contribute to rendered miRNA expression pattern? If yes, to what extent and in which way is it beneficial for an athlete would be open questions for coming studies. A similar study should be conducted to women. Since sex has an influence on several immune reactions, a miRNA expression pattern from female subjects would be of special interest since a comparison with male subjects would further highlight the diversity between women and men. A major issue would be to find a way for satisfying sample collection and preparation in the field. Thus, a more practicable protocol for whole blood sample collection must be adopted, allowing the separation of individual cells from the circulatory system by preventing total RNA from degradation directly after samples were obtained.

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10 Appendix

Curriculum Vitae

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Personal data

Born on the 11th of April 1985 in Eberstalzell, Austria

Working Experience

03/ 2011 – 06/ 2012 **Diploma thesis, Vienna**

- „Exhaustive Exercise such as an Ironman Alters MicroRNA Expression Pattern."
- Total RNA isolation and microRNA expression profiling from human whole blood samples

04/ 2012 – 06/ 2012 **Dialog<>Gentechnik, Vienna**
Project assistant (project „Wer forscht mit?")

- Development of the content and experiments of the project
- Supervision and support of the kids conducting the experiments
- Training and coordination of the interns, involved in the project

11/ 2011 – 03/ 2012 **ViennaOpenLab, Vienna**

- Tutor

03/ 2010 – 05/ 2010 **Internship at the Institute of Sports Medicine Copenhagen (ISMC), Copenhagen**

- Total RNA isolation from human muscle sections and subsequent mRNA RT-qPCR analysis

02/ 2010 – 06/ 2010 **Semester abroad in Copenhagen, Denmark**

06/ 2009 – 08/ 2009 **Internship Max F. Perutz Laboratories (MFPL) – Group Josef Loidl, Vienna**

- Handling and establishment of *Tetrahymena* mutants

05/ 2009 – 06/ 2009 **Internship Institute of Molecular Biotechnology (IMBA) – Group Jürgen Knoblich, Vienna**

- Handling and establishment of *Drosophila* mutants
- Dissection and mutant selection (immunoflourescence microscopy)

03/ 2009 **Internship Max F. Perutz Laboratories (MFPL) – Group Roland Foisner, Vienna**

- Cell culture, establishment and analysis of mutant cell lines

02/ 2009 **Internship Max F. Perutz Laboratories (MFPL) – Group Georg Weitzer, Vienna**

- Cell culture, establishment and analysis of mutant cell lines

09/ 2001 – 06/ 2004 **6 months internship as an electrician in the course of my education at the HTBLA Steyr.**

Education

Since 10/ 2005 **Study „Molecular Biology" at the University of Vienna**

- Emphasis on molecular medicine, cell biology and genetics
- Diploma thesis: „Exhaustive Exercise such as an Ironman Alters MicroRNA Expression Pattern."

09/ 2004 – 04/ 2005 **Military duty in the „Julius-Raab-Kaserne", Mautern as well as in the „Birago-Kaserne", Melk**

09/ 1999 – 06/ 2004 **Federal Secondary College of Engineering (electronics, informatics) „HTBLA Steyr", Steyr**

- Matura

09/ 1995 – 07/ 1999 **High School „BRG Hamerlingstraße", Linz**

Advanced Education

09/ 2008 – 10/ 2008 **Presentation techniques (with focus on scientific content)**

- Presentation and rhetoric skills

03/ 2011 – 10/ 2011 **Science communications (in context of an Open Laboratory)**

- Scientific communication
- Particularities in dealing with a younger audience

Related Skills

Languages	**German:** native tongue **English:** fluent **Danish:** basal
IT	Excellent in MS Word, MS Excel and MS Power Point as well as in SigmaPlot and EndNote
Interests	Beachvolleyball, volleyball, tennis, running as well as cycling

Vienna, 11th June 2012