UNIVERSITY OF FOGGIA

PhD RESEARCH IN

"TRANSLATIONAL MEDICINE AND FOOD:
INNOVATION, SAFETY AND MANAGEMENT"

(cycle XXXII)

"Life style, medicine and anabolic steroids: organ damages, new molecular biomarkers, epidemiology, pathology and toxicology"

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A c a d e m i c T r i e n n i u m

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STATEMENT OF ORIGINALITY

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution, and contains no material previously published or written by another person, except where due reference has been made in the text.

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November 10, 2019

ABSTRACT

Background: To date, even if anabolic androgenic steroid (AAS) abuse is clearly associated with a broad spectrum of collateral effects, adolescents and athletes frequently use a large group of synthetic derivatives of testosterone, both for esthetical purposes and for improving their performance. AAS use is frequently associated with different substances such as energy drinks and dietary supplements. The combined use of different substances, such as AASs, energy drinks, and dietary supplements, could worsen each adverse effect, worsening the risk for several pathologies such as cardiovascular disease. Moreover, the presence of other silent substrates (for example, the genetic predisposition to cardiovascular diseases, the presence of the Metabolic Syndrome or physical inactivity) combined with the use of this kind of substance can represent an explosive mix for the users, causing severe consequences and, in particular cases, death.

Open questions: One common factor between detecting each of these performance-enhancing drugs is that the traditional methods of drug testing were taken at a single time point. An enhanced method of detection has been required to counter the increasingly sophisticated doping regimens and the ongoing development of new substances.

The contemporary use of these substances could represent an important warning for public health, worsening the cost of health care.

Aims: The goal of this PhD project was to analyze several important aspects linked to the use of AASs, defining the following: 1 - organ damage; 2 - new molecular biomarkers; 3 - epidemiology; 4 - pathology; 5 - toxicology.

Research plan: (i) During the project, two complete literature reviews were performed using the most used scientific databases. (ii) All samples were selected analyzing the documentation of all autopsies performed by the Institute of Legal Medicine of Foggia from 2001 to July 2018 (about 1700 autopsies): - 5 cases of young men positive for AAS use were selected for the “AAS group”; - 6 cases of men who died of heart failure were enrolled as the "HF (heart failure) group", with the aims to evaluate the heart damages; - 15 cases composed the three groups enrolled to analyze brain injury (5 for the “Stroke group (SG)”, 5 for the “Drug group (DG)”, and 5 for the “Aging group (AG)”); - 5 cases of men who died of cardiac arrest who had suffered from cirrhosis caused by non-alcoholic fatty liver disease (NAFLD group), were selected to evaluate the liver damages; - 5 cases of men who had died of cardiac arrest after a long period (more than 10 years) of Chronic kidney disease (CKD group), were enrolled to evaluate kidney damages; - 6 cases of healthy men, who had died in car accidents were enrolled to compose the control groups: 4 cases (age mean 28 ± 7.4

with negative anamnesis for heart suffering and negative for the toxicological examination were selected as Control Group in the Quantitative Real-Time PCR (qRT-PCR) reactions for the miRNA tested in cardiac and musculoskeletal tissues, and 2 cases (mean age 41.5 ± 2.12 years), who died in car accidents for causes other than brain trauma, NAFLD, CKD and negative for toxicological examination were selected as Control group in the qRT-PCR reactions performed on brain, liver, and kidney. (iii) Histological and toxicological examinations were performed in all groups; (iv) miRNA quantification through qRT-PCR was performed; (v) for each miRNA the prediction effect analysis was carried out through both with the literature review and "*in silico*", using several informatics tools; (vi) two surveys using online data collection tools were performed: "Multidimensional Body-Self Relations Questionnaire (MBSRQ)" and "in house" questionnaire to define the voluptuary habits of the interviewees; (vii) Statistical analysis was carried out through the software Statistica for Windows.

Results: The literature reviews were very important to define the main adverse effects linked to AAS use/abuse and to identify the "candidate miRNAs" to test on the tissue samples of the selected cases. The main adverse effects concerning AAS use were strictly linked to the reproductive systems, particularly in adolescents. Moreover, other organs that are severely damaged by AAS use were heart, kidney, liver, and brain. For these reasons, several miRNAs related to these organs were selected and tested in the selected groups. For heart and musculoskeletal tissues, the miRNA expression values of miRNA has-133a-3p, hsa-miR-208a-3p, hsa-miR-499a-3p, and hsa-miR-1-3p were tested. The expression levels of miR-133 in heart tissue (HF and AAS groups) and muscle tissue (AAS group) resulted upregulated compared to controls. The expression levels of miR-208 in heart tissue (HF and AAS groups) resulted upregulated compared to controls. These results were not confirmed in muscle tissue (AAS group), where expression levels were similarly to controls. The expression levels of miR-499 resulted upregulated in the HF group, while in the AAS group, the expression levels of this miRNA were similar to controls. The expression levels of miR-1_3 both in HF and AAS groups resulted upregulated compared to controls. Moreover, this miRNA resulted overexpressed in the muscle (*vastus lateralis*) of the AAS group.

In kidney tissue, miR-21 and miR-205 were tested. The expression values of miR-21 in kidney tissues were upregulated compared to controls, even if the CDK values were significantly higher than the AAS group. The expression levels of miR-205 were higher in the CKD group compared to the AAS group, even if they were overexpressed in both groups compared to controls.

Analyzing the results on the liver tissue, comparing the expression values of miR-21 in the

two tested groups (AAS vs NAFDL), even if the values were higher compared to controls, this miRNA resulted overexpressed in the AAS group with a statistical significant difference. Concerning the expression levels of miR-122 in each group, even if the values were overexpressed compared to controls, the NAFDL values were significantly higher than the AAS group. Analyzing the expression levels of miRNA-132, there were no significant differences comparing the AAS group to the NAFDL group, even if both miRNAs were higher compared to controls. The same results were obtained analyzing the data about miR-155.

On brain tissue, several miRNAs were tested (miR-21, miR-34, miR-124, miR-132, and miR-200b) in four groups (SG, AG, DG, and AAS groups). For miR-21 the expression values of the AAS group were similar to the expression values of the SG and AG groups and was significantly higher compared to DG. For miR-34, the AAS group showed expression levels of this miRNA similar to the DG group, resulting higher compared to the AG and SG groups. The tissue levels of miR-124 were higher in the AAS and SG groups compared to the DG and AG groups. miR-132 was higher in the DG and AAS groups compare to the AG and SG groups. Finally, miR-200b was significantly overexpressed in the AAS group, even if this miRNA was higher in all groups than in controls.

The interpretation of miRNA expression was performed through several web tools ("*in silico*" analysis) ascertaining the possible effects of the aberrant expression at cellular levels. In this way, it was possible to achieve the main goal of this project, to ascertain the pivotal roles for several miRNAs expressed in specific tissue, linking them to specific organ damage.

Finally, from the analysis of the questionnaires it was possible to create the first Italian map on the phenomenon linked to the use of several substances, such as AASs, Energy Drinks and Supplements. The collected data demonstrated that there was a significant association ($p < 0.05$) between the use of anabolic steroids and consumption of other substances, such as energy drinks and supplements. The potential health risks related to heavy consumption of these products have largely gone unaddressed. For these reasons, the effort of the scientific community both for a better understand and to better communicate the risks linked to the use of these substances should be improved.

Significance and Impact of research: In the light of the results discussed in this project, it would appear that concerns in the scientific community and among the public regarding the potential adverse health effects of the increased consumption of energy drinks, supplements and AASs are broadly valid. As extensively discussed, AAS use is not limited

to athletes, but concerns young people who use these kinds of substances for esthetical purposes. For these reasons, this ongoing field of research is very challenging for the scientific community, in particular for the forensic field, involving a large number of people. As demonstrated in this PhD project, the main organs involved as targets of the adverse effects of AAS use are heart, kidney, liver, and brain; in several cases, the pathologies could be fatal for the abusers as described in the discussed cases. The identification of new molecular biomarkers can be considered of interest for the scientific community, not only for anti-doping purposes but for public health, too.

Indeed, considering the results of the questionnaires, the combined use of AASs with other substances such as supplements or energy drinks, is significantly higher compared to non-users, demonstrating the idea of polypharmacy for AAS users. These substances can be anonymously supplied by the internet, where they are sold with alleged effects on mental, physical and sexual performance without any real evidence-based research. Obviously, the adverse effects of each substance are combined with each other, increasing the health risks for users and the public health cost for assistance in severe cases such as cardiovascular diseases.

Future trends: The potential health risks related to a heavy consumption of these products have largely gone unaddressed. For these reasons, the effort of the scientific community both for a better understanding and to better communicate the risks linked to the use of these substances should be improved.

Key words: Anabolic Androgenic Steroids (AASs), Adverse effects, Abuse, Kidney, Liver, Heart, Brain, miRNAs, new molecular biomarkers, Molecular mechanisms

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PUBLICATIONS ARISING FROM THIS PhD PROJECT

- BERTOZZI, G., SALERNO, M., POMARA, C., **SESSA, F. Neuropsychiatric and Behavioral Involvement in AAS Abusers. A Literature Review** (2019) Medicina (Lithuania), 55 (7), art. no. 396. DOI: 10.3390/medicina55070396

- BERTOZZI, G., **SESSA, F.,** MAGLIETTA, F., CIPOLLONI, L., SALERNO, M., FIORE, C., FORTAREZZA, P., RICCI, P., TURILLAZZI, E., POMARA, C. **Immunodeficiency as a side effect of anabolic androgenic steroid abuse: a case of necrotizing myofasciitis** (2019) Forensic Science, Medicine and Pathology, DOI: 10.1007/s12024-019-00144-z *in press*

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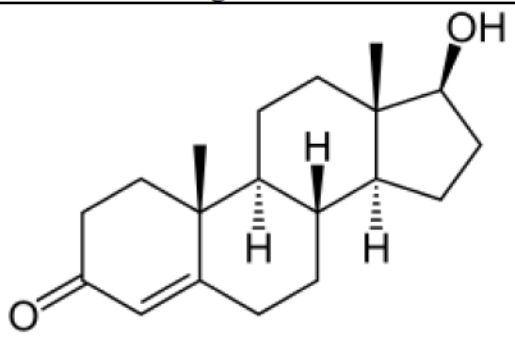
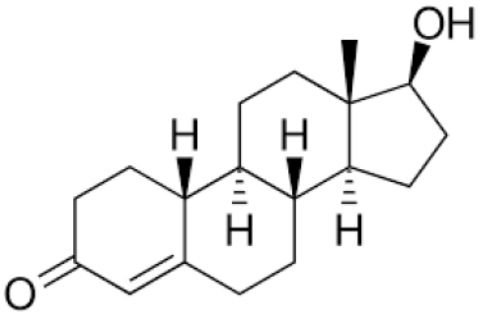
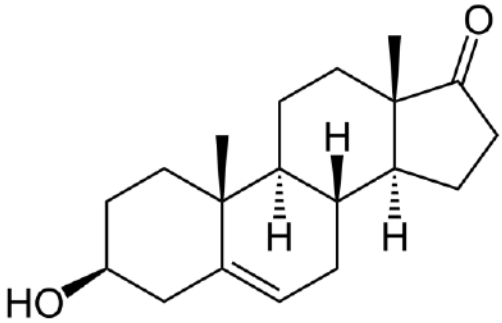
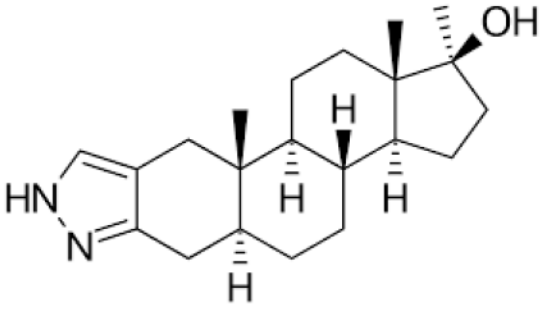
Chapter 1. INTRODUCTION

1.1. The use of Anabolic Androgenic Steroids (AASs)

To date, even if anabolic androgenic steroid (AAS) abuse is clearly associated with a broad spectrum of collateral effects, frequently adolescents and athletes use a large group of synthetic derivatives of testosterone, both for esthetical use and to improve their performance (Bailey et al., 2013; Dickinson et al., 2014). The market for performance-enhancing drugs is continuously increasing. Indeed, the phenomenon of doping no longer affects only professional athletes, but it is continually expanding to subjects practicing sports activities at the amateur level (Reardon and Creado, 2014).

The main AASs are summarized in Table 1. AASs are administered, both orally and by injection, cyclically, for periods ranging from 6-8 weeks followed by equivalent periods of abstinence.

Endogenous AAS	Exogenous AAS
 TESTOSTERONE	 NANDROLONE
 DEHYDROEPIANDROSTERONE	 STANOZOLOL

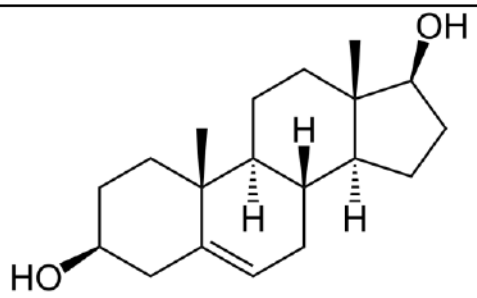
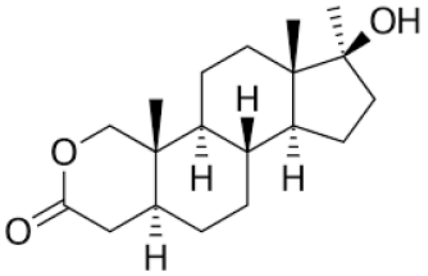
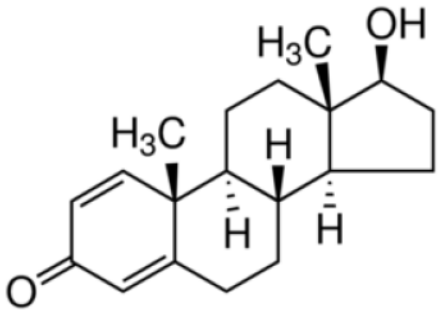
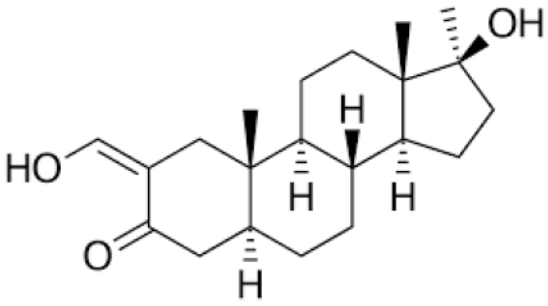
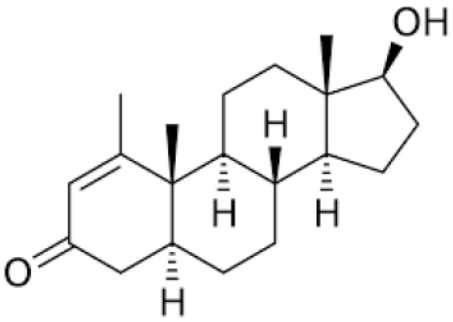
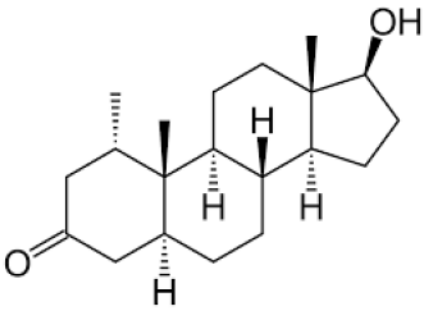
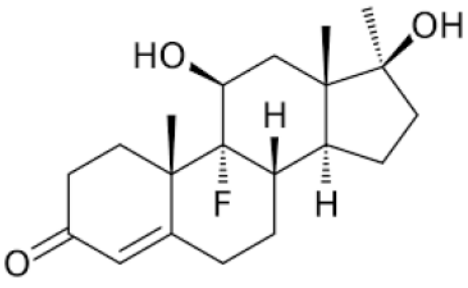
 ANDROSTENEDIOL	 OXANDROLONE
 DEHYDROTESTOSTERONE	 OXYMETHOLONE
	 METHENOLONE
	 MESTEROLONE
	 FLUOXYMESTERONE

Table 1. The main AASs: the endogenous AASs are produced by the human body, the exogenous ones can be obtained synthetically and are commonly used in therapy and doping

AAS use is frequently associated with different substances such as energy drinks and dietary supplements. The use of these products has become widespread: while it is known that acute overdose has been associated with toxicity, there is growing concern that chronic use of energy drinks and dietary supplements may cause neurologic and cardiovascular toxicity, particularly when they are combined with AAS use (Grasser et al., 2014; Hajsadeghi et al., 2016; Rottlaender et al., 2012).

Performance-enhancing drugs is an umbrella term used to describe those substances that give an athlete an unfair physical or mental advantage over a competitor. Prohibited substances or techniques are categorized by their target outcome: to improve strength, endurance or mental tolerance (Lippi and Plebani, 2011). Athletes involved in drug doping may employ a masking agent to avoid detection. Diuretics are by far the most common and are used to lower the concentration of the drug or its metabolite in the urine by increasing urinary volume. Although diuretics are detectable with multi-analytic screening, other masking agents including desmopressin and glycerol require more sophisticated methods (World Anti-Doping Agency,2014; Cadwallader et al., 2010).

One common factor in detecting each of these performance-enhancing drugs is that the traditional methods of drug testing were taken at a single time point. An enhanced method of detection has been required to counter the increasingly sophisticated doping regimens and the ongoing development of new substances. The athlete's biological passport is the evolution of drug testing techniques that WADA has developed for this purpose (Bucknall et al., 2014). For these reasons, the identification of new molecular biomarkers remains an ambitious target for the scientific community.

In recent years, energy drinks and dietary supplement use have increased, especially among young people, because these substances are sold as cognitive enhancing supplements. However, many of these new products produce altered psychological states, like

amphetamine effects (fatigue resistance, mood increase) and performance enhancement (Cole et al., 2018; Enriquez and Frankel, 2017; Grgic and Mikulic, 2017; Pomportes et al., 2017).

The combined use of different substances, such as AASs, energy drinks, and dietary supplements, could worsen each single adverse effect, increasing the risk for several pathologies such as cardiovascular disease. Moreover, the presence of other silent substrates (for example, genetic predisposition to cardiovascular diseases, the presence of the Metabolic Syndrome or physical inactivity) combined with the use of this kind of substance can represent an explosive mix for users, causing severe consequences and, in particular cases, death.

For these reasons, it is very challenging for the scientific community to improve our knowledge in this research field.

1.2. Anabolic Androgenic Steroids: Mechanisms of Action

AASs are synthetic derivatives of testosterone and their pharmacodynamics are similar to all other steroid hormones. Testosterone exerts different functions: in particular, it has a pivotal role in sexual differentiation, and it is produced more in men than in women. The biochemical pathway to obtain testosterone starts from cellular cholesterol, which after enzymatic cleavage of the lateral chain becomes pregnenolone. This molecule can then follow two different metabolic patterns. In the first 3-beta-hydroxysteroid dehydrogenase transforms pregnenolone into progesterone, then 17-a-hydroxylase adds an oxidrile group to pregnenolone and progesterone, synthesizing, respectively, 17-a-OH-pregnenolone and 17-a-OH-progesterone. Acting on each of these molecules, 17,20-lyase produces dehydroepiandrosterone (DHEA) and androstenedione. Androstenediol and testosterone are produced by the action of 17-beta-hydroxysteroid dehydrogenase on the two molecules. Androstenediol, moreover, can be transformed into testosterone by 3-beta-hydroxysteroid dehydrogenase (Bremner et al., 1983; Saad et al., 2013).

Testosterone can exert its action itself but, more commonly, via the work of two enzymes called 5-a-reductase and CYP19-aromatase; in fact, it is possible to distinguish active (di-hydro-testosterone and estradiol respectively) and inactive (androsterone and etiocholanolone respectively) testosterone metabolites. In particular, di-hydro-testosterone and testosterone, acting through the binding androgen receptor, have a different affinity, the first being higher than the second. As has been stated above, testosterone is the primary androgen in men, primarily produced by Leydig cells and, in less quantity, by adrenal glands, in a relatively constant amount, with some diurnal variation that is age correlated. Indeed, daily variations of testosterone are well documented with specific levels that peak between 05:30 and 08:00 and trough levels occurring approximately 12 h later (de la Torre et al., 1981; Montanini et al., 1988; Walker, 2009; Wittert, 2014).

Androgens have several actions in the human body, in particular, in males, they deal with body growth and sexual differentiation acting on all tissues and organs. Testosterone production, after a role in fetal development, starts its effect during puberty, under the metabolic stimuli of gonadotropins, regulating, above all, spermatogenesis (Unger, 2014; Walia et al., 2011).

AASs are membrane-permeable and influence the nucleus of cells by direct action. Synthetically, when the exogenous hormone penetrates the membrane of the target cell, the first step is their link to an androgen receptor (AR) located in the cytoplasm of the cell. From there, the compound hormone-receptor diffuses into the nucleus, it either alters gene expression (Sanchez et al., 2012), or activates processes that send signals to other parts of the cell (Hampl et al., 2016).

Thanks to drug designers, to date, more than 100 AAS compounds have been synthesized. Analyzing their chemical structure, metabolic half-life, and physiological effects, three classes of AASs can be identified. The first class was obtained by the esterification of the 17β-hydroxyl group of testosterone and includes testosterone propionate and testosterone cypionate. The second class is composed of esterified AASs, connected with the long side chain moieties, with a substitution of hydrogen for the methyl group at C19. The third class of AASs comprises those compounds that are alkylated at C17, such as 17α-methyltestosterone and stanozolol (Oberlander et al., 2012).

The most frequently abused androgens are nandrolone, testosterone, stanozolol, methandienone, and methenolone (Kicman, 2008; Sessa et al., 2018).

The anabolic androgenic effects are linked to the androgen receptor (AR)-signaling action. Androgen receptors are expressed in myosatellite cells (also named satellite cells); these are the precursors of skeletal muscle cells (Sinha-Hikim et al., 2004).

AASs exert their action by several different mechanisms: (i) they modulate androgen

receptor expression as a consequence of intracellular metabolism; (ii) they affect the androgen receptor directly and thus subsequent interactions with co-activators and transcriptional activity; (iii) they interfere with the glucocorticoid receptor expression eliciting an anticatabolic effect; and (iv) they act on the Central Nervous System (CNS) resulting in behavioral changes, following genomic and non-genomic pathways (Kicman, 2008; Parkinson and Evans, 2006).

In particular, free testosterone is transported into target tissue cell cytoplasm; binding to the androgen receptors (AR) takes place either directly or after conversion to 5α-dihydrotestosterone (DHT) by the cytoplasmic enzyme 5-alpha reductase. In the cell nucleus, both free and bound testosterone act on specific nucleotide sequences of the chromosomal DNA. The produced mRNA can activate DNA transcription of specific responsive genes (Handelsman et al., 2015).

AASs mimic testosterone physiological effects, and primarily act via the androgen receptor (figure 1).

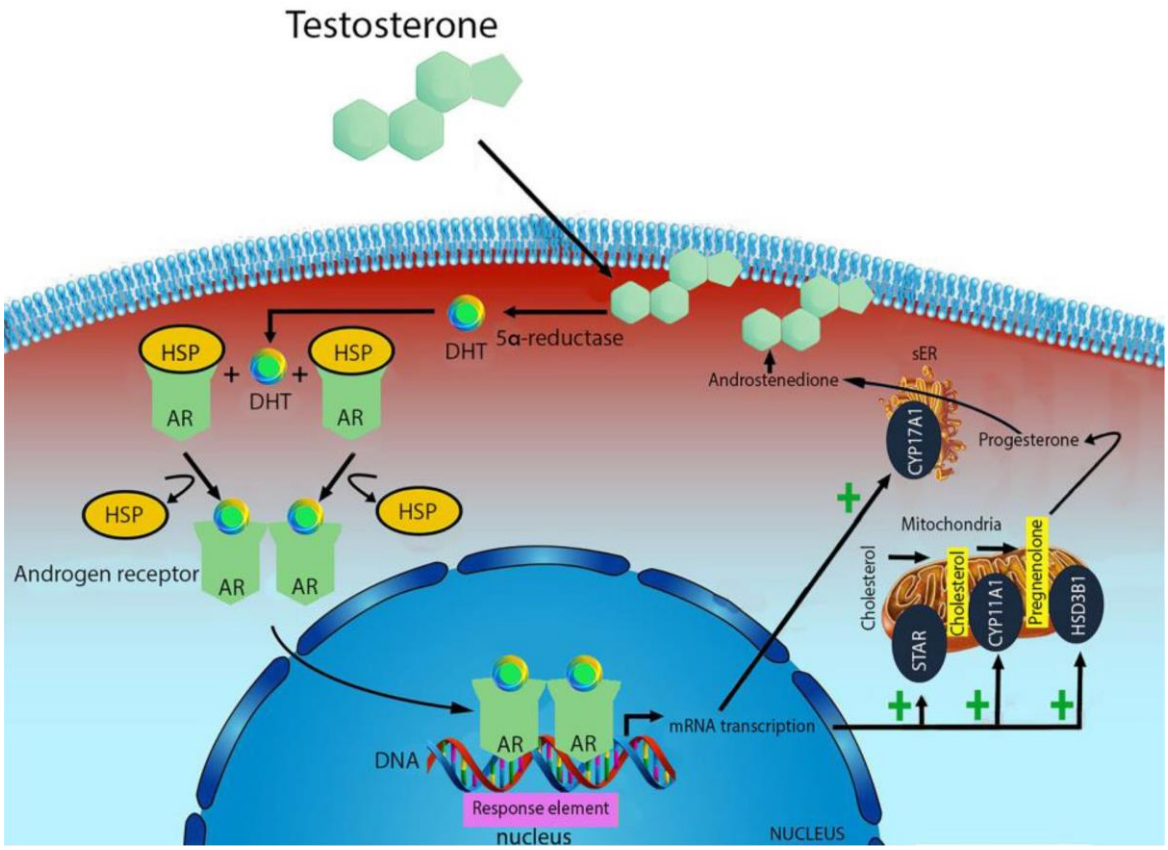


Figure 1: Mechanism of testosterone action. Free testosterone is transported into the target tissue cell cytoplasm, where it can either bind the androgen receptor, or be reduced to 5α-di-hydro-testosterone (DHT) by the cytoplasmic enzyme 5-alpha reductase. The T-receptor complex undergoes a structural change that allows its translocation into the cell nucleus, where it directly binds to specific nucleotide sequences of the chromosomal DNA. The produced mRNA promotes the biosynthesis of testosterone: our own picture, previously published (Salerno et al., 2018).

1.3. Adverse Effects of Androgen Abuse

AAS use/abuse is frequently linked to widespread serious health damage: indeed, even in cases of a one-time cycle (use over a specific time-period) at very low doses can cause irreversible harmful effects after the cycle is completed. Moreover, recreational users and/or athletes use AASs in association with other drugs, such as stimulants and/or depressants. In this way, the correct attribution of adverse effects on AAS use/abuse becomes very difficult. Furthermore, when a side effect occurs in athletes or bodybuilders, it is complicated to elucidate if the adverse effects were linked to androgen use or to "athlete" status (Pope et al., 2014a, 2014b).

Nonetheless, as illustrated in figure 2, a large number of side effects related to AAS use/abuse has been described. The illicit use of AASs provokes or favors the development of serious pathological conditions, such as hypertension, atherosclerosis, hepatic damage and tumors, tendon ruptures, reduced libido, and psychiatric/behavioral disorders such as aggressiveness and irritability (Bertozzi et al., 2017; Junior et al., 2018; Mewis et al., 1996; Stannard and Bucknell, 1993; White and Noeun, 2017; Yesalis and Bahrke, 1995). The effects on mood and behavior are also well established: depressive, hypomanic or manic episodes, sometimes associated with psychotic symptoms, increased risk of suicidal or homicidal death have been observed in AAS users (Bertozzi, Salerno, Pomara, & Sessa, 2019; Kanayama, Hudson, & Pope, 2008; Piacentino et al., 2015; H.G. Pope & Katz, 1994). Some studies reported adverse renal, immunologic and musculoskeletal effects (Kanayama et al., 2010; Pope et al., 2014b). The main adverse effects linked to AAS use/abuse are summarized in figure 2.

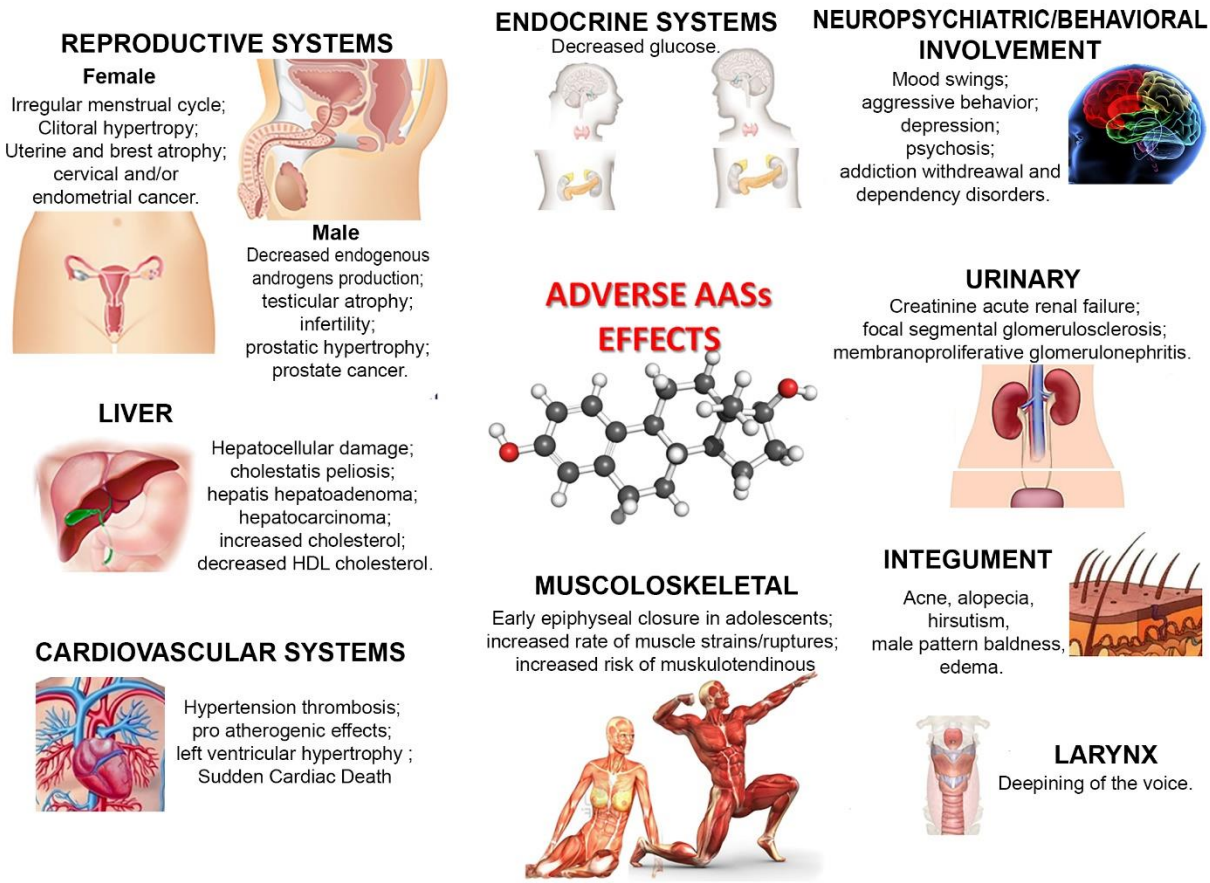


Figure 2. Principal adverse effects of AAS use/abuse: own picture, previously published (Sessa et al., 2018)

Therefore, even if there are well-known consequences of AAS abuse because of their particular chemical structure that is similar to corticosteroids and their use at supraphysiological doses, their full pathway of action is still under study. In this context, a recently published case report discussed and provided evidence on unusual adverse effects linked to immunodeficiency in an AAS abuser. This kind of chronic complication, even if not usually considered, may lead to sudden death (Bertozzi et al., 2019b). For these reasons, further studies are needed to completely clarify the main adverse effects linked to AAS use/abuse.

1.4. The role of miRNA in the research project

Over the last few years, the development of MicroRNA (miRNA) technologies has become an essential part of research projects and their role as potential molecular biomarkers is being investigated by the scientific community.

MicroRNAs (miRNAs) are 20–22 nucleotide non-coding RNA molecules, which regulate gene expression at the post-transcriptional level, located in intergenic or intronic regions as individual or clustered genes (Bartel, 2004). Several steps must occur before miRNAs can act, including the action of many enzymes, such as RNA polymerase II, Drosha, Exportin 5, Dicer and Argonaute (Ago). Figure 3 shows a schematic description of miRNA biogenesis (MacFarlane and Murphy, 2010; Slezak-Prochazka et al., 2010).

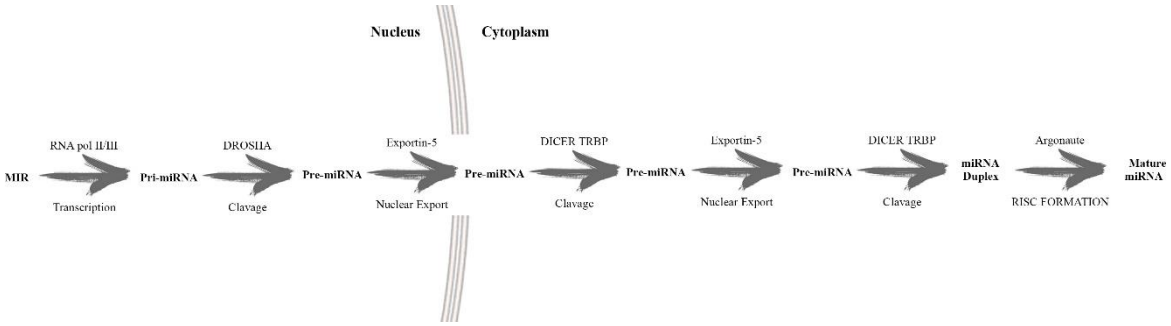


Figure 3. Schematic description of miRNA biogenesis. In the nucleus, RNA polymerase II transcribed the long primary miRNA, which is later converted by the endonuclease Drosha intopre-miRNA. This pre-miRNA is exported into the cytoplasm and further cleaved by Dicer, developing mature miRNAs. This latter recognizes 3' untranslated regions, guided by RNA, inducing silencing complexes (RISC). The result is the silencing of target expression.
This picture is our own, previously published (Sessa et al., 2018)

miRNA nomenclature was based simply on the sequence of discovery, with few notable exceptions (such as let-7 and lin-4). Subsequently, the identifiers indicating the species (hsa, human; mmu, murine; etc.) were added. Moreover, other symbols (such as "*") or numbers (-5p or 3p) could be added, identifying better the miRNAs (for example indicating the homology, the guide miRNA strand, etc...) (Desvignes et al., 2015).

These small noncoding RNAs regulate gene expression by RNA-RNA interactions, but this is not the only mechanism to control protein production; other mechanisms are: ribosomal RNA modifications, repression of mRNA expression by RNA interference, and alternative

splicing (Catalanotto et al., 2016).

Main clinical applications of miRNA dosage and deregulation are: i) cancer characterization and prediction of the course of a disease (Segura et al., 2010; Yu et al., 2008); ii) viral infection diagnosis (Lecellier et al., 2005); iii) implications in nervous system development (Mehler and Mattick, 2007); iv) cardiovascular disorder diagnosis (Ai et al., 2010; Van Rooij et al., 2006; Wang et al., 2010a); v) identification of specific patterns in primary muscular disorders (Eisenberg et al., 2007); vi) differences among diagnosed type 1 diabetes and healthy control diabetes (Nielsen et al., 2012). These associations demonstrate that using these abnormally expressed miRNAs as biomarkers for diseases is a valuable diagnostic strategy.

The discovery of miRNAs has opened up new avenues of research in biomedicine. It is remarkable to note the fast rate of fundamental discoveries made in two decades (illustrated by the exponentially growing number of publications). These molecules indeed harbor specific features (stability, easy manipulation, reasonably simple detection, tissue specificity) that make them appealing candidates as diagnostic, prognostic, or theranostic biomarkers and even therapeutic targets (Leuenberger and Saugy, 2015).

However, some uncertainties remain that may prevent their immediate large-scale exploitation: for these reasons, collective efforts made by clinicians, academic and industrial researchers are needed to better clarify possible miRNA applications.

1.5 Circulating MicroRNAs: New Molecular Biomarkers in the fight against doping

Circulating miRNAs are highly stable in several body fluids, including plasma and serum. Moreover, emerging evidence demonstrates that serum miRNAs remain stable at different temperature conditions if compared to other sources of miRNAs. Hence, in view of their potential use as novel, non-invasive biomarkers, the profiles of circulating miRNAs have been explored in the field of anti-doping (Chana et al., 2013; Leuenberger and Saugy, 2015). To date, several papers have described the potential use of circulating miRNAs as specific biomarkers in the anti-doping field. For example, the use of a continuous erythropoietin receptor activator induces higher plasma levels of miR-144: the plasma dosage of this miRNA could be used for the detection of erythropoiesis-stimulating agents (Zhou et al., 2015). Another study described a relationship between the expression of 4 miRNAs and the use of recombinant human GH (Keane et al., 2015). Moreover, the study of Leuenberger et al. highlighted the importance of circulating miRNAs as biomarkers for autologous blood transfusions (Leuenberger et al., 2013b).

The use of circulating miRNAs to detect performance-enhancing agents, such as AASs, could become very important in the near future, considering that currently the gold standard method of detecting exogenous testosterone administration is gas chromatography–mass spectrometry analyses of urine samples (Leuenberger and Saugy, 2015).

Quantitative RT-PCR is the gold standard method of quantifying circulating miRNAs. This technique has high sensitivity and specificity and is suitable for quantification across a wide dynamic range (the ratio between the largest and smallest values of a changeable quantity). Two major strategies enable the specific amplification of mature miRNAs via RT-PCR. In the first strategy, a stem-loop-shaped primer binds specifically to the mature miRNA to generate a unique template for RT (Chen et al., 2005). Alternatively, in poly(A)-tailed PCR, a poly(A) tail is added to the 3′ UTR of a mature miRNA by a polyadenylating enzyme, and

then universal primers containing 5′ terminal oligo (dT) sequences are used to initiate the RT reaction (Kroh et al., 2010).

Although the technical variability of circulating miRNA extraction can be normalized by using a non-human spike-in control, no absolute internal controls exist due to the cell-free condition of the sample. Invariant (non-changing) miRNAs are sometimes selected as endogenous controls; however, the biological significance of specific miRNAs can be underestimated using this approach (Kroh et al., 2010).

In the near future, new molecular biomarkers it could be incorporated into the adaptive model of the Athlete Biological Passport (ABP), considering their high stability in blood and unmodified characteristics when exposed to environmental factors (Ponzetto et al., 2016). Furthermore, the possibility to detect miRNAs not only in serum and plasma but also in urine, saliva and other body fluids (Gilad et al., 2008), makes these new molecular biomarkers the new frontier in the fight against doping.

Chapter 2. AIMS AND PLANNING OF RESEARCH

The goal of this work was to analyze several important aspects linked to the use of anabolic androgenic steroids, defining the following: - organ damage; - new molecular biomarkers; - epidemiology; - pathology; - toxicology. The research hypothesis at the base of the present project is summarized in figure 4.

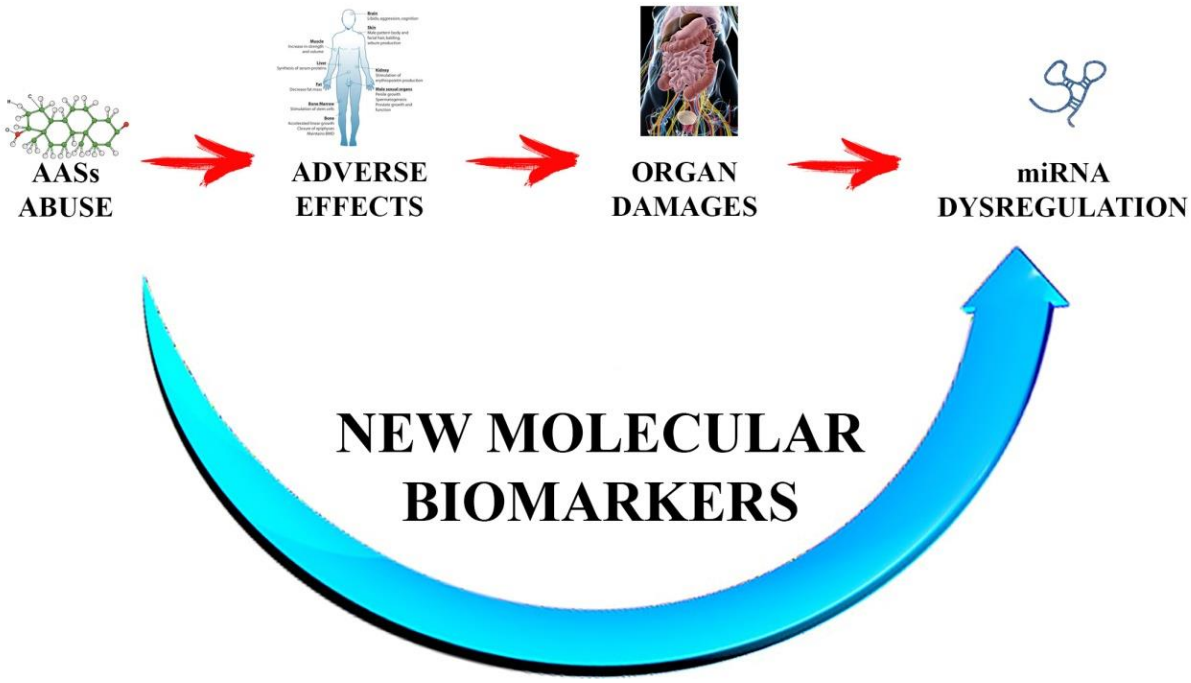


Figure 4: The research hypothesis of the project is that AAS abuse has side effects on the health of the user; after a middle term use/abuse, organ damage could occur, with a dysregulation of miRNA expression. These miRNAs could become new molecular biomarkers of AAS use/abuse. This picture is my own, previously published (Sessa et al., 2018)

In particular, during the first year of this project, the evaluation of adverse effects generated by AAS use/abuse was performed. To achieve these goals, a literature review, histological and toxicological examinations on selected cases (5 young people who had died with toxicological positive tests) were performed. This step was very important in order to better define the adverse effects linked with AAS use/abuse.

In the light of these findings, the second year activities of the project were performed. Firstly, a systematic literature review and meta-analysis on miRNA expression dysregulated in organ

damage related to AAS use/abuse was performed. This activity allowed us to select the "candidate miRNAs" as new molecular biomarkers linked to each adverse effect of AAS use/abuse. Finally, a miRNA quantification in heart and musculoskeletal tissues was performed in two groups, "AAS group" (composed of 5 cases, positive for toxicological analysis for AAS abuse), and "Heart Failure (HF) group" (composed of 6 cases who had died of heart failure, with negative toxicological analysis).

Finally, the third year activities of this PhD project were performed both at the University of Foggia and the University of Malta:

- Detection of new molecular biomarkers ("miRNA signatures") in other tissues sampled from the "AAS group": kidney, liver, and brain. For each tissue, subjects who had died of pathologies concerning the main organs tested as listed below were enrolled.

  The data about miRNA expression in kidney tissue were compared between the "AAS group" and CKD group.

  The data obtained in the liver tissue of the "AAS group" were compared with the data collected in the "NAFLD" group.

  Finally, the CNS was extensively studied. Indeed, on brain tissue, miRNA expression was evaluated in four selected groups of cadavers: "AAS group", Drug group (DG), ischemic stroke-related deaths (SG group), aging damage in older people who died from other causes (AG group).

- Realization of the first map of the phenomenon through anonymous questionnaires to evaluate the voluptuary practices linked to AAS use, in the South of Italy. To achieve this goal, two questionnaires were administered:
  - *Survey 1:* composed of the Multidimensional Body-Self Relations Questionnaire (MBSRQ);

- *Survey 2*: composed of 48 questions in order to determine the voluptuary habits of the interviewees about their use of "Smart Drugs", "Energy drinks" and "AASs", their mental status and the interactions with doctors regarding performance-enhancing drugs.

Chapter 3. MATERIALS AND METHODS

3.1. Literature Review

During the project, two complete literature reviews were performed at different times: firstly, at the beginning of the research project, and secondly, before the second year activities.

The first review was conducted using PubMed, Scopus, Web of Science, and Google Scholar databases. On these database websites, I searched for articles from 1 January 1980 to March 2019 using the following key terms: “Anabolic Androgenic Steroid”, “death”, “toxicity”, and “side/adverse effects”. The main keyword “Anabolic Androgenic Steroid” was individually searched for in association with each of the others. Articles were excluded by title, abstract, or full text for irrelevance to the investigated issues. Lastly, to identify further studies that met the inclusion criteria, the references of the selected articles were also reviewed.

For the second review, some databases, from 1990 to June 2017, were searched: Medline, Cochrane Central, Scopus, Web of Science, Science Direct, EMBASE and Google Scholar, using the following keywords: “miRNA”, “Anabolic Androgenic Steroid”, “side/adverse effects”, organs and systems (“Cardiovascular system”, “reproductive system”, “central nervous system”, “liver”, “kidney”, and “skin”). The main keywords, “Anabolic Androgenic Steroid”, organs and systems (Cardiovascular system, reproductive system, central nervous system, liver, kidney, and skin), and “miRNA” were individually searched for in association with each of the others.

3.2. Case selection

All samples were selected analyzing the documentation of all autopsies performed by the Institute of Legal Medicine of Foggia from 2001 to July 2018 (about 1700 autopsies). All procedures were performed in accordance with international guidelines and were approved by the Scientific Committee of the University of Foggia.

3.2.1 AAS group

Five cases of young men (29 - 40 years), with toxicological positive test for anabolic agents, were selected (age mean 32.4 ± 4.39 years; mean body mass index (BMI) 26.9 ± 1.26).

For cases 1, 2 and 3 (table 2) all the information was obtained analyzing the medico-legal

documentation produced for juridical purposes and/or through the analysis of the case report listed in the references. On the Formalin-Fixed Paraffin-Embedded (FFPE) tissues, collected during the autopsies, miRNA quantifications were performed. On the other hand, all investigation concerning cases 4 and 5 were performed during activities, considering that each case occurred between 2016 and 2019.

Case 1 - The case of a 29-year-old bodybuilder who suddenly collapsed after dinner in his apartment (weight 72 kg, height 166 cm; BMI= 26.1) was selected. He had a weightlifting workout at the gymnasium a few hours before. Cardiopulmonary resuscitation, initiated by an emergency physician, was unsuccessful. According to his friends, he had been taking anabolic steroids (testosterone, nandrolone, and stanozolol) parenterally for several years, mainly to improve his appearance. Several glass vials were found in his room and were labeled Winstrol (stanozolol), Deca (nandrolone), Testoviron Depot, and Andriol (testosterone) (Fineschi et al., 2007).

Case 2 - The case of 30-year-old male, first an amateur, and later a competitive bodybuilder, who worked out regularly at the gymnasium, suddenly collapsed at home and was found dead (weight 90 kg, height 178 cm; BMI=28.4). He had a weightlifting workout at the gymnasium a few hours before. In an ashtray near the body, a 2-ml vial of nandrolone decanoate was found along with a used 2.5-ml syringe. The apartment contained other drugs too, most of which fell into the AAS and supplements categories. All witnesses confirmed that the subject had started using AASs approximately 6 months prior to his death. Baseline blood tests, performed a few days before death, were unremarkable except for a slight increase in hepatic enzymes (Fineschi et al., 2007).

Case 3 – The case of 32-year-old bodybuilder was selected (weight 90 kg, height 189 cm; BMI= 25.19). He suddenly lost consciousness during a weight lifting workout at the gymnasium and died. Two weeks earlier, he had experienced an episode of chest pain for a few minutes, with pain radiating to his left arm. According to relatives and friends, the patient did not consult a doctor and continued training, believing that his physical activity had caused the pain. For several months, he had been taking testosterone propionate (700 mg/ wk) and nandrolone (200 mg/wk) parenterally, and he had been taking stanozolol per os (70 mg/wk). His medical history was unremarkable. (Fineschi et al., 2001).

Case 4 – the case of a 40-year-old man was selected: the corpse was found by his wife in his covered garage (weight 83 kg, height 175 cm, and BMI=27.1). His t-shirt was completely sweaty. At the external examination, three acupuncture signs were found on the super-external quadrant of the left gluteal. No other significant signs were reported. Nearby, a

blood-stained 10-ml syringe was found, even if the cap was on. Finally, a small glass vial labeled as Trenbolone Acetate was found.

Case 5 – The case of a 31-year-old male, competitive bodybuilder, was selected (weight 80 kg, height 170 cm; BMI=27.7). He went to hospital for a trauma on his leg. At blood tests, liver damage was described, but he went home against medical advice. After two days, his health conditions got worse and he returned to the hospital. In only a few hours he went into a coma due to crush syndrome, a medical condition characterized by major shock and renal failure after a crushing injury to skeletal muscle. Crush injury is compression of extremities or other parts of the body that causes muscle swelling and/or neurological disturbances in the affected areas of the body, while crush syndrome is localized crush injury with systemic manifestations. After 10 days he died. All witnesses confirmed that the subject had started using AASs approximately 18 months prior to his death (Bertozzi et al., 2019b).

The main characteristics of the AAS group are summarized in table 2.

GROUP	AGE	CAUSE OF DEATH	MACROSCOPIC FINDINGS	MICROSCOPIC HEART FINDINGS
AAS				
1	29	Heart failure secondary to AAS assumption	The heart had a normal shape and weighed 380 g	Segmentation of the myocardial cells, widening of intercalated discs, and foci of contracted myocardium alternated with bundles, distended myocardium with granular disruption of the myocytes.
2	30	Heart failure secondary to AAS assumption	The heart had a normal shape and weighed 400 g	Histopathologic examination of the heart revealed focal myocardial fibrosis.
3	32	Heart failure secondary to AAS assumption	The heart had a normal shape and weighed 450 g	Histologically, the grayish area corresponded to typical infarct necrosis with a histologic age of approximately 15 days. The lesion was characterized by hyperdistended myocardial cells with sarcomeres in registered order, circumscribed at its periphery by young collagen tissue containing numerous macrophages.
4	40	Heart failure secondary to AAS assumption	The heart had a normal shape and weighed 380 gr.	Histopathologic examination of the heart revealed widespread fibrotic areas in myocardial tissue.
5	31	Septic shock subsequent to necrotizing myofasciitis	The heart had a normal shape and weighed 350 g.	Histopathologic examination revealed at the myocardium level limited areas of fibrosis. Finally, in some fields there were aspects of dissolving myofibers (colliquative necrosis).

Table 2. The main characteristics of the AAS group (subjects died with toxicological positive tests for anabolic androgenic steroids). These samples were tested for all miRNA experimentation. Each case was tested for all selected miRNAs, changing the tissue (Heart, Liver, Kidney, Brain, Musculoskeletal).

3.2.2 Heart failure (HF) group

Six cases of men who died of heart failure with negative toxicological analysis were enrolled

as the "HF (heart failure)" group (mean age 54.33 ± 9.97 years; BMI mean 26.9 ± 1.4). The main information for each case is summarized in table 3.

GROUP	AGE	CAUSE OF DEATH	MACROSCOPIC FINDINGS	MICROSCOPIC FINDINGS
			CARDIAC TISSUE	
HF				
1	72	Sudden cardiac death (SCD) in a patient with coronary heart disease (CHD)	Abnormal heart form (such as "ball" kind) with increased dimension (542 gr). The epicardium appeared strongly at the left ventricular wall, carton consistency.	Extensive areas of colliquative myocytolysis (grade I); extended areas of necrosis; arteriolosclerosis.
2	58	Acute heart failure (AHF)	Increased heart dimensions (510 gr) of a red-brownish color, with abundant subepicardial fat. The cardiac cavities contained abundant dark-red fluid blood. Aortic arch affected by atheromatous alterations.	Significant areas of necrosis on the left ventricle; areas of connective tissue damage, important presence of leukocytes.
3	54	SCD caused by Myocardial Ischemia	Regular heart in form and dimensions (weight 557gr).	Contraction band necrosis, coronary sclerosis, colliquative myocytolysis. Presence of recent thrombus in posterior descending artery (PDA).
4	51	SCD due to cardiac degeneration	Normal heart in dimension and form (480 gr).	Contraction band necrosis, fibrosis, stasis, and colliquative myocytolysis.
5	47	Cardiac failure with histopathological aspects of severe heart failure	Regular size of the heart (measuring 12x11.5x7 cm, weight 440 gr). Normal vascular structures.	Classical signs of chronic pericarditis; contraction-band necrotic areas, perivascular and interstitial fibrosis, stasis, grade III colliquative myocytolysis, arteriolosclerosis.
6	44	Acute myocardial necrosis with electromechanical dissociation with several ischemic areas	Regular heart (640 gr), with moderate subepicardial adipose tissue.	Contraction band necrosis,, acute stasis, coronary sclerosis.

Table 3. The main characteristics of the HF group (subjects died of heart failure with negative toxicological analysis). These samples were tested for cardiac and musculoskeletal tissues.

3.2.3 Brain injury: Stroke group (SG), Drug group (DG), and Aging group (AG)

To perform the analysis on the brain tissue, 3 groups were tested: 5 cases of men who died of brain stroke were selected (mean age 57.8 ± 6.7 years), making up the SG group; 5 cases of men who died with a toxicological test positive for drug abuse (cocaine) were selected (mean age 29.2 ± 5.6 years), forming the DG group; 5 cases of older men who died of Sudden Cardiac arrest (mean age 75.8 ± 6.9 years), generating the AG group (Table 4).

GROUP	AGE	CAUSE OF DEATH	MACROSCOPIC FINDINGS	MICROSCOPIC FINDINGS
			BRAIN TISSUE	
SG				
1	80	ischemic stroke	brain edema; reddish punctiform areas of the white matter.	perineuronal and perivasal edema, red neurons, malacic areas.
2	79	ischemic stroke from a vertebrobasilar embolism	brain edema; reddish punctiform areas of the white matter.	perineuronal edema, red neurons.
3	34	ischemic stroke in a subject with atheromatous formation at the Willis polygon	atherosclerotic alterations in the Willis polygon.	vasogenic edema, red neurons.
4	24	ischemic stroke from a systemic massive bleeding	post-hypoxic malacic areas of the white matter.	perivasal edema, red neurons, hemorrhages of Duret.
5	72	ischemic stroke	brain edema; reddish punctiform areas of the white matter.	vasogenic edema, ischemic areas, "red neurons".
DG				
1	29	sudden cardiac death secondary to cocaine intake	brain edema.	cortical edema.
2	32	fatal ventricular arrhythmia secondary to cocaine intake	brain edema.	vasogenic edema.
3	30	sudden cardiac death secondary to cocaine intake	brain edema.	perineuronal and perivasal edema.
4	20	fatal cardiac arrhythmia secondary to cocaine intake	brain edema.	cortical edema, arteriolosclerosis.
5	35	fatal ventricular arrhythmia secondary to cocaine intake	brain edema, stasis.	perineuronal edema, small perivascular hemorrhages
AG				
1	80	fatal cardiac arrhythmia	brain edema.	vasogenic edema, arteriolosclerosis.
2	83	ventricular arrhythmia	brain edema.	perineuronal and perivasal edema.
3	77	cardiac failure	brain edema.	vasogenic edema, small periventricular hemorrhages.
4	65	sudden cardiac death	brain edema.	cortical edema.
5	74	fatal cardiac arrhythmia	brain edema.	perineuronal and perivasal edema, small perivascular hemorrhages.

Table 4. The main characteristics of three groups tested for brain tissues: SG, stroke group; DG, drug group; AG, aged group.

3.2.4 Non-alcoholic fatty liver disease (NAFLD) group

Furthermore, to perform the liver tissue analysis, 5 cases of men who died of cardiac arrest (mean age 47.4 ± 4.03 years). During their life, all subjects had suffered from cirrhosis caused by NAFLD. The main characteristics are summarized in table 5.

GROUP	AGE	CAUSE OF DEATH	MACROSCOPIC FINDINGS	MICROSCOPIC FINDINGS
			LIVER TISSUE	
NAFLD				
1	43	Heart failure in a subject suffering from cirrhosis caused by NAFLD	The presence of micronodular cirrhosis in the liver with fatty change was detected by small, yellow nodules.	Presence of regenerative nodules of hepatocytes surrounded by connective tissue that bridges between portal tracts. Within this collagenous tissue lymphocytes were detected as well as a proliferation of bile ducts.
2	53	Heart failure in a subject suffering from cirrhosis caused by NAFLD	The presence of micronodular cirrhosis in the liver with fatty change was detected by small, yellow nodules.	Presence of regenerative nodules of hepatocytes surrounded by connective tissue that bridges between portal tracts.
3	49	Heart failure in a subject suffering from cirrhosis caused by NAFLD	The presence of micronodular cirrhosis in the liver with fatty change was detected by small, yellow nodules.	Presence of regenerative nodules of hepatocytes surrounded by fibrous connective tissue that bridges between portal tracts was reported. Within this collagenous tissue lymphocytes were detected as well as a proliferation of bile ducts.
4	44	Heart failure in a subject suffering from cirrhosis caused by NAFLD	The presence of micronodular cirrhosis in the liver with fatty change was detected by small, yellow nodules.	Presence of regenerative nodules of hepatocytes surrounded by connective tissue that bridges between portal tracts.
5	48	Heart failure in a subject suffering from cirrhosis caused by NAFLD	The presence of micronodular cirrhosis in the liver with fatty change was detected by small, yellow nodules.	Presence of regenerative nodules of hepatocytes surrounded by fibrous connective tissue that bridges between portal tracts.

Table 5. The main characteristics of the NAFLD group.

3.2.5 Chronic kidney disease (CKD) group

To analyze the kidney tissue, 5 cases of men who died of cardiac arrest after a long period of time (from 2 to 5 years) of Chronic kidney disease (CKD) were selected (mean age 66.2 ± 5.4 years). The main characteristics are summarized in table 6.

GROUP	AGE	CAUSE OF DEATH	MACROSCOPIC FINDINGS	MICROSCOPIC FINDINGS
			KIDNEY TISSUE	
CKD				
1	67	Heart failure in a subject suffering from Chronic kidney disease (CKD)	Abnormal kidney dimensions (reduced).	Diffuse hypocellular interstitial fibrosis of the cortex as well as of the medullae with tubular atrophy and global glomerular obsolescence. Moderate amounts of interstitial lymphocytes in the less fibrotic areas mainly located in the medullary rays and at the cortico-medullary junction.
2	69	Heart failure in a subject suffering of Chronic kidney disease (CKD)	Abnormal kidney dimensions (reduced).	Diffuse hypocellular interstitial fibrosis of the cortex as well as of the medullae with tubular atrophy and global glomerular obsolescence. Moderate amounts of interstitial lymphocytes in the less fibrotic areas mainly located in the medullary rays and at the cortico-medullary junction.
3	59	Heart failure in a subject suffering of Chronic kidney disease (CKD)	Abnormal kidney dimensions (reduced).	Perihilar focal segmental sclerotic lesion affecting enlarged glomeruli. Area of scarring with interstitial fibrosis, tubular atrophy, glomerulosclerosis.
4	73	Heart failure in a subject suffering of Chronic kidney disease (CKD)	Abnormal kidney dimensions (reduced).	Area of scarring and interstitial fibrosis, tubular atrophy.
5	63	Heart failure in a subject suffering of Chronic kidney disease (CKD)	Abnormal kidney dimensions (reduced).	Mild to moderate interstitial fibrosis and enlarged glomerulus.

Table 6. The main characteristics of the CKD group.

3.2.6 Control groups

Four cases of healthy men (age mean 28 ± 7.4), died from a car accident with negative anamnesis for heart suffering. The toxicological examination was negative for all subjects. This group was selected as reference samples in the Real-Time PCR reactions for the miRNA tested in cardiac and musculoskeletal tissues (Table 7).

GROUP	AGE	CAUSE OF DEATH	MACROSCOPIC FINDINGS	MICROSCOPIC FINDINGS
CONTROL				
1	29	Hypovolemic shock in polytrauma	Regular heart in form and dimensions	No pathological signs on the heart tissues
2	38	Hypovolemic shock in polytrauma	Regular heart in form and dimensions	No pathological signs on the heart tissues
3	24	Hypovolemic shock in polytrauma	Regular heart in form and dimensions	No pathological signs on the heart tissues
4	21	Hypovolemic shock in polytrauma	Regular heart in form and dimensions	No pathological signs on the heart tissues

Table 7. The main characteristics of the CONTROL group (subjects died from a car accident with negative anamnesis for heart suffering), utilized for miRNA quantification in the heart and musculoskeletal tissues.

Finally, 2 cases of healthy men (mean age 41.5 ± 2.12 years), who died in car accidents, for causes other than brain trauma, NAFLD, CKD and negative for toxicological examination were selected as controls in the Real-Time PCR reactions. The main data are reported in table 8.

GROUP	AGE	CAUSE OF DEATH	MACROSCOPIC FINDINGS	MICROSCOPIC FINDINGS
			BRAIN, LIVER, AND KIDNEYS TISSUES	
CONTROL				
1	40	Hypovolemic shock in polytrauma	Regular brain, liver and kidneys both in form and dimensions	No microscopic alteration on brain, liver and kidneys
2	43	Hypovolemic shock in polytrauma	Regular brain, liver and kidneys both in form and dimensions	No microscopic alteration on brain, liver and kidneys

Table 8. The main characteristics of the CONTROL group (subjects died in car accidents with negative anamnesis for brain, liver, and kidney diseases), used for miRNA quantification in the brain, liver, and kidney tissues.

3.3. Histological examination

The histological examination was performed for all cases. Histology is of value in confirming, evaluating and sometimes revising the course of natural disease processes that may have contributed to the cause of death. The sections were obtained from different tissues (Heart, Muscoloskeleton -Vastus lateralis-, Brain, Liver, Kidney) as indicated above. All slides were stained with hematoxylin and eosin, mounted with coverslips and finally observed with a Leica DM5000 upright microscope (Leica Microsystems).

3.4. Toxicological examination

All chemicals were analytical grade and were supplied by Analyticals Carlo Erba, Milan, Italy. AAS standards were supplied by Sigma and SALARS, Como, Italy. The solvents were purchased from Fluka Chemie. The extraction was performed according to Varian's procedure for C18 Bond-Elut used in AAS separation from the biological sample.

From each sample, 1 µl was injected into a gas chromatography/mass spectrometry (GC/MS) Hewlett Packard 6890/5973 system. The method was previously described (Pomara et al., 2016).

3.5. miRNA quantification through quantitative real-time PCR (qRT-PCR)

Total RNA, including miRNAs, was isolated from formaldehyde-fixed paraffin-embedded (FFPE) samples (four 20µm sections) using the Recover All Total Nucleic Acid Isolation Kit (Life Technologies) with minor modifications. Briefly, before RNA extraction, all samples were deparaffinated and processed as indicated in the protocol. Finally, the purified RNA was eluted with 65 µL RNAse-free-water. Quantification was performed in kidney, liver and central nervous system tissues.

The quantification of RNA was performed with the Qubit RNA HS Assay Kit (Life Technologies), using the Qubit Fluorometer; it provides an accurate and selective method for the quantitation of low-abundance RNA samples.

For miRNA profiling, the TaqMan Advanced miRNA Assay (Applied Biosystems Darmstadt, Germany) was used. This kit is composed of a pre-formulated primer and probe sets that are designed for the analysis of miRNA expression levels. The assays were selected at thermofisher.com/advancedmirna.

The miRNAs tested on the selected samples are summarized in table 9:

Tissue	Assay Name	Mature miRNA Sequence:	Chromosome Location
Heart and Muscle	hsa-miR-1-3p	UGGAAUGUAAAGAAGUAUGUAU	Chr.18: 21829015 - 21829036
	hsa-miR-133a-3p	UUUGGUCCCCUUCAACCAGCUG	Chr.18: 21825712 - 21825733
	hsa-miR-208a-3p	AUAAGACGAGCAAAAAGCUUGU	Chr.14: 23388602 – 23388623
	hsa-miR-499a-3p	AACAUCACAGCAAGUCUGUGCU	Chr.20: 34990445 - 34990466
Kidney	hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA	Chr.17: 59841266 - 59841337
	hsa-miR-205-5p	UCCUUCAUUCCACCGGAGUCUG	Chr.1: 209432133 - 209432242
Liver	hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA	Chr.17: 59841266 - 59841337
	hsa-miR-122-5p	UGGAGUGUGACAAUGGUGUUUG	Chr.18: 58451074 - 58451158
	hsa-miR-132-3p	UAACAGUCUACAGCCAUGGUCG	Chr.17: 2049908 - 2050008
	hsa-miR-155-3p	CUCCUACAUAUUAGCAUUAACA	Chr.21: 25573980 - 25574044
Brain	hsa-miR-132-3p	UAACAGUCUACAGCCAUGGUCG	Chr. 17 - 2049908 - 2050008
	hsa-miR-200b-3p	UAAUACUGCCUGGUAAUGAUGA	Chr.1: 1167104 - 1167198
	hsa-miR-21-5p	UAGCUUAUCAGACUGAUGUUGA	Chr.17: 59841266 - 59841337
	hsa-miR-34a-5p	UGGCAGUGUCUUAGCUGGUUGU	Chr.1: 9151668 - 9151777
	hsa-miR-124-5p	CGUGUUCACAGCGGACCUUGAU	Chr.8: 9903388 - 9903472
		Endogenous control genes	
(for all tissues)	hsa-miR-186-5p	CAAAGAAUUCUCCUUUUGGGCU	Chr.1: 71067631 - 71067716
(only for heart and muscle)	hsa-miR-361-5p	UUAUCAGAAUCUCCAGGGGUAC	Chr.X: 85903681 - 85903702

Table 9. Tested miRNAs with relative tissues.

cDNA was obtained following TaqMan Advanced miRNA Assays User Guide (Applied Biosystems, Publication number 100027897 Rev. C). Quantitative real-time PCR (qRT-PCR) was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems Darmstadt, Germany), and raw data were analyzed using the relative software (version 2.3). As described in the user's guide for Fast reaction plate, the qRT-PCR reactions (in triplicates) were performed with an incubation at 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. A negative control without cDNA was also included in parallel.

The data obtained were compared with the data from endogenous controls; as described in the user guide, they showed that gene expression was relatively constant across tissues. Normalization to endogenous control genes is currently the most accurate method to correct for potential biases that are caused by sample collection, variation in the amount of starting material, RT efficiency, and RNA preparation and quality. For this experiment "has-miR-186-5p" (TaqMan Advanced miRNA Assays, ThermoScientific) was used as an endogenous control for all tests, while "hsa-miR-361-5p" (TaqMan Advanced miRNA Assays, ThermoScientific) was used for heart and muscle tissues (table 9).

Expression fold changes were computed using the $2^{-\Delta\Delta Ct}$ calculation (Livak and Schmittgen, 2001), where ΔCt = Ct (test miRNA) – Ct (mir-186-5p/mir-361-5p) and $\Delta\Delta Ct$ = ΔCt (individual sample) - ΔCt (control median samples).

3.6. MiRNAs database and target prediction tools

The analysis of each miRNA was performed through a literature review, and several informatics tools: miRBase (available to http://www.mirbase.org/), TargetScanVert (available to http://www.targetscan.org/) and miRDB (available to http://mirdb.org/).

3.7. Questionnaires

Two surveys using online data collection tools were performed (google forms online survey, available at www.google.com):

- Survey 1, entitled "Multidimensional Body-Self Relations Questionnaire (MBSRQ)" (Cash, 2000a);
- Survey 2: was composed of 48 questions to know voluptuary habits.

Both questionnaires were filled in by a group of university students. Both surveys were performed between October 2018 and March 2019.

Guidelines from our local research and development department determined that the project

was an audit project and formal ethical approval was therefore not required. No personally identifiable data were collected or stored by the investigators.

3.7.1. Multidimensional Body-Self Relations Questionnaire (MBSRQ)

The MBSRQ was used to assess attitudes towards body image, physical activity, and health. These attitudes were evaluated in nine different scales, and then a tenth scale was added to the revised scoring system (Brown et al., 1990; Cash, 2000b, 2017).

To assess attitudes towards physical appearance, two main scales were used: "the appearance evaluation" scale that evaluates how attractive or unattractive and how happy or unhappy the person feels with his physical appearance and "the appearance orientation" scale to assess the time and effort spent by the person to "look good" (Brown et al., 1990; Cash, 2000b, 2017).

As for physical activity, the following scales were used: "the fitness evaluation" scale is how the person rates himself/herself as fit or not and how he/she feels involved in physical activities and the "fitness orientation" scale to assess the time and effort spent to improve his/her physical fitness (Brown et al., 1990; Cash, 2000b, 2017).

For attitudes towards health, the "health evaluation" scale was used to identify whether the person felt healthy or susceptible to disease, "the health orientation" scale was used to identify whether a person was health alert or neglected his/her health and "illness orientation scale" was used to assess how cautious she/he was to physical health and how he/she reacted to medical signs and symptoms (Brown et al., 1990; Cash, 2000b, 2017).

Finally to assess attitudes towards body weight, the "overweight preoccupation" scale tested fat anxiety, attention paid to body weight, dieting and controlled eating behaviors, the "self-classified weight" scale was used to assess how the person perceived his/her weight and the "body area satisfaction scale" was how satisfied or dissatisfied he/she was with specific parts of his/her body (Brown et al., 1990; Cash, 2000b, 2017).

This questionnaire was made up of 69 items. Each of the MBSRQ scales had its corresponding items. These items could be answered by a primary number ranging from 1 to 5; a low score of "1" indicated that the participant highly disagreed with the given statement, whereas a high score of 5 indicated that the participant highly agreed with it (Brown et al., 1990; Cash, 2000b, 2017). There were two scoring systems for the MBSRQ, a conceptual scoring system and a revised version (Brown et al., 1990; Cash, 2000b, 2017). The main differences between the two scoring systems fall into the following three scales: fitness evaluation, fitness orientation and health orientation (which was divided into two scales

health orientation and illness orientation in the revised scoring system). However, the conceptual scoring and the revised scoring were similar for the six remaining MBSRQ subscales (Brown et al., 1990; Cash, 2000b, 2017). In this study, the conceptual scoring system was used to score the MBSRQ.

3.7.2 Survey 2: voluptuary habits

Survey 2 was composed of 48 questions in order to know the voluptuary habits of the interviewees about the use of "Smart Drugs and Energy drinks", “Supplements” and "anabolic-androgenic steroids (AAS)".

Each part of Survey 2 was further subdivided into three parts. The first part concerned the knowledge of specific substances and their use. For the "Smart Drugs and Energy drinks" use/abuse about these substances were questioned: Cannabinoids, Synthetic cations (similar to amphetamines), Sexual performance-enhancing drugs, Coffee, Tea, Classic cola, WhoopAss, VitaminWaterEnergy Citrus, Bomba Energy, HiBall Energy, Red Bull, Powershot, Full Throttle, Monster, RockStar, V, Ammo, Fuel Cell, Blow Energy Brink Mix, NOS, Jolt Cola, Cocaine Energy Drink, Spike Shooter, Viso Energy Vigor, RedLine Power rush, Bookoo Energy, Fixx, and Wired X505.

For the "Supplements" use/abuse these substances were questioned: Soy protein, Oxygen, Creatine monohydrate, Carnosine, Organic Iron, APL, Magnesium Liquid, Thermo Fit 2, Arginine, GTS (Glucose Transport System), Mega Ram Plus, Vitamin C, Maltodex, Organic Magnesium, Carbo Burner, B Complex, Lipo Burner, Amino Drink, BCAA. For each question, the answers could be chosen between 4 with a relative score-value: - Never / Unknown (0 points); - Sometimes (1 points); - In training (2 points); - Near to the competition (3 points); Always (4 points).

For “AAS” use, an open answer was used to respond to this question:

- Which kind of AASs have you used?

In the case of a positive answer, other questions were given in order to ascertain the frequency, the cycles, the training, and other useful information.

The second part of the questionnaire allowed us to understand mental status during use. The questions are listed below:

- Does your mental state improve using (“Smart Drugs”/"Energy Drink", “Supplements”, “AASs”)? For example, in cognitive functioning, are you happier, more attentive, more concentrated and generally better? (Score: - 0, No; -1, no worse, no better; - 2, better).
- How would you classify your mental status during the use of (“Smart Drugs”/"Energy

Drink", “Supplements”, “AASs”)? (scale score from 0 to 10: 0, not worrying; 10, worrying). The last part of the questionnaire concerned physical perception during the use of the substances. The questions are listed below:

- Does your physical condition improve using (“Smart Drugs”/"Energy Drink", “Supplements”, “AASs”)? For example, you feel stronger, leaner, faster, fitter, healthier. (Score:- 0, No; -1, no worse, no better; - 2, better).
- How would you classify your physical condition during the use of (“Smart Drugs”/"Energy Drink", “Supplements”, “AASs”)? (scale score from 0 to 10: 0 “not healthy”, 10 “very healthy”).

3.8. Statistical analysis

Unless specified otherwise, data are expressed as means ± SEM versus baseline. The results were analyzed through student’s t-test (paired comparison) or one-way ANOVA using Tukey’s test as the post-hoc test (multiple comparisons) ($P<0.05$). Statistics were performed through the software Statistica for Windows (Statsoft, Tulsa, and Okhla.). Descriptive statistical analysis was performed using Microsoft Office Excel 2007.

Chapter 4. RESULTS

4.1. Literature review

During the three years of activity, two important literature reviews were performed. The first literature review ascertained the main adverse effects of AAS use/abuse. Even if this topic has been extensively investigated by the scientific community, to date, it is very important to better define the mechanisms of action that underline the adverse effects.

The main adverse effect with relative differences are reported in table 10.

Targets	Adverse Effects
Cardiovascular system	- myocardial infarction due to premature atherosclerosis (Montisci et al., 2012); - left ventricular hypertrophy (Büttner and Thieme, 2010); - impaired left ventricular function (D'Andrea et al., 2007; Thiblin et al., 2009); - arterial thrombosis (Parkinson and Evans, 2006) - pulmonary embolism (Payne et al., 2004) - fibrosis (Sader et al., 2001); - SCD (Fineschi et al., 2007; Montisci et al., 2012); - myocardial infarction without significant atherosclerotic coronary artery (Ferenchick and Adelman, 1992; Kierzkowska et al., 2005)
Genitourinary, reproductive complications, and sexual secondary factors	**Female:** - Hirsutism and Lowered voice tone (Damrose, 2009) - disruption of the ovarian function (Christou and Tigas, 2018); - delayed menarche, clitoromegaly, dysmenorrhoea, oligomenorrhoea, secondary amenorrhoea, anovulation and, as their consequence, infertility, (Nieschlag and Vorona, 2015); - breast reduction ((Demers et al., 1991)) - the female athlete triad (low energy intake, menstrual disorders and low bone mass) (Nieschlag and Vorona, 2015) - male-pattern baldness in women (Glaser and Dimitrakakis, 2013)
	Male: - suppression of spermatogenesis (Christou et al., 2017); - testicular atrophy (El Osta et al., 2016); - infertility and erectile dysfunction (Rahnema et al., 2014); - gynaecomastia (Sansone et al., 2017) - acceleration of baldness in men (Pan and Kovac, 2016)
Bone and muscles	- premature closure of the epiphysis in children(Hoffman and Ratamess, 2006)
Central nervous system	- aggressive behavior, anxiety, extreme mood swings from depression to mania or hypomania (Bertozzi et al., 2019a);

	- increased aggression and hostility (van Amsterdam et al., 2010); - destructive impulses and/or self-destructive impulses (Harmer, 2010);
Liver	- hepatic cholestasis (bile canal obstruction) causing jaundice (Elsharkawy et al., 2012; Ibrahim et al., 2018); - peliosis hepatitis (blood-filled sacs in the liver)(Bond et al., 2016; Solimini et al., 2017); - liver tumors (increased risk)(Niedfeldt, 2018)
Skin	- cystic acne (Goldman and Basaria, 2018)
Immune system	- immunosuppressive effects (Bertozzi et al., 2019b; Demling and DeSanti, 2003); - reducing immune cell number and function (Marshall-Gradisnik et al., 2009)

Table 10. Adverse effects generated by the use/abuse of anabolic androgenic steroids.

Moreover, to achieve the goals of the present research project, another literature review was performed with the aim of analyzing the literature production about all side effects related to AAS abuse, searching for the candidate miRNAs investigated in previous studies for their possible use as molecular biomarkers. Notably, the research hypothesis underlying this research project is that the same miRNAs investigated for similar damage generated by AAS use could be useful for developing new anti-doping tools. All miRNAs linked to specific damage are summarized in table 11.

This review was published during the activities of this project in 2018:

SESSA, F., SALERNO, M., DI MIZIO, G., BERTOZZI, G., MESSINA, G., TOMAIUOLO, B., PISANELLI, D., MAGLIETTA, F., RICCI, P. AND POMARA C. **Anabolic androgenic steroids: searching new molecular biomarkers.** (2018) Front. Pharmacol. 9:1321. doi: 10.3389/fphar.2018.01321

Cardiovascular System and Heart	**Differentially Expressed miRNAs**		**Reference**
	Upregulated	**Downregulated**	
ischemia		miR-1 miR-133	(Song et al., 2015)
hypertrophy	miR-208a; miR-150; miR-23a; miR-24; miR-21; miR-195; miR-199	miR-1; miR-26b; miR-27a; miR-143; miR-29; miR-133	(Hata, 2013; Joladarashi et al., 2014; Wong et al., 2016)
cardiac fibrosis	miR-21; miR-133	miR-29	(Joladarashi et al., 2014)
arrhythmia	miR-1; miR-133; miR-133a; miR-212;miR-17- miR-92;miR-106bb-; miR-25	miR-150	(Joladarashi et al., 2014)
Musculoskeletan	**Differentially Expressed miRNAs**		**Reference**
	Upregulated	**Downregulated**	
after 7 days of mechanical overload		miR-1; miR-133	(Kirby et al., 2015)
slow twitch fibers	miR-208b; miR-499		(Kirby et al., 2015)
Reproductive system disease (Male)	**Differentially Expressed miRNAs**		**Reference**
	Upregulated	**Downregulated**	
prostate cancer	miR-200c, miR-20a, miR-20b, miR-182	miR-222, miR-221, miR-145, miR-214, miR-125b, miR-143, miR-29a, miR-24, miR-199a	(Afshar et al., 2014)
	miR-375; miR-17; miR-93; miR-106a; miR-141; miR-720; miR-7a; miR-200b; miR-21; miR-106b; miR-375; miR-663b; miR-615-3p; miR-425-5p; miR-663a; miR-182-5p; miR-183-5p	miR-205-5p; miR-221-3p; miR-222-3p; miR-376c-3p; miR-136-5p; miR-455-3p; miR-455-5p; miR-154-5p	(Kristensen et al., 2016; Porkka et al., 2007; Volinia et al., 2006)
	miR-let-7a-2, miR-let-7i, miR-16-1, miR-17-5p, miR-20a, 21, miR-24-1, miR-25, miR-27a, miR-29a, miR-29b-2, miR-30c, miR-32, miR-34a, miR-92-2, miR-93-1, miR-95, miR-101-1, miR-106a, miR-124a-1, miR-126a-1, miR-135-2, miR-146, miR-149, miR-181b-1, miR-184, miR-187, miR-191, miR-196-1, miR-197, miR-199a-1, miR-214, miR-128a, miR-195, miR-198, miR-199a-1, miR-199a-2, miR-203, miR-206, miR-2014, miR-2018-2, miR-223, miR-202, miR-210, miR-296, miR-320, miR-370, miR-373, miR- 498, miR-503	Let 7a, let- 7b, let-7c, let-7d, let-7g, 16, 23a, 23b, 26a, 92, 99a, 103, 125a, 125b, 143, 145, 195, 199a, 221, 222, 497	

	miR-Let-7a-5p, miR-let-7d-3p, miR- let-7d-5p, miR- 7b-5p, miR-20a- 5p, miR-21-3p, miR-25-3p, miR-29b-2-5p, miR-30d-3p, miR-92a-3p, miR-92b-3p, miR-93-3p, miR-96-5p, miR-103b-3p, miR-182-5p, miR-183-5p, miR-375, miR-421, miR-423-3p, miR-423-5p, miR- 425-5p, miR-484, miR-615-3p, miR-663a, miR-663b, miR-664a-3p, miR-1248, miR-1260a		(Kristensen et al., 2016)
Reproductive system diseases (female)	**Differentially Expressed miRNAs**		**Reference**
	Upregulated	**Downregulated**	
premature ovarian failure (Blood)	miR-202; miR-146a; miR-125b-2; miR-139-3p; miR-654-5p; miR-27a; miR-765; miR-23a; miR-342-3p; miR-126	miR-Let-7c; miR-144	(McGinnis et al., 2015)
follicle atresia	miR-936; miR-26b; miR-149; miR-10b; miR-574-5p; miR-149; miR-1275; miR-99a	miR-Let-7i; miR-92b; miR-92a; miR-1979; miR-1308; miR-1826	(Li et al., 2015)
ovarian cancer	miR-21; miR-203; miR-205	miR-200 (ovarian cancer cell migration)	(Donadeu et al., 2017)
Central nervous system	**Differentially Expressed miRNAs**		**Reference**
	Upregulated	**Downregulated**	
depression (LH)	miR-96, miR-141, miR182, miR-183, miR-183*, miR-198, miR-200a, miR-200a*, miR-200b, miR-200b*, miR-200c, and miR-429.		(Dwivedi, 2014)
non depression (NLH)		miR-96, miR-141, miR182, miR-183, miR-183*, miR-198, miR-200a, miR-200a*, miR-200b, miR-200b*, miR-200c, and miR-429.	(Dwivedi, 2014)
Liver	**Diferentially Expressed miRNAs**		**Reference**
	Upregulated	**Downregulated**	
hepatic hypercholesterol and hyperlipid metabolism	miR-122; miR-21; miR-23		(Szabo and Bala, 2013)
inflammatory response hyperinflammation	miR-155, miR-132, miR-125b, miR-146a, miR-150, miR-181, let-7 and miR-21.20,21		(Szabo and Bala, 2013)
drug-induced liver injury (DILI)	miR-710 and miR-711 miR-16a, miR-328 and miR-299-5p	miR-122 and miR-192 miR-122a	(Szabo and Bala, 2013)
Kidney	**Diferentially Expressed miRNAs**		**Reference**
	Upregulated	**Downregulated**	
kidney fibrosis	miR-21		(Badal and Danesh, 2015)

renal fibrosis, tubular hypertrophy, glomerular alterations	miR-200a, miR-200b, miR-141, miR-429, miR-205, and miR-192.		(Wei et al., 2013)
acute kidney injury (AKI)	mir-21, mir-205, mir-127 and mir-494		(Wei et al., 2013)
Skin	**Diferentially Expressed miRNAs**		**Reference**
	Upregulated	**Downregulated**	
dermatomyositis	miR-146b and miR-155	miR-1, miR-133, miR-206, miR-11040; miR-30a-3p	(Luo and Mastaglia, 2015)

Table 11. Organ damage and miRNA expression profiles investigated in the literature.

4.2. Autopsy, histological and toxicological examination on AAS abuser

For each selected case the autopsy examination combined with the histological and toxicological investigation were performed.

The main characteristics relative to each case are reported in table 12.

	Case 1	Case 2	Case 3	Case 4	Case 5
Age	29	30	32	40	31
Body weight	72 kg	90 kg	90 kg	83 kg	80 kg
Height	166 cm	178 cm	189 cm	175 cm	170
BMI	26,1	28,4	25.19	27.1	27,7
Bodybuilder	Yes	Yes	Yes	No	Yes
Hospitalization	No (he died at home)	No (he died at home)	No (he died at the gym)	No (he died in his own garage)	Yes (10 days)
Cause of death	Heart failure secondary to AAS assumption	Heart failure secondary to AAS assumption	Heart failure secondary to AAS assumption	Heart failure secondary to AAS assumption	Septic shock, starting as necrotizing myofasciitis

Table 12. In this table the main characteristics about each case were summarized.

4.2.1 Autopsy and Histological examination

Case 1

At the autopsy, all organs were macroscopically normal, except the heart. It had a regular shape and weighed 380 g, with a predicted weight of 304 g (range 215–429 g). No pathological signs were detected analyzing the coronary arteries, the myocardium, and the valvular apparatus. Both ventricular wall thicknesses were normal.

Microscopic examinations were performed on the ventricles, revealing numerous foci of contraction band necrosis. There were two foci of fibrosis, one at the subendocardial anterior left ventricle and another at the interventricular septum. These findings were noted in all myocardial sections: segmentation of the myocardial cells and/or widening of intercalated discs and bundles of contracted myocardium alternating; bundles of distended myocardium with granular disruption of the myocytes (Figure 5). No pathological signs were noted on the coronary arteries, reporting only the physiologic intimal thickening typical of the subepicardial arterial vessels.

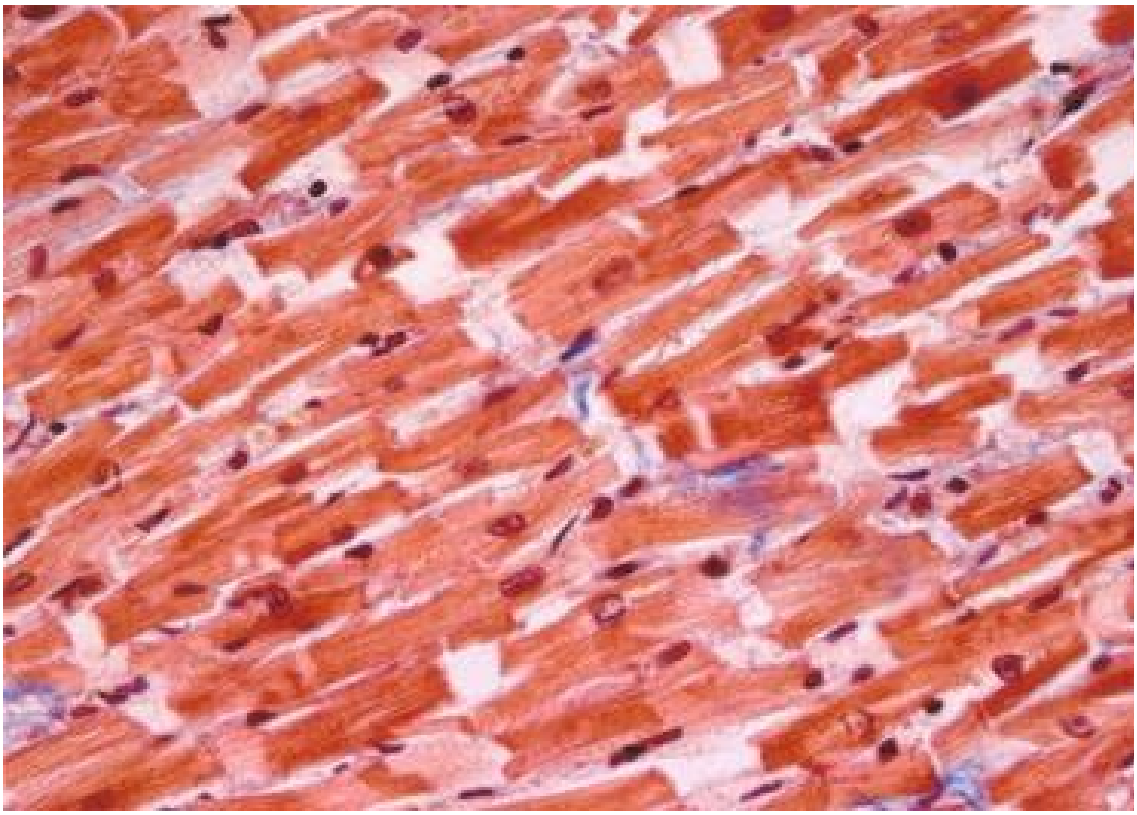


Figure 5: Segmentation of the myocardial cells, widening of intercalated discs, and bundles of contracted myocardium alternating with bundles of distended myocardium with granular disruption of myocytes (H & E ×250). This picture is my own picture, previously published (Fineschi et al., 2007)

Case 2

The autopsy revealed abnormal muscle development, testicular atrophy, and hepatomegaly. The heart was normal in shape (weight was 400 g).

Histopathologic examination of the heart revealed focal myocardial fibrosis (Figure 6); no alterations were detected on the conduction system. The liver showed cholestasis and vascular gaps compatible with the diagnosis of peliosis hepatis (Drug abuse handbook, 1998).

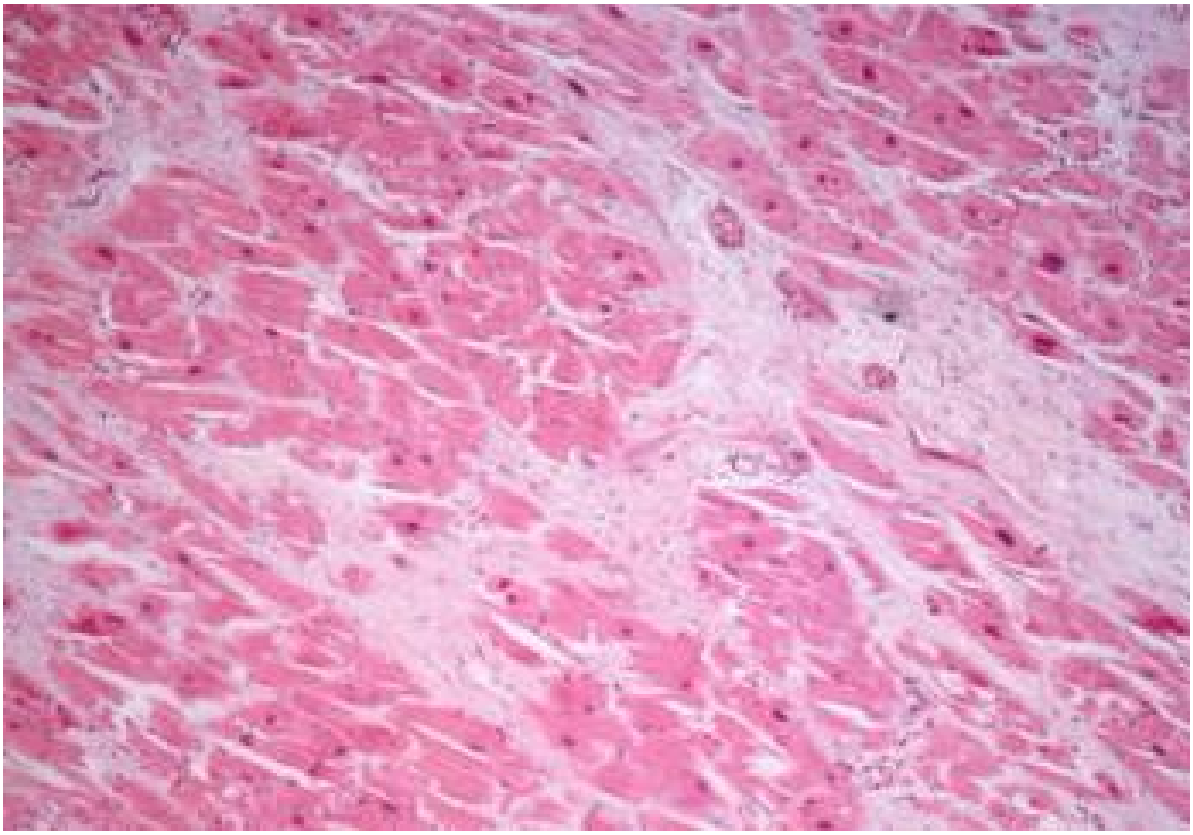


Figure 6. Scar fibrosis (H & E ×100)

Case 3

The external examination showed a male body with prominent muscular masses. At the autopsy, the heart weight was 450 g, normal for dimension and form. Heart structures (pericardium, cardiac valves, endocardium, and coronary arteries) were normal. No thrombi were detected both in vessels and in the cardiac cavities. Several samples were taken for histologic examination.

A grayish area similar to typical infarct necrosis with a histologic age of approximately 15 days was detected. The lesion was characterized by hyperdistended myocardial cells surrounded by sarcomeres, circumscribed at its periphery by young collagen tissue containing numerous macrophages (Figure 7). At other cardiac sites, the pathologic findings were represented by occasional foci of contraction band necrosis and few fibrotic microfoci in the internal portion of the posterior left ventricle and interventricular septum.

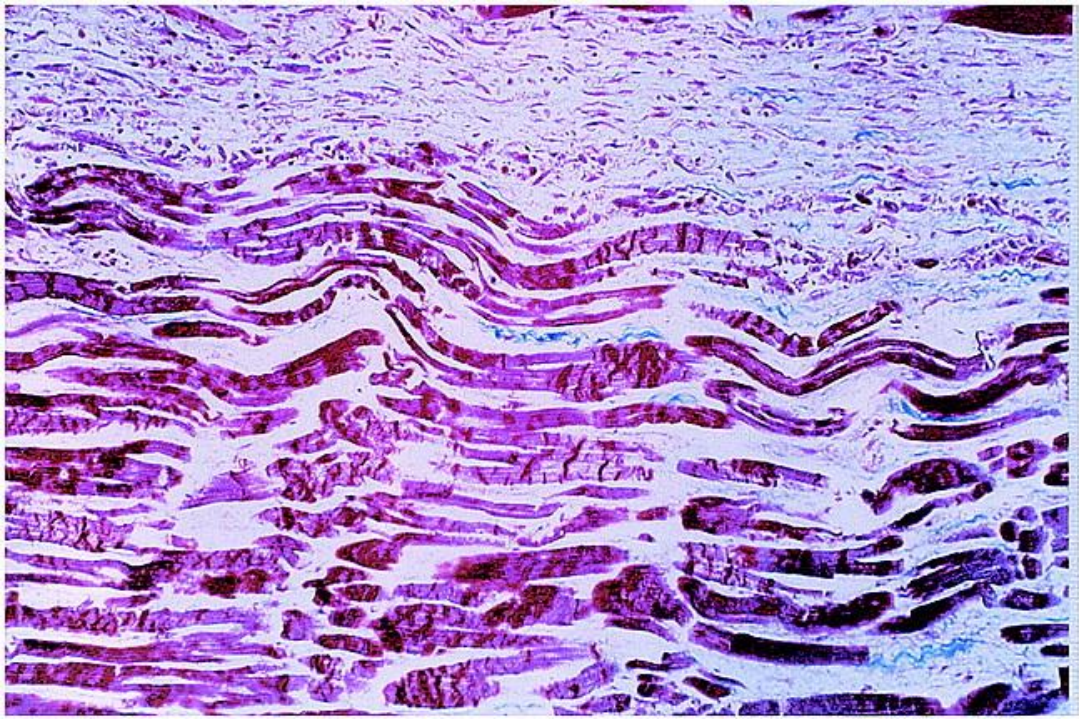


Figure 7. Many hypercontracted, deeply eosinophilic myocardial cells (early contraction band necrosis) external to a 15-day-old infarct already surrounded by collagen tissue plus macrophage reaction (phosphotungstic acid hematoxylin ×80).

A segmentation of the myocardial cells was found in all myocardial samples. Granular disruption of the myocardial cells was occasionally observed. No other signs of pathologic change were reported.

Case 4

The external examination showed a regular heart shape both in consistency and color (the weight was 380 g). At the autopsy, no pathological signs were found: the identification of the macroscopic cause of death was not possible.

Histopathologic examination of the heart revealed fibrotic areas of myocardial tissue substitution with interruption in the muscular continuity (Figure 8). Liver slides were characterized by the presence of micro and macrocytic steatosis areas.

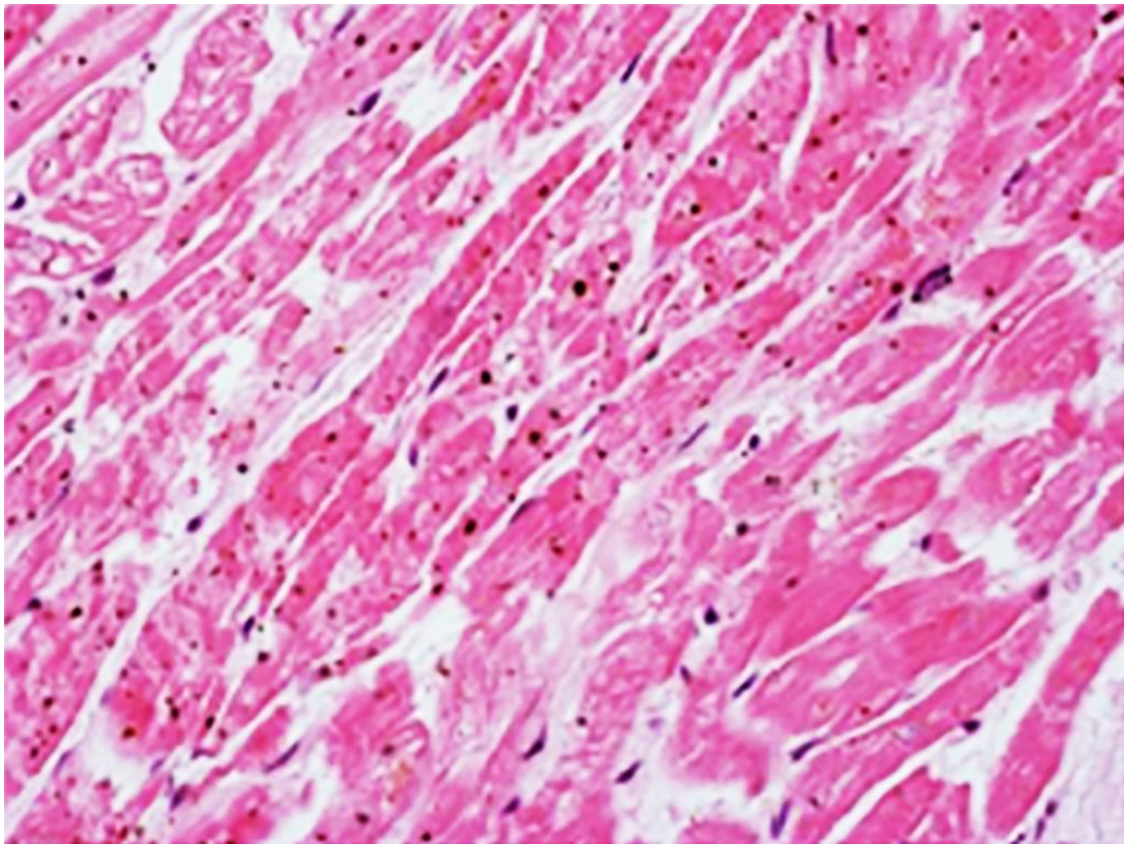


Figure 8. Heart slide showing grade II colliquative myocytosis, contraction bands, necrosis foci and perivasal fibrosis (H & E ×100).

Case 5

The autopsy revealed abnormal muscle development, testicular atrophy, and hepatomegaly. The heart weight was 350 g.

The heart showed regular thickness of the subepicardial fat layer and arteriole walls. The myocardium presented, in some fields, limited areas of fibrosis as well as scattered interstitial hemorrhages. Finally, in some fields, there were aspects of dissolving myofibers (colliquative necrosis). Sampling from rectus femoris and vastus lateralis was remarkable for the abundant presence of polymorphonuclear cells and erythrocytes both at the perimysial level and between the fascicles (Figure 9a). In some fields, the muscular structure appeared completely subverted with large spaces occupied by erythrocytes and myocellular residue (Figure 9b, 9c). Immunohistochemical anti-myoglobin showed myofibrillar rexis (Figure 9d).

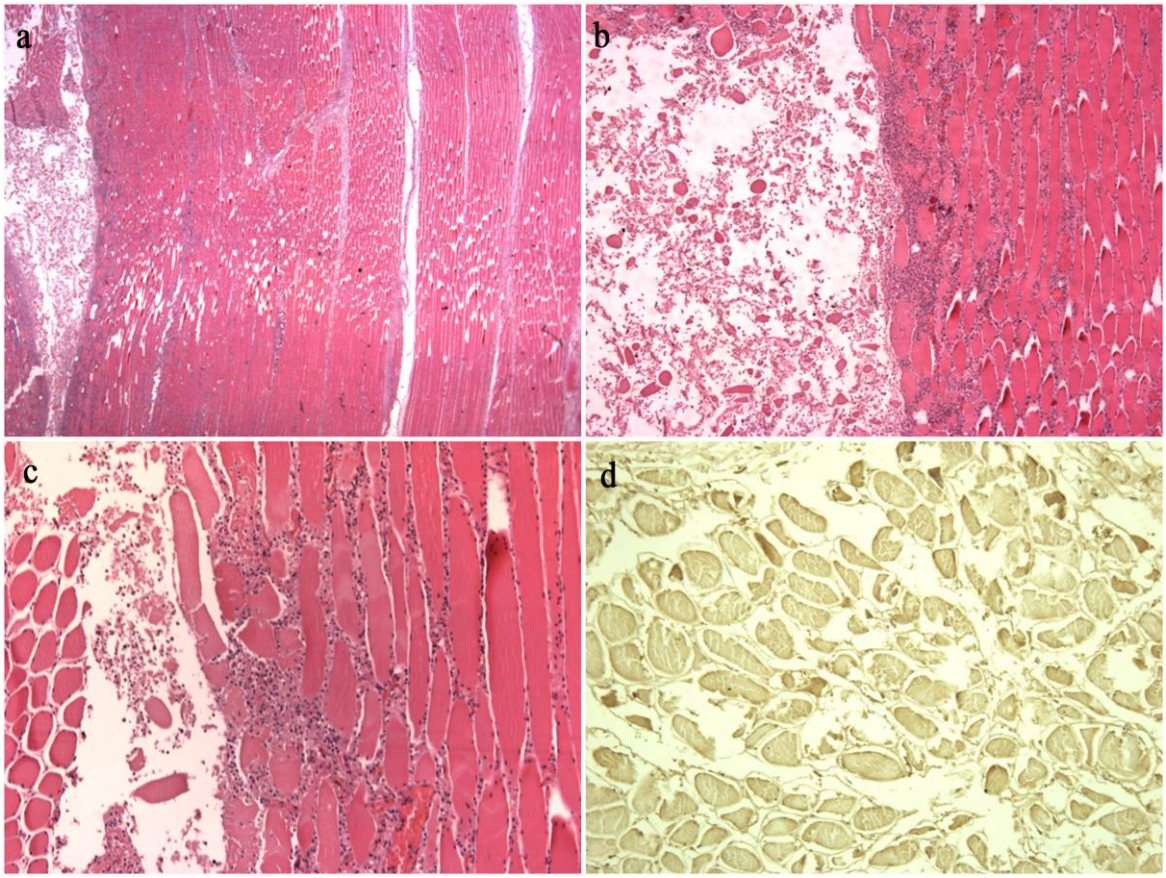


Figure 9. Muscle tissue: abundant polymorphonuclear cells and erythrocytes both at the perimysal level and between the fascicles themselves (a). In some fields, the muscular structure appears completely subverted with large spaces occupied by erythrocytes and myocellular residue (b, c). Immunohistochemical anti-myoglobin staining showing myofibrillar rexis (d).

4.2.2 Toxicological examination

For all cases, abuse substances tests were performed with negative results.

In case 1 the urine concentration for nandrolone was not measurable, stanozolol 43 µg/l, and testosterone/epitestosterone ratio (T/E)=28.7. Complete toxicologic examination was negative for drugs of abuse, including ethanol.

In case 2 the blood concentration for nandrolone was 19 ng/ml; in the urine the concentration for Norandrosterone was 208.4 ng/ml, while testosterone/epitestosterone ratio (T/E) = 42.

In case 3 the urine concentration for nandrolone was 16 ng/ml, stanozolol 33 µg/l, and testosterone/epitestosterone ratio (T/E)=23.4. The Gas chromatographic-mass spectrography of urine showed 19-nor-androsterone, 19-nor-etiocholanolone, and nor-epiandrosterone (metabolites of nandrolone), as well as 3-idrossi-stanozolol and 3-idrossi-17-epistanozolol (metabolites of the anabolic steroid stanozolol).

In case 4, the toxicological assessments performed on the blood sample showed a positive result for stanozolol 53 ng/mg and tamoxifen 19 ng/mg. Moreover, toxicological investigations were also performed on other items found at the crime scene: a blood-stained 10-ml syringe. The analysis performed on non-biological items showed only traces of trenbolone acetate in the syringe.

In case 5, the toxicological examination was carried out on hair: in toxicology it is an important bio-monitor that can extend the window of drug detection to weeks, months or even years. In this biological matrix the concentration of testosterone propionate was 24 pg/mg, clenbuterol was 26 pg/mg, stanozolol 66 pg/mg, trenbolone 28 pg/mg, oxandrolone 33 pg/mg and tamoxifen 84 pg/mg. Furthermore, the blood sample was analyzed: there was positivity for stanozolol 46 ng/mg and tamoxifen 15 ng/mg.

4.3. miRNA quantification through qRT-PCR

The quantitative analysis step aimed to evaluate the expression levels of several miRNAs in the AAS group, compared to other groups composed of subjects suffering from related pathologies.

For heart and musculoskeletal tissues, the miRNA expression values of miRNA has-133a-3p, hsa-miR-208a-3p, hsa-miR-499a-3p, and hsa-miR-1-3p were tested. The results were analyzed using the expression values of two different endogenous controls: hsa-miR-186 and miR-361.

For kidney tissue, miRNA expressions of hsa-miR-21-5p and hsa-miR-205-5p were evaluated in the AAS group compared to the CKD group.

hsa-miR-21-5p, hsa-miR-122-5p, hsa-miR-132-3p and hsa-miR-155-3p expression values were evaluated in liver tissue and compared with the AAS group and NAFLD group.

Finally, in the brain tissue samples, the quantitative analysis was performed to evaluate the expression levels of several miRNAs: hsa-miR-21-5p, hsa-miR-132-3p, hsa-miR-200b-3p, hsa-miR-34a-5p, and hsa-miR-124-5p, in the four groups (AASs, SG, DG, and AG).

For the kidney, liver and brain tissues, the expression values were compared with the expression levels of the endogenous control, hsa-miR-186.

Moreover, a statistical analysis was performed analyzing the expression values of each miRNA tested, comparing the tested groups.

4.3.1. Heart tissue and muscoleskeletal system

The first step of quantitative analysis evaluated the expression levels of the tested miRNAs (has-133a-3p, hsa-miR-208a-3p, hsa-miR-499a-3p, and hsa-miR-1-3p), in the HF group. A box plot analysis was performed for each endogenous control, hsa-miR-186 and has-miR-361 (Figures 10 and 11).

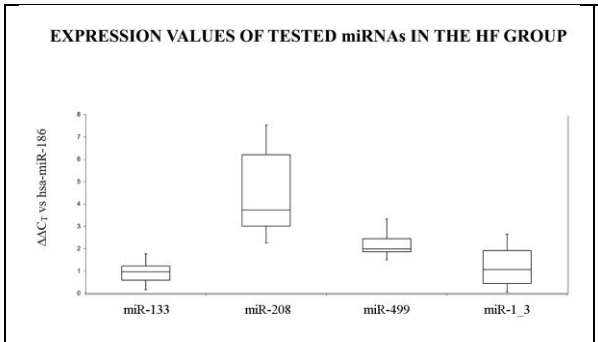


Figure 10. The box plot represents the expression levels of the target miRNAs (endogenous control miR-186) analyzed in the HF group (heart tissue).

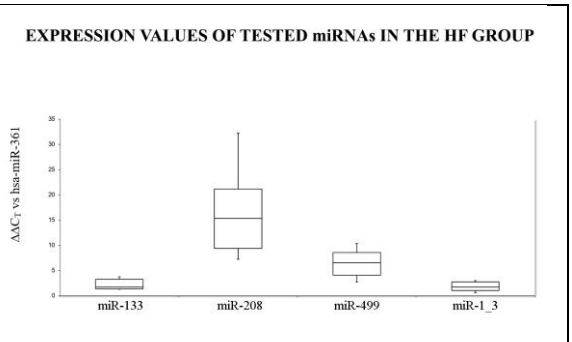


Figure 11. The box plot represents the expression levels of the target miRNAs (endogenous control miR-361) analyzed in the HF group (heart tissue).

As illustrated in the figures 10 and 11, all tested miRNAs were upregulated compared to endogenous controls. In table 13 the expression levels are reported.

Mean Expression levels (HF group) (endogenous control: miR-186)			
miR-133	**miR-208**	**miR-499**	**miR-1-3**
0.94 ± 0.57	4.51 ± 2.21	2.32 ± 0.89	1.21 ± 1.02
Mean Expression levels (HF group) (endogenous control: miR-361)			
2.22 ± 1.17	16.75 ± 9.73	6.43 ± 3.01	1.81 ± 1.04

Table 13: Expression levels of the tested miRNAs in the HF group.

Comparing the expression values for each tested miRNA, there were statistically significant differences: miR-208 expression was significantly higher compared to the other tested miRNAs [F(3,20) = 8.88, p = 0.0006(has-miR-186); F(3,20) = 10.93, p = 0.00018 (has-miR-361)].

The second step is the quantification of the expression levels of miRNA has-133a-3p, hsa-miR-208a-3p, hsa-miR-499a-3p, hsa-miR-1-3p, in the AAS group. A box plot analysis was performed for each endogenous control (Figures 12 and 13).

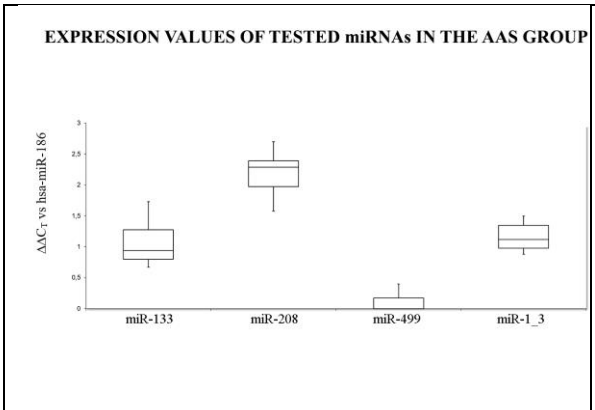


Figure 12. The box plot represents the expression levels of the target miRNAs (endogenous control miR-186) analyzed in the AAS abuser group (heart tissue).

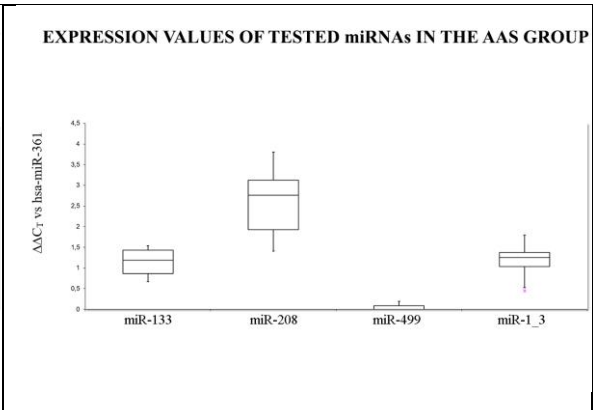


Figure 13. The box plot represents the expression levels of the target miRNAs (endogenous control miR-361) analyzed in the AAS abuser group (heart tissue).

Table 14 summarizes the data indicating the expression levels of each tested miRNA.

Mean Expression levels (AAS group) (endogenous control: miR-186)			
miR-133	**miR-208**	**miR-499**	**miR-1-3**
1.07 ± 0.4	2.19 ±0.39	0.1 ± 0.17	1.16 ± 0.24
Mean Expression levels (AAS group) (endogenous control: miR-361)			
1.14 ± 0.36	2.6 ± 0.91	0.05 ± 0.08	1.19 ± 0.45

Table 14: Expression levels of miRNAs analyzed in the AAS group, in heart tissue.

Comparing the expression values for each tested miRNA, there were statistically significant differences: the expression of miR-208 was significantly higher compared to the other tested miRNAs [$F(3,20) = 42.23$, $p = 7.67 \times 10^{-9}$(has-miR-186); $F(3,20) = 22.21$, $p = 1.41 \times 10^{-6}$ (has-miR-361)].

Moreover, the statistical analysis was performed comparing the data of each miRNA between the two groups (AASs vs HF). Analyzing the expression values of miR-133, using mir-186 as endogenous control, it was expressed similarly in two tested groups (t-test, p=0.33); no significant differences were found analyzing the expression values of miR-1_3 (t-test, p=0.33). The expression values were significantly higher in the HF group compared to the AAS group both for miR-208 and miR-499: indeed, even if these miRNAs were overexpressed in both groups compared to controls, the t-test highlighted a statistical difference in the results (HFvsAASs: miR-208, t-test, p=0.02; miR-499, t-test, p=0.001). Approximately the same results were reported using the other endogenous control, miR-361, even if, in this case, the statistical analysis showed that miR-133 (t-test, p=0.03) was overexpressed in the HF group more than in the AAS group. No statistical differences were reported analyzing the data from miR-1_3 (t-test, p=0.11), while both miR-208 and miR-499 were expressed higher in the HF group (miR-208, t-test, p=0.008; miR-499, t-test, p=0.001).

Moreover, the quantification of the expression levels of miRNAs has-133a-3p, hsa-miR-208a-3p, hsa-miR-499a-3p, and hsa-miR-1-3p, in the AAS group, was performed testing muscle tissue (vastus lateralis) (figures 14 and 15).

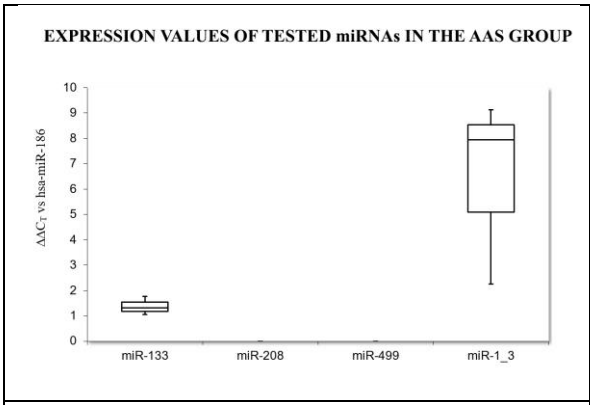


Figure 14. The box plot represents the expression levels of the target miRNAs (endogenous control miR-186) analyzed in the AAS group in muscle tissue (*vastus lateralis*).

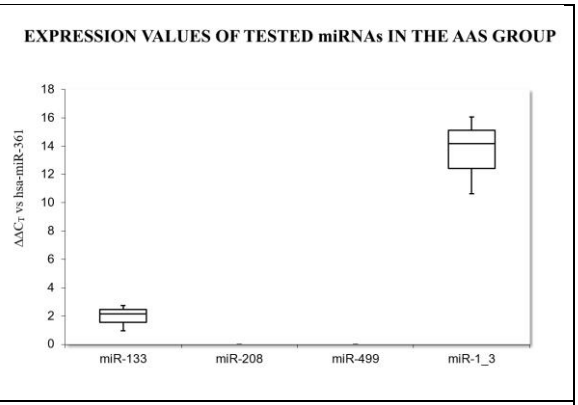


Figure 15. The box plot represents the expression levels of the target miRNAs (endogenous control miR-361) analyzed in the AAS group in muscle tissue (*vastus lateralis*).

Table 15 summarizes the data indicating the expression levels of each tested miRNAs.

Mean Expression levels (AAS group) (endogenous control: miR-186)			
miR-133	**miR-208**	**miR-499**	**miR-1-3**
1.37 ± 0.35	$1.39^{-10} \pm 2.58^{-11}$	$1.42^{-10} \pm 6.93^{-10}$	6.44 ± 3.67
Mean Expression levels (AAS group) (endogenous control: miR-361)			
1.94 ± 0.91	$4.59^{-10} \pm 3.3^{-10}$	$1.95^{-10} \pm 1.37^{-10}$	13.63 ± 2.76

Table 15: Expression levels of miRNAs analyzed in the AAS group, in muscle tissue (vastus lateralis).

Finally, all data for each tested miRNA are summarized in box plots elaborated for each single tissue analyzed (figures 16-19). These schematic representations are more useful to compare the expression level variations of each tested miRNA.

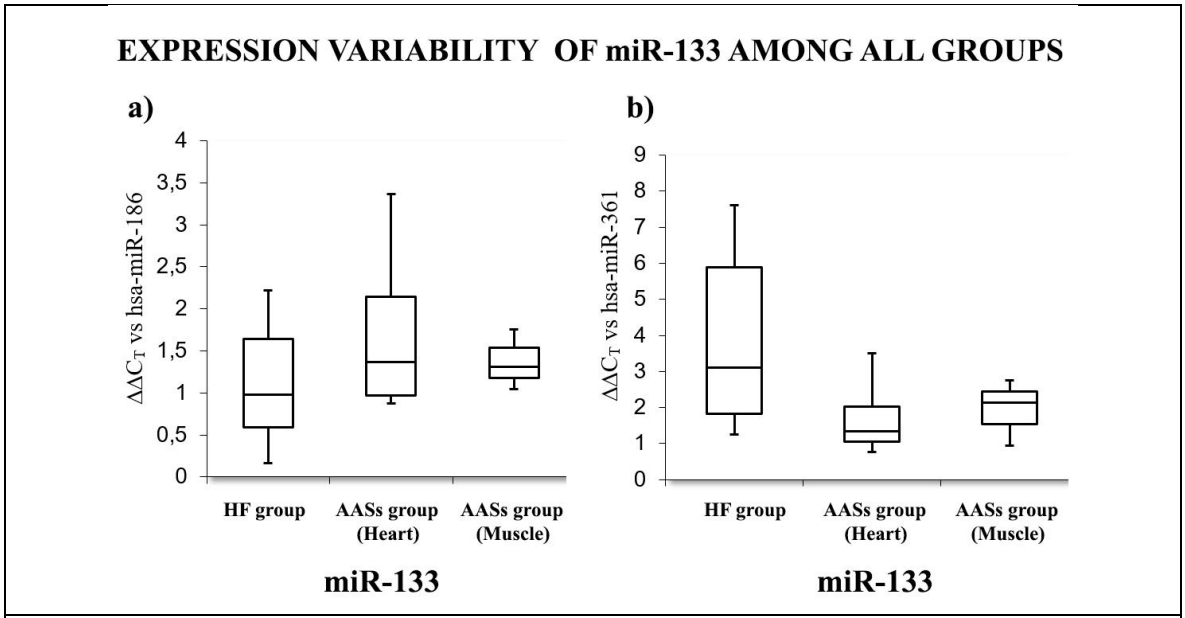


Figure 16a. The box plot represents the expression levels of miR-133 (endogenous control miR-186) in heart tissue (HF and AAS groups) and muscle tissue (AAS group).
Figure 16b. The box plot represents the expression levels of miR-133 (endogenous control miR-361) in heart tissue (HF and AAS groups) and muscle tissue (AAS group).

The expression levels of miR-133 in heart tissue (HF and AAS groups) and muscle tissue (AAS group) were upregulated compared to controls. No statistical differences were reported analyzing data both with miR-186 [$F(2,15) = 1.59$, $p = 0.23$] and with miR-361 [$F(2,15) = 2.48$, $p = 0.11$].

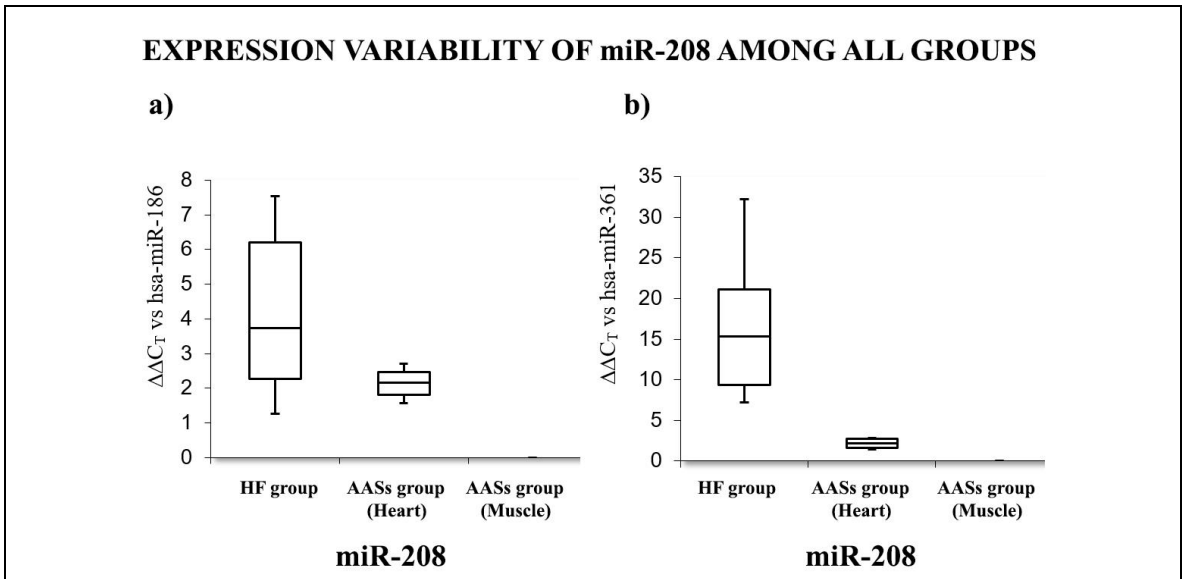


Figure 17a. The box plot represents the expression levels of miR-208 (endogenous control miR-186) in heart tissue (HF and AAS groups) and muscle tissue (AAS group).
Figure 17b. The box plot represents the expression levels of miR-208 (endogenous control miR-361) in heart tissue (HF and AAS groups) and muscle tissue (AAS group).

The expression levels of miR-208 in heart tissue (HF and AAS groups) were upregulated compared to controls. These results were not confirmed in the muscle tissue (AAS group),

where it was expressed similarly to controls. The differences reported were statistically significant with both endogenous controls, miR-186 [$F(2,15) = 17.35$, $p = 0.0001$] and with miR-361 [$F(2,15) = 15.19$, $p = 0.0002$].

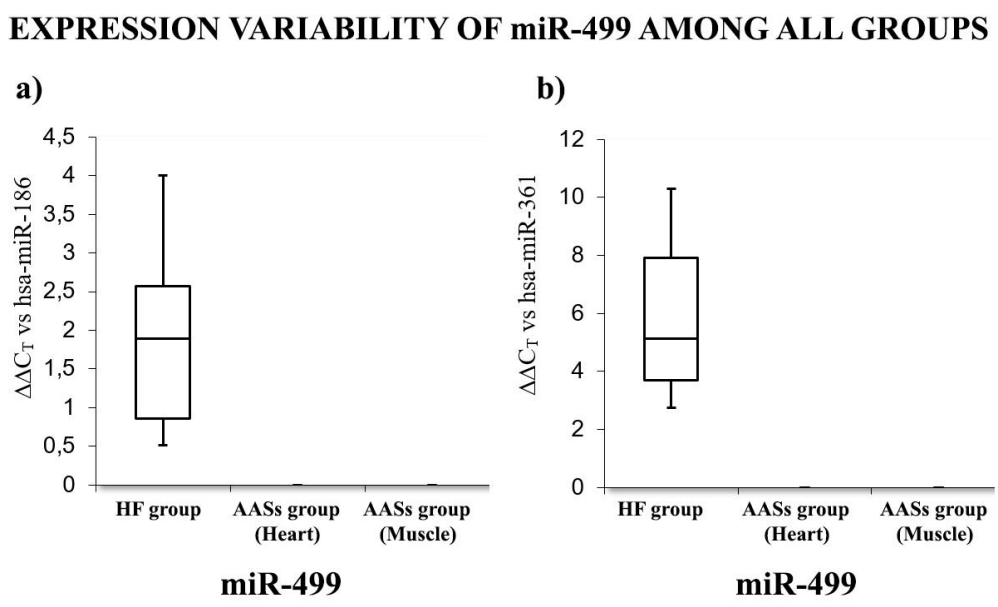


Figure 18a. The box plot represents the expression levels of miR-499 (endogenous control miR-186) in heart tissue (HF and AAS groups) and muscle tissue (AAS group).
Figure 18b. The box plot represents the expression levels of miR-499 (endogenous control miR-361) in heart tissue (HF and AAS groups) and muscle tissue (AAS group).

The expression levels of miR-499 were upregulated in heart tissue in the HF group. In the heart and muscle tissues of the AAS group, the expression levels were similar to controls. The differences reported were statistically significant with both endogenous controls, miR-186 [$F(2,15) = 35.97$, $p = 1.89 \times 10^{-6}$] and with miR-361 [$F(2,15) = 26.87$, $p = 1.1 \times 10^{-5}$].

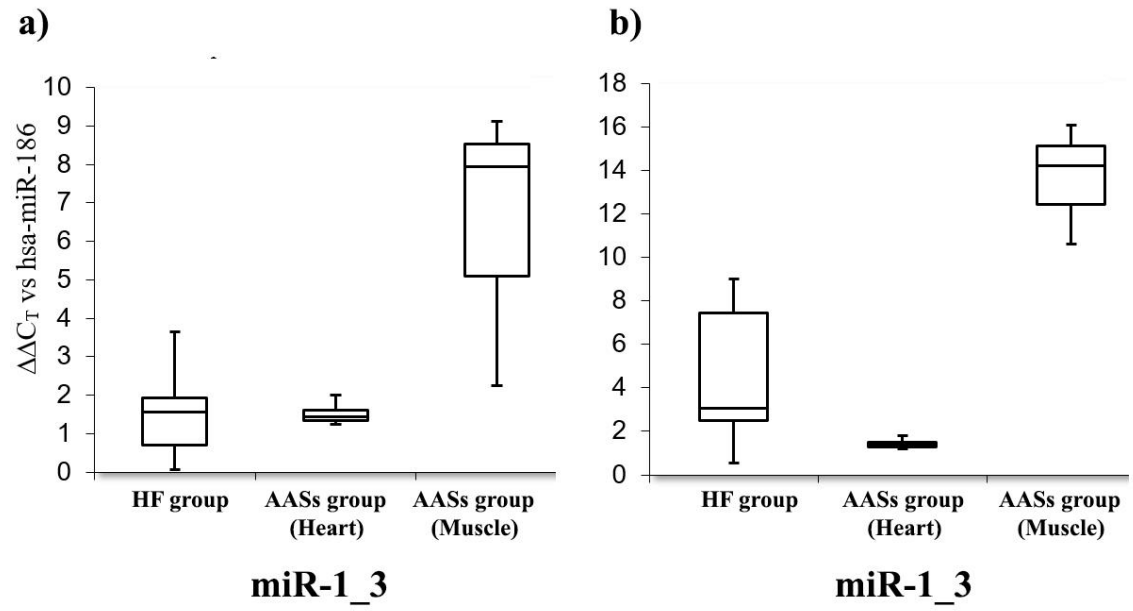


Figure 19a. The box plot represents the expression levels of miR-1_3 (endogenous control miR-186) in heart tissue (HF and AAS groups) and muscle tissue (AAS group).
Figure 19b. The box plot represents the expression levels of miR-1_3 (endogenous control miR-361) in heart tissue (HF and AAS groups) and muscle tissue (AAS group).

The expression levels of miR-1_3 in heart tissue (HF and AAS groups) and muscle tissue (AAS group) were upregulated compared to controls. This miRNA was overexpressed in the muscle tissue (vastus lateralis). The described differences were statistically significant with both endogenous controls, miR-186 [$F(2,15) = 12.52$, $p = 0.0006$] and with miR-361 [$F(2,15) = 113.34$, $p = 8.83 \times 10^{-10}$].

4.3.2 Kidney tissues

The expression values of miR-21 in kidney tissues of all tested groups are summarized with a box plot analysis (Figure 20).

Comparing the expression values in the two tested groups (AASs vs CKD), even if the values were improved compared to controls, the CDK values were statistically significantly higher than the AAS group [$F(1,8) = 5.31$, $p = 0.013$].

Performing a box plot analysis of the expression levels of miR-205 in each group (figure 21), even if it was higher in the CKD group compared to the AAS group, it was not statistically significant [$F(1.8) = 5.31$, $p = 0.21$].

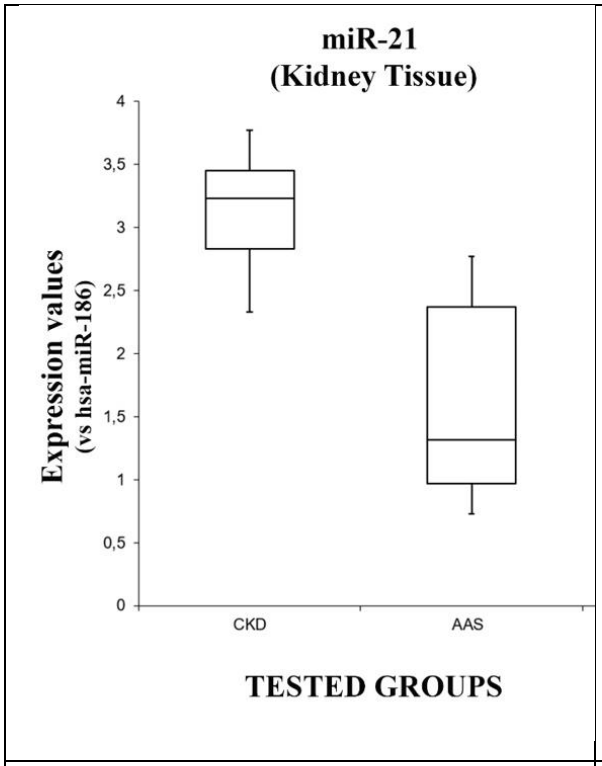


Figure 20. Box plot analysis comparing expression levels of miR-21 (endogenous control miR-186) in each group.

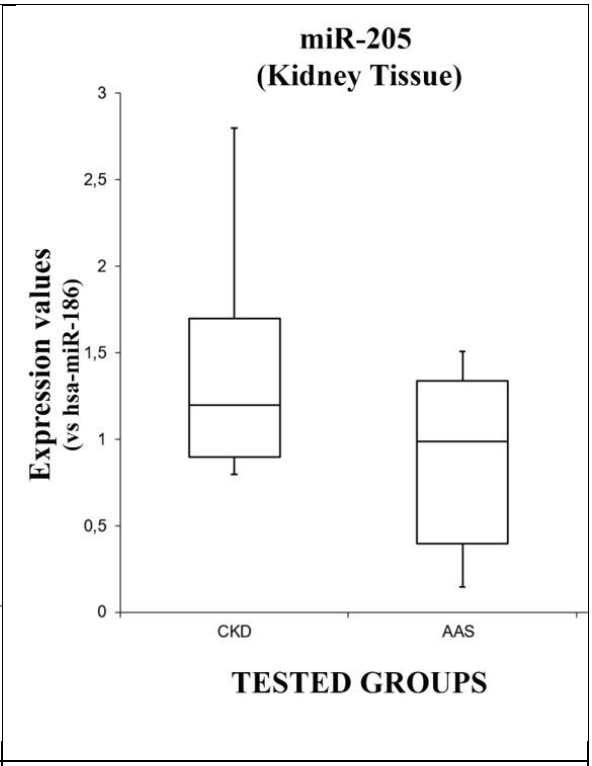


Figure 21. Box plot analysis comparing expression levels of miR-205 (endogenous control miR-186) in each group.

4.3.3 Liver tissues

The expression values of miR-21 in the liver tissues of all tested groups are summarized with a box plot analysis in Figure 22. Comparing the expression values in the two tested groups (AASs vs CKD), even if the values were higher compared to controls, this miRNA was overexpressed in the AAS group with a statistically significant difference [$F(1,8) = 5.31$, $p = 0.023$].

Performing a box plot analysis of the expression levels of miR-122 in each group (figure 23), even if the values were improved compared to controls, the NAFDL values were significantly higher than the AAS group [$F(1,8) = 5.31$, $p = 0.017$].

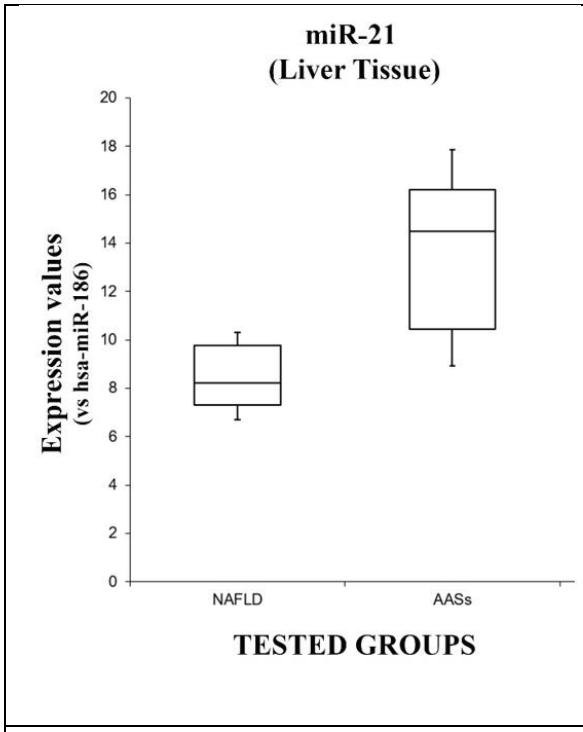


Figure 22. Box plot analysis comparing expression levels of miR-21 (endogenous control miR-186) in each group.

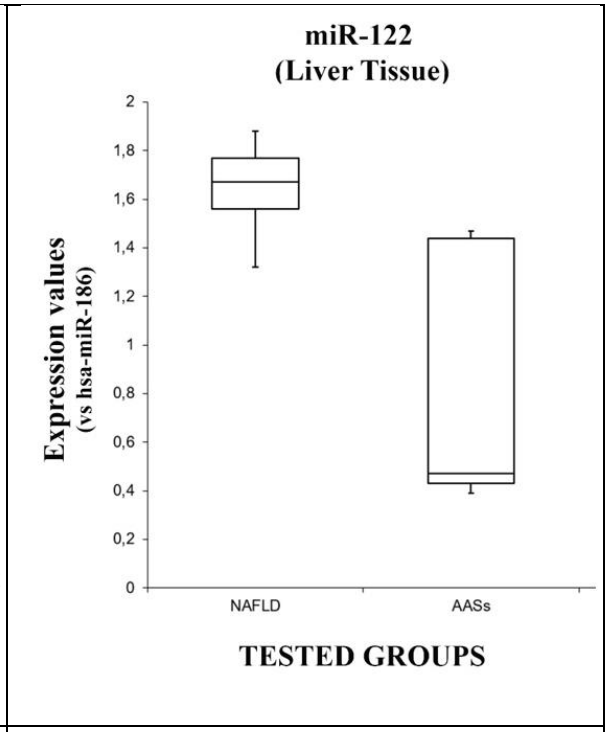


Figure 23. Box plot analysis comparing expression levels of miR-122 (endogenous control miR-186) in each group.

Analyzing the expression levels of miRNA-132, there were no significant differences comparing the AAS Group to the NAFDL group [$F(1,8) = 5.31$, $p = 0.54$] and AG [$F(1,8) = 5.31$, $p = 0.59$] (figure 24).

Finally, analyzing the expression levels of miRNA-155, there were no significant differences comparing the AAS Group with the NAFDL group [$F(1,8) = 5.31$, $p = 0.59$] (Figure 25).

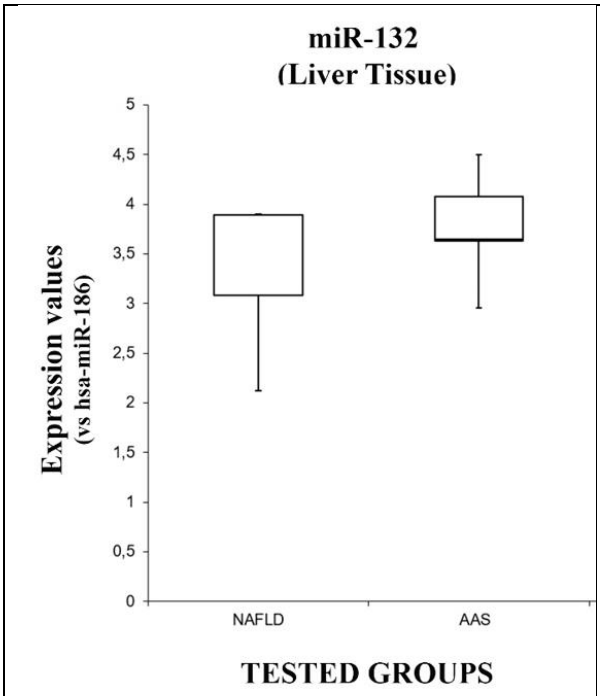


Figure 24. Box plot analysis comparing expression levels of miR-132 (endogenous control miR-186) in each group.

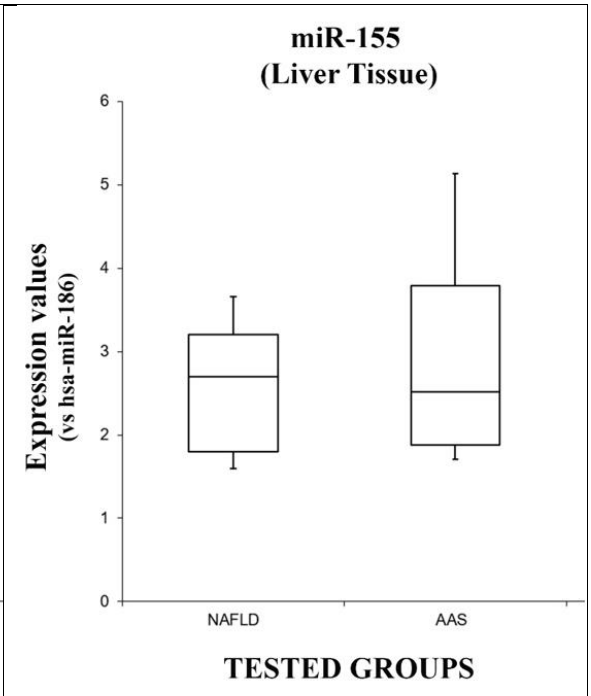


Figure 25. Box plot analysis comparing expression levels of miR-155 (endogenous control miR-186) in each group.

4.3.4 Brain tissues

The expression values of miR-21 in brain tissues of all tested groups are summarized with a box plot analysis in Figure 26.

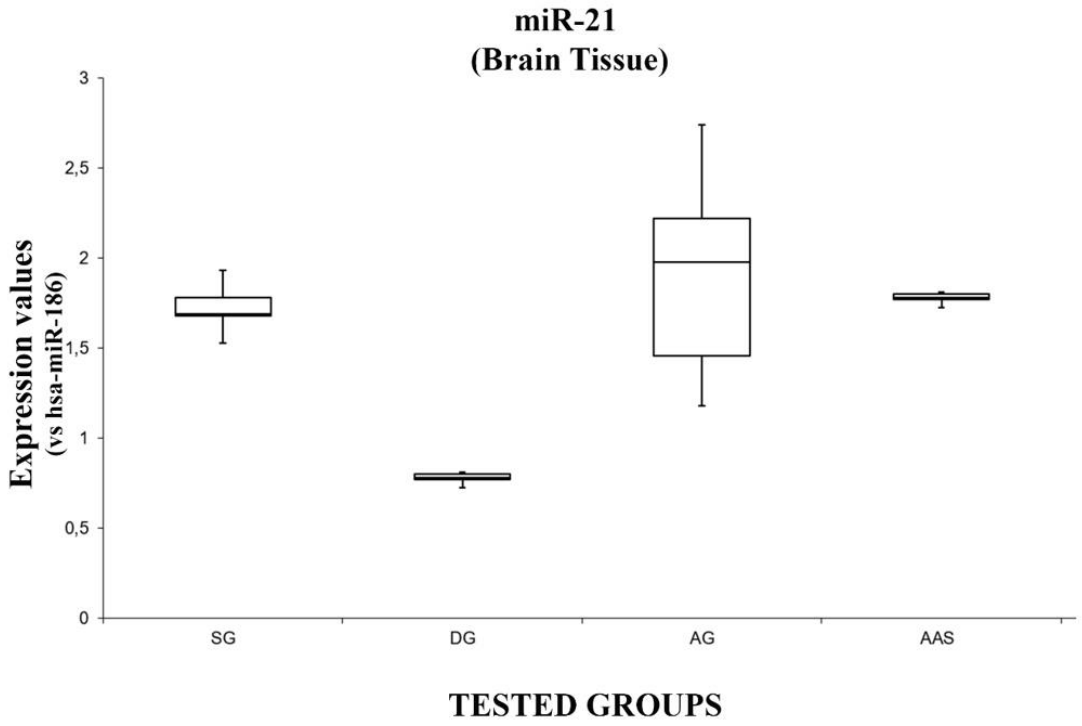


Figure 26. Box plot analysis comparing expression levels of miR-21 (endogenous control miR-186) in each group.

There were statistically significant differences among group means as determined by one-way ANOVA [$F(3,16) = 3.23$, $p = 0.00019$]. Moreover, comparing the expression levels of miRNA-21 singularly (AAS group vs each other group), there were no significant differences comparing the AAS group to the SG [$F(1,8) = 5.31$, $p = 0.54$] and AG [$F(1,8) = 5.31$, $p = 0.59$] groups. The expression levels were significantly higher in the AAS group compared to the DG group [$F(1,8) = 5.31$, $p < 0.05$ (8.27×10^{-9})].

Analyzing the expression values of miR-34 (figure 27), the data showed that this miRNA was significantly higher in the AAS and DG groups with respect to the SG and AG groups [$F(3,16) = 3.23$, $p < 0.05$ (1.23×10^{-9})]. Analyzing the AAS group values compared to each other group, there were no statistical differences in the mir-34 expression values between AAS and DG groups [$F(1,8) = 5.31$, $p = 0.09$], while the expression levels were significantly higher with respect to both the AG group [$F(1,8) = 5.31$, $p < 0.05$ (1.08×10^{-6})] and the SG group [$F(1,8) = 5.31$, $p < 0.05$ (4.09×10^{-5})].

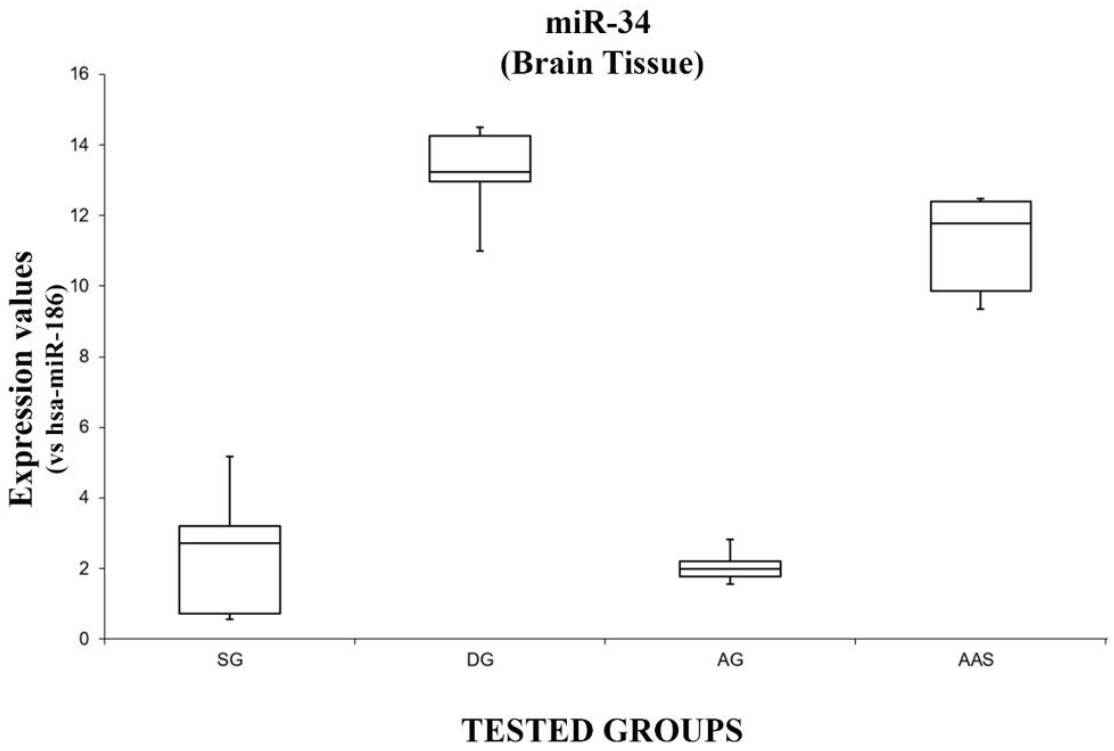


Figure 27. Box plot analysis showing the expression levels of miR-34 (endogenous control miR-186) in each group.

The data of miR-124 showed a significant difference among the tested groups [$F(3.16) = 3.23$, $p < 0.05$ (9.05×10^{-12})] (figure 28); comparing singularly (AAS groups vs other groups), its expression levels were significantly lower compared to the SG group [$F(1.8) = 5.31$, $p = 0.000262$], while they were significantly higher with respect to both the DG group [$F(1.8) = 5.31$, $p = 0.000152$] and the AG group [$F(1.8) = 5.31$, $p = <0.05(1.36^{-5})$].

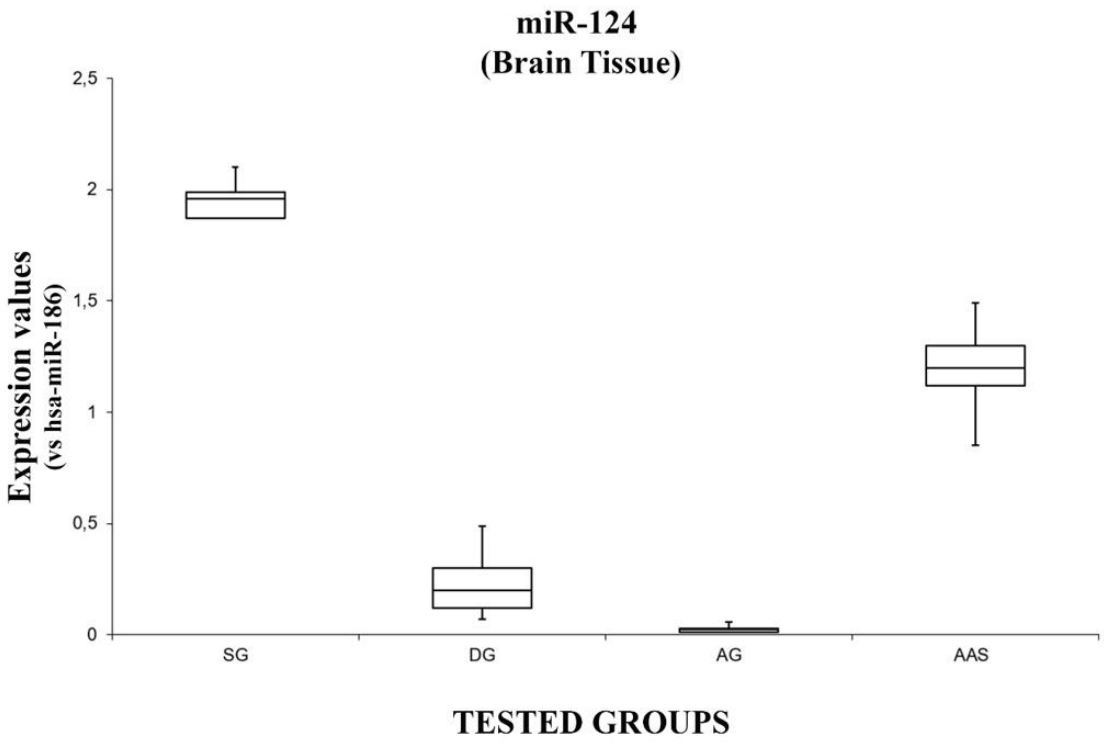


Figure 28. Box plot analysis showing the expression levels of miR-124 (endogenous control miR-186) in each group.

Analyzing the expression levels of miR-132, there were statistical differences among group means as determined by one-way ANOVA [F (3.16) = 3.23, p = $<0.05(3.95^{-7})$]. Moreover, comparing the expression levels of miRNA-132 singularly, there were no significant differences between the DG and AAS groups [F (1.8) = 5.31, p = 0.41], even if the DG values were higher. Contrariwise, analyzing the data of the AAS group with the other groups, there were significantly higher expression levels compared to both the SG group [F (1.8) = 5.31, p = 0.000149], and the AG group [F (1.8) = 5.31, p = 0.000264] (figure 29).

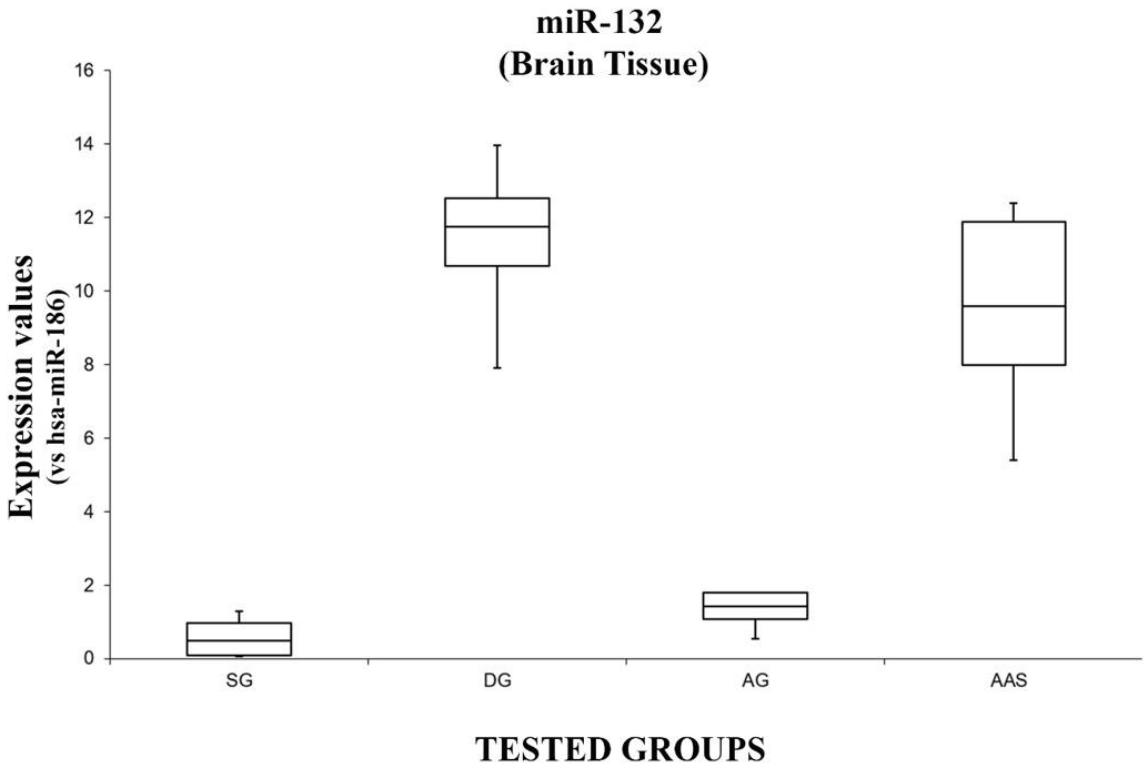


Figure 29. Box plot analysis showing the expression levels of miR-132 (endogenous control miR-186) in each group.

Performing a box plot analysis of the expression levels of miR-200b in each group (figure 30), its expression levels were higher in the AAS group compared to the other groups [F (3.16) = 3.23, p = 0.000234]. Furthermore, comparing singularly the expression values of miR-200b with the AAS group and the other groups, there are significant differences both with DG [F (1.8) = 5.31, p = 0.000397] and AG [F (1.8) = 5.31, p = 0.0169] groups, while no significant differences were found between the expression values of this miRNA comparing the AAS group vs the SG group [F (1.8) = 5.31, p = 0.06].

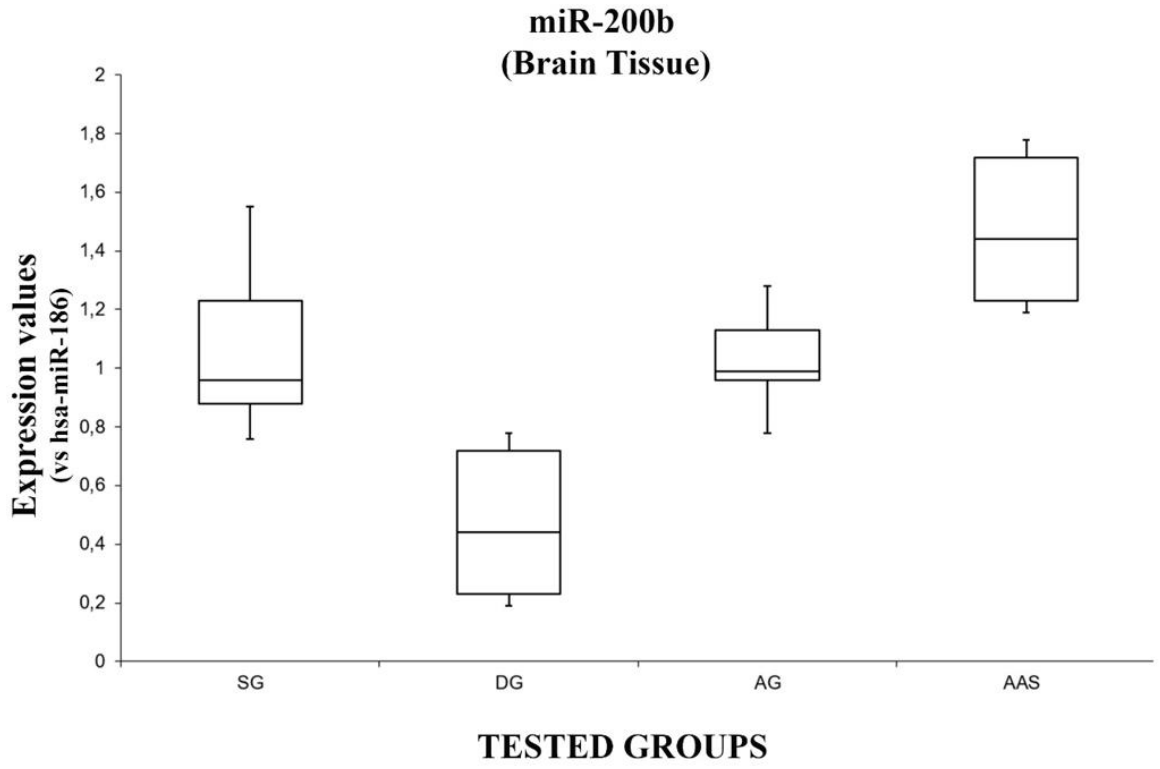


Figure 30. Box plot analysis showing the expression levels of miR-200b (endogenous control miR-186) in each group.

4.4 MicroRNAs database and target prediction tools

The analysis of each miRNA was performed through a literature review, and several informatics tools: miRBase (available to: http://www.mirbase.org/), TargetScanVert (available to: http://www.targetscan.org/) and miRDB (available to: http://mirdb.org/).

On cardiac and muscle tissues the following miRNAs were tested: hsa-miR-1-3p, hsa-miR-133a-3p, hsa-miR-208a-3p, and hsa-miR-499a-3p.

Figure 31 screens of each database interrogated for hsa-miR-1-3p.

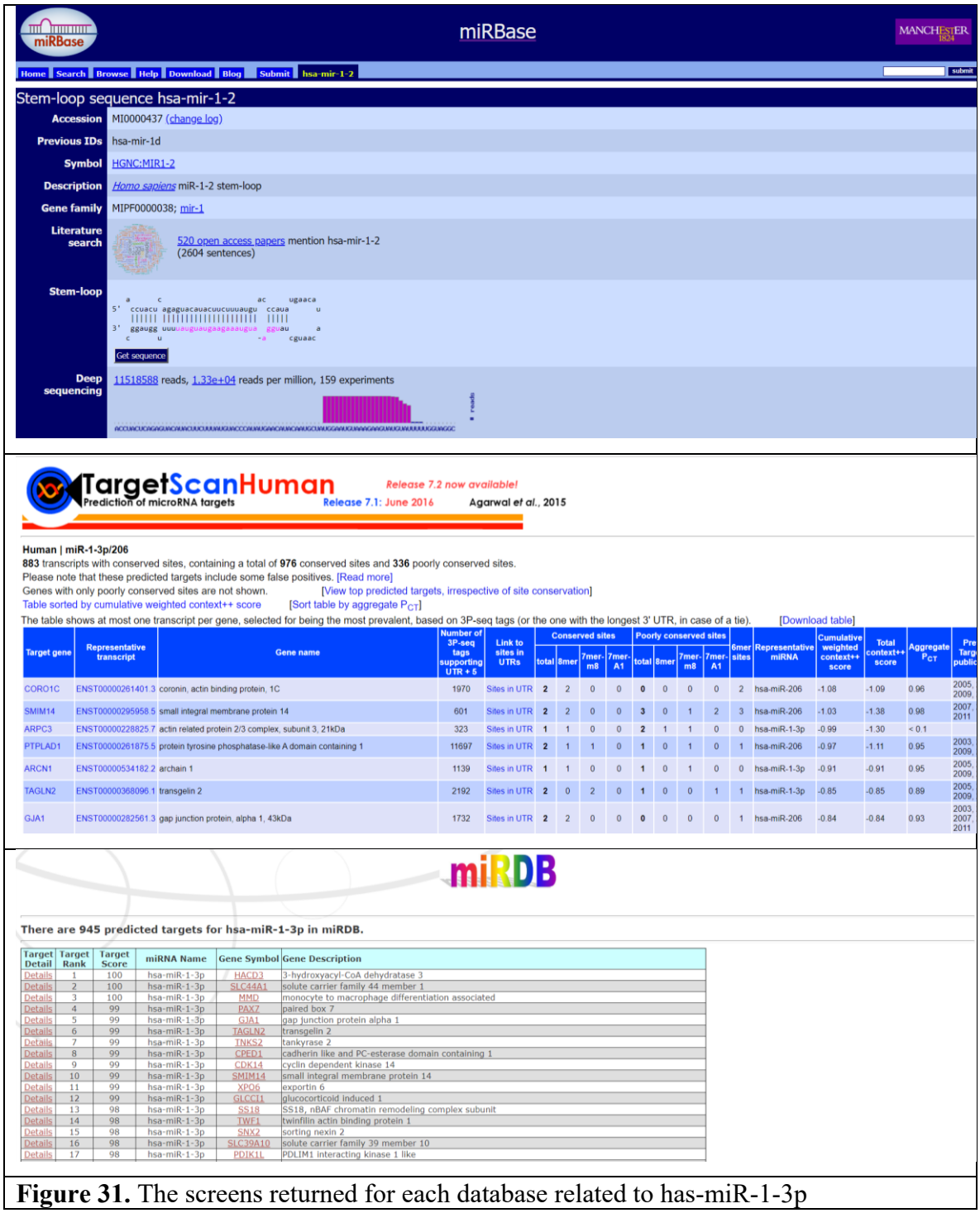


Figure 31. The screens returned for each database related to has-miR-1-3p

Figure 32 screens of each database interrogated for the hsa-miR-133a-3p.

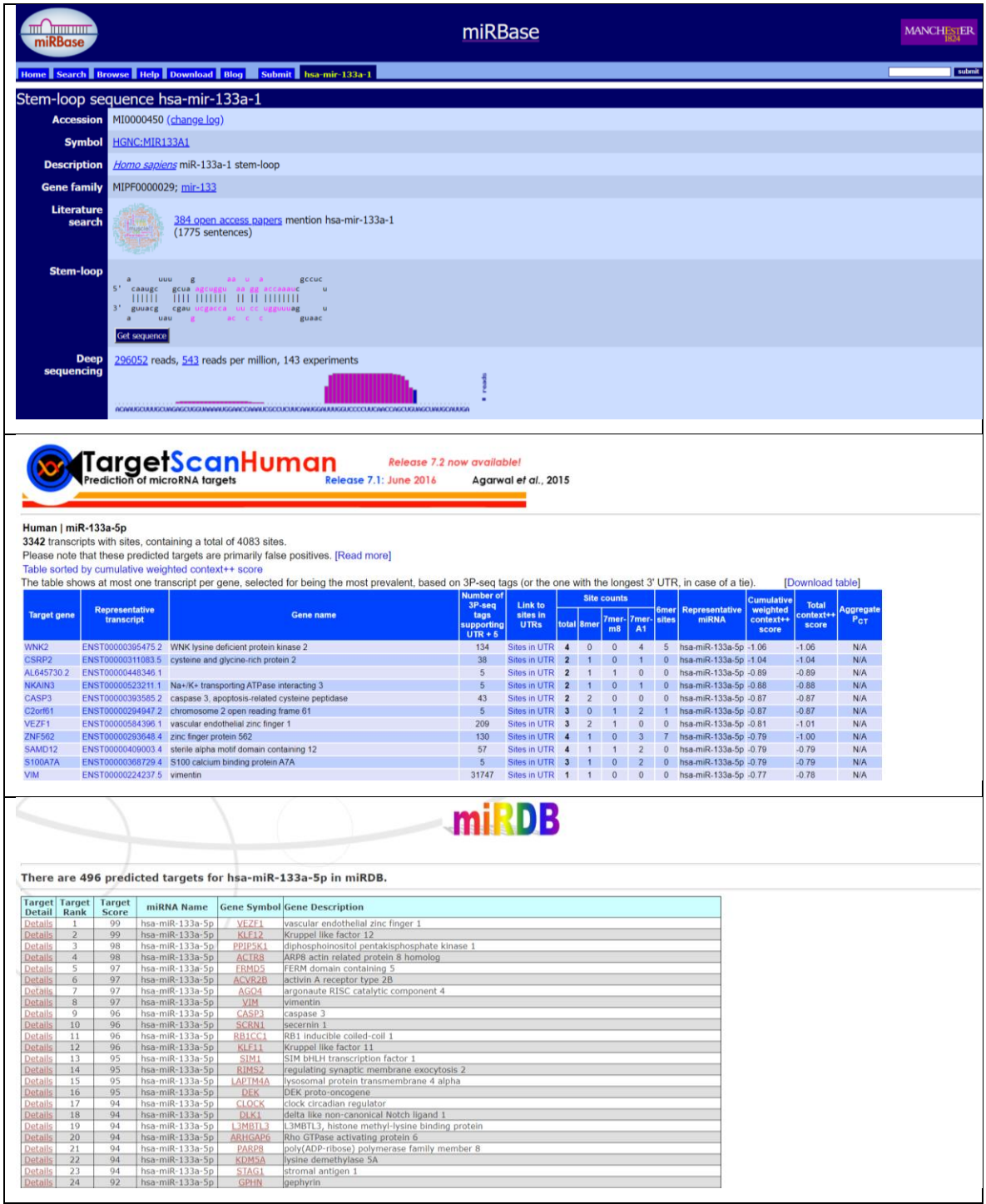


Figure 32. The screens returned for each database related to hsa-miR-133a-3p.

Figure 33 screens of each database interrogated for hsa-miR-208a-3p.

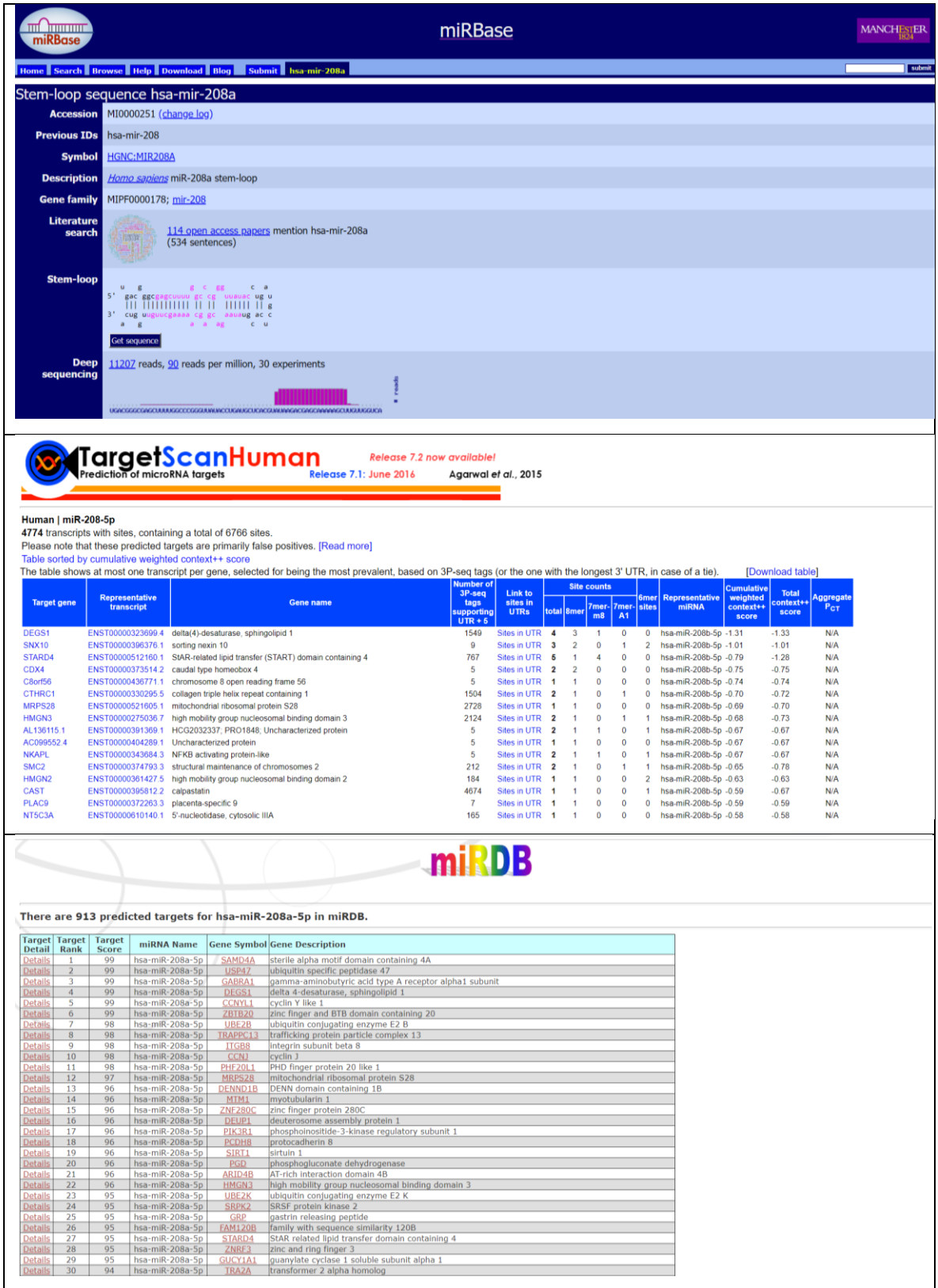
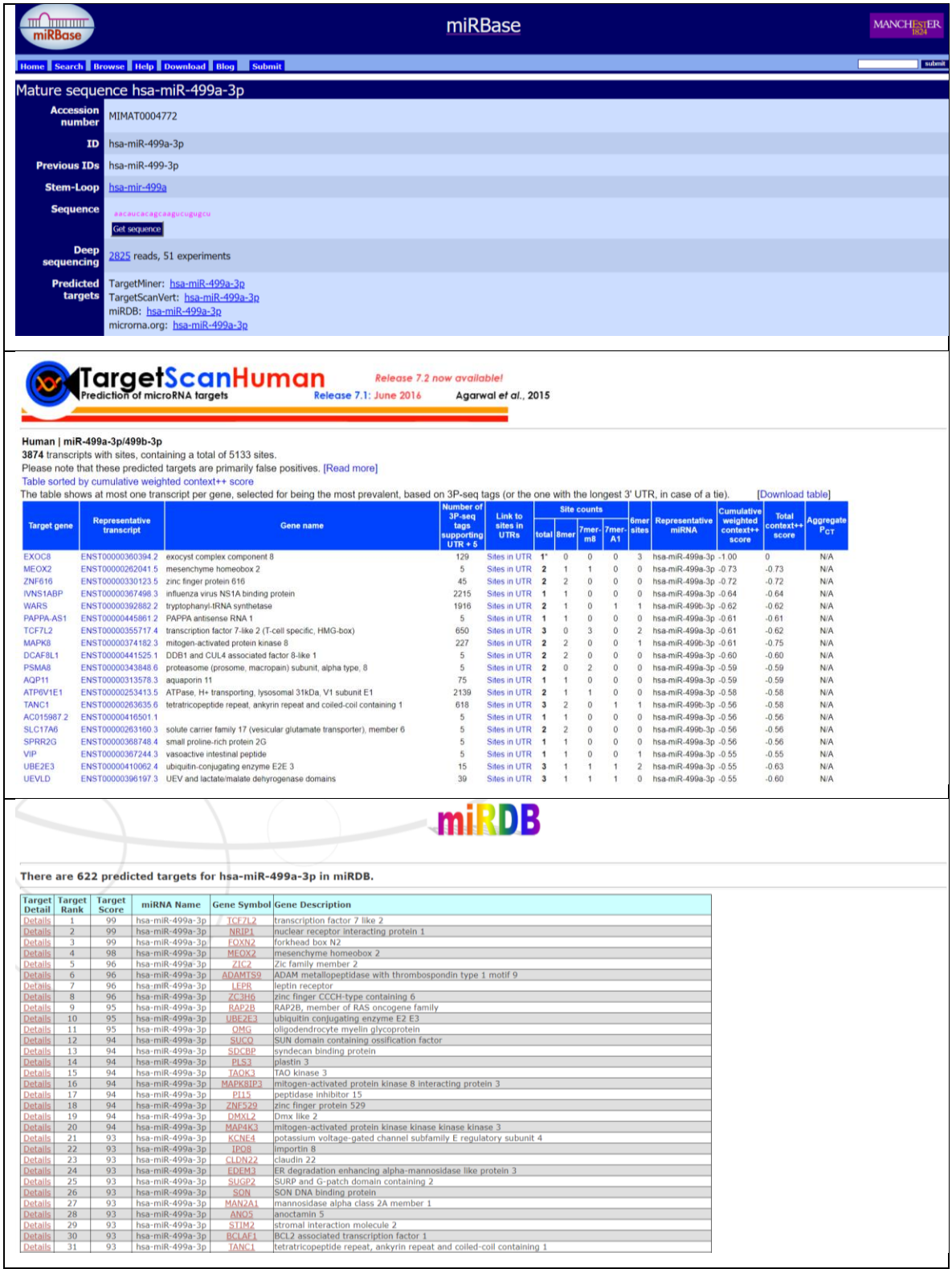


Figure 33. The screens returned for each database related to hsa-miR-208a-3p.

Figure 34 screens of each database interrogated for hsa-miR-499a-3p.



miRBase

Home | Search | Browse | Help | Download | Blog | Submit

Mature sequence hsa-miR-499a-3p

Accession number	MIMAT0004772
ID	hsa-miR-499a-3p
Previous IDs	hsa-miR-499-3p
Stem-Loop	hsa-mir-499a
Sequence	aacaucacagcaagucugugcu Get sequence
Deep sequencing	2825 reads, 51 experiments
Predicted targets	TargetMiner: hsa-miR-499a-3p TargetScanVert: hsa-miR-499a-3p miRDB: hsa-miR-499a-3p microrna.org: hsa-miR-499a-3p

TargetScanHuman — Prediction of microRNA targets — Release 7.2 now available! — Release 7.1: June 2016 — Agarwal *et al.*, 2015

Human | miR-499a-3p/499b-3p

3874 transcripts with sites, containing a total of 5133 sites.

Please note that these predicted targets are primarily false positives. [Read more]

Table sorted by cumulative weighted context++ score

The table shows at most one transcript per gene, selected for being the most prevalent, based on 3P-seq tags (or the one with the longest 3' UTR, in case of a tie). [Download table]

Target gene	Representative transcript	Gene name	Number of 3P-seq tags supporting UTR + 5	Link to sites in UTRs	Site counts				6mer sites	Representative miRNA	Cumulative weighted context++ score	Total context++ score	Aggregate P_{CT}
					total	8mer	7mer-m8	7mer-A1					
EXOC8	ENST00000360394.2	exocyst complex component 8	129	Sites in UTR	1*	0	0	0	3	hsa-miR-499a-3p	-1.00	0	N/A
MEOX2	ENST00000262041.5	mesenchyme homeobox 2	5	Sites in UTR	2	1	1	0	0	hsa-miR-499a-3p	-0.73	-0.73	N/A
ZNF616	ENST00000330123.5	zinc finger protein 616	45	Sites in UTR	2	2	0	0	0	hsa-miR-499a-3p	-0.72	-0.72	N/A
IVNS1ABP	ENST00000367498.3	influenza virus NS1A binding protein	2215	Sites in UTR	1	1	0	0	0	hsa-miR-499a-3p	-0.64	-0.64	N/A
WARS	ENST00000392882.2	tryptophanyl-tRNA synthetase	1916	Sites in UTR	2	1	0	1	1	hsa-miR-499b-3p	-0.62	-0.62	N/A
PAPPA-AS1	ENST00000445861.2	PAPPA antisense RNA 1	5	Sites in UTR	1	1	0	0	0	hsa-miR-499a-3p	-0.61	-0.61	N/A
TCF7L2	ENST00000355717.4	transcription factor 7-like 2 (T-cell specific, HMG-box)	650	Sites in UTR	3	0	3	0	2	hsa-miR-499a-3p	-0.61	-0.62	N/A
MAPK8	ENST00000374182.3	mitogen-activated protein kinase 8	227	Sites in UTR	2	2	0	0	1	hsa-miR-499b-3p	-0.61	-0.75	N/A
DCAF8L1	ENST00000441525.1	DDB1 and CUL4 associated factor 8-like 1	5	Sites in UTR	2	2	0	0	0	hsa-miR-499a-3p	-0.60	-0.60	N/A
PSMA8	ENST00000343848.6	proteasome (prosome, macropain) subunit, alpha type, 8	5	Sites in UTR	2	0	2	0	0	hsa-miR-499a-3p	-0.59	-0.59	N/A
AQP11	ENST00000313578.3	aquaporin 11	75	Sites in UTR	1	1	0	0	0	hsa-miR-499a-3p	-0.59	-0.59	N/A
ATP6V1E1	ENST00000253413.5	ATPase, H+ transporting, lysosomal 31kDa, V1 subunit E1	2139	Sites in UTR	2	1	1	0	0	hsa-miR-499a-3p	-0.58	-0.58	N/A
TANC1	ENST00000263635.6	tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 1	618	Sites in UTR	3	2	0	1	1	hsa-miR-499b-3p	-0.56	-0.58	N/A
AC015987.2	ENST00000416501.1		5	Sites in UTR	1	1	0	0	0	hsa-miR-499a-3p	-0.56	-0.56	N/A
SLC17A6	ENST00000263160.3	solute carrier family 17 (vesicular glutamate transporter), member 6	5	Sites in UTR	2	2	0	0	0	hsa-miR-499b-3p	-0.56	-0.56	N/A
SPRR2G	ENST00000368748.4	small proline-rich protein 2G	5	Sites in UTR	1	1	0	0	0	hsa-miR-499a-3p	-0.56	-0.56	N/A
VIP	ENST00000367244.3	vasoactive intestinal peptide	5	Sites in UTR	1	1	0	0	1	hsa-miR-499a-3p	-0.55	-0.55	N/A
UBE2E3	ENST00000410062.4	ubiquitin-conjugating enzyme E2E 3	15	Sites in UTR	3	1	1	1	2	hsa-miR-499a-3p	-0.55	-0.63	N/A
UEVLD	ENST00000396197.3	UEV and lactate/malate dehydrogenase domains	39	Sites in UTR	3	1	1	1	0	hsa-miR-499a-3p	-0.55	-0.60	N/A

miRDB

There are 622 predicted targets for hsa-miR-499a-3p in miRDB.

Target Detail	Target Rank	Target Score	miRNA Name	Gene Symbol	Gene Description
Details	1	99	hsa-miR-499a-3p	TCF7L2	transcription factor 7 like 2
Details	2	99	hsa-miR-499a-3p	NRIP1	nuclear receptor interacting protein 1
Details	3	99	hsa-miR-499a-3p	FOXN2	forkhead box N2
Details	4	98	hsa-miR-499a-3p	MEOX2	mesenchyme homeobox 2
Details	5	96	hsa-miR-499a-3p	ZIC2	Zic family member 2
Details	6	96	hsa-miR-499a-3p	ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif 9
Details	7	96	hsa-miR-499a-3p	LEPR	leptin receptor
Details	8	96	hsa-miR-499a-3p	ZC3H6	zinc finger CCCH-type containing 6
Details	9	95	hsa-miR-499a-3p	RAP2B	RAP2B, member of RAS oncogene family
Details	10	95	hsa-miR-499a-3p	UBE2E3	ubiquitin conjugating enzyme E2 E3
Details	11	95	hsa-miR-499a-3p	OMG	oligodendrocyte myelin glycoprotein
Details	12	94	hsa-miR-499a-3p	SUCO	SUN domain containing ossification factor
Details	13	94	hsa-miR-499a-3p	SDCBP	syndecan binding protein
Details	14	94	hsa-miR-499a-3p	PLS3	plastin 3
Details	15	94	hsa-miR-499a-3p	TAOK3	TAO kinase 3
Details	16	94	hsa-miR-499a-3p	MAPK8IP3	mitogen-activated protein kinase 8 interacting protein 3
Details	17	94	hsa-miR-499a-3p	PI15	peptidase inhibitor 15
Details	18	94	hsa-miR-499a-3p	ZNF529	zinc finger protein 529
Details	19	94	hsa-miR-499a-3p	DMXL2	Dmx like 2
Details	20	94	hsa-miR-499a-3p	MAP4K3	mitogen-activated protein kinase kinase kinase kinase 3
Details	21	93	hsa-miR-499a-3p	KCNE4	potassium voltage-gated channel subfamily E regulatory subunit 4
Details	22	93	hsa-miR-499a-3p	IPO8	importin 8
Details	23	93	hsa-miR-499a-3p	CLDN22	claudin 22
Details	24	93	hsa-miR-499a-3p	EDEM3	ER degradation enhancing alpha-mannosidase like protein 3
Details	25	93	hsa-miR-499a-3p	SUGP2	SURP and G-patch domain containing 2
Details	26	93	hsa-miR-499a-3p	SON	SON DNA binding protein
Details	27	93	hsa-miR-499a-3p	MAN2A1	mannosidase alpha class 2A member 1
Details	28	93	hsa-miR-499a-3p	ANO5	anoctamin 5
Details	29	93	hsa-miR-499a-3p	STIM2	stromal interaction molecule 2
Details	30	93	hsa-miR-499a-3p	BCLAF1	BCL2 associated transcription factor 1
Details	31	93	hsa-miR-499a-3p	TANC1	tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 1

Figure 34. The screens returned for each database related to hsa-miR-499a-3p.

On kidney tissues, the following miRNAs were tested: hsa-miR-21-5p, hsa-miR-205-5p.

Figure 35 screens of each database interrogated for hsa-miR-21-5p.

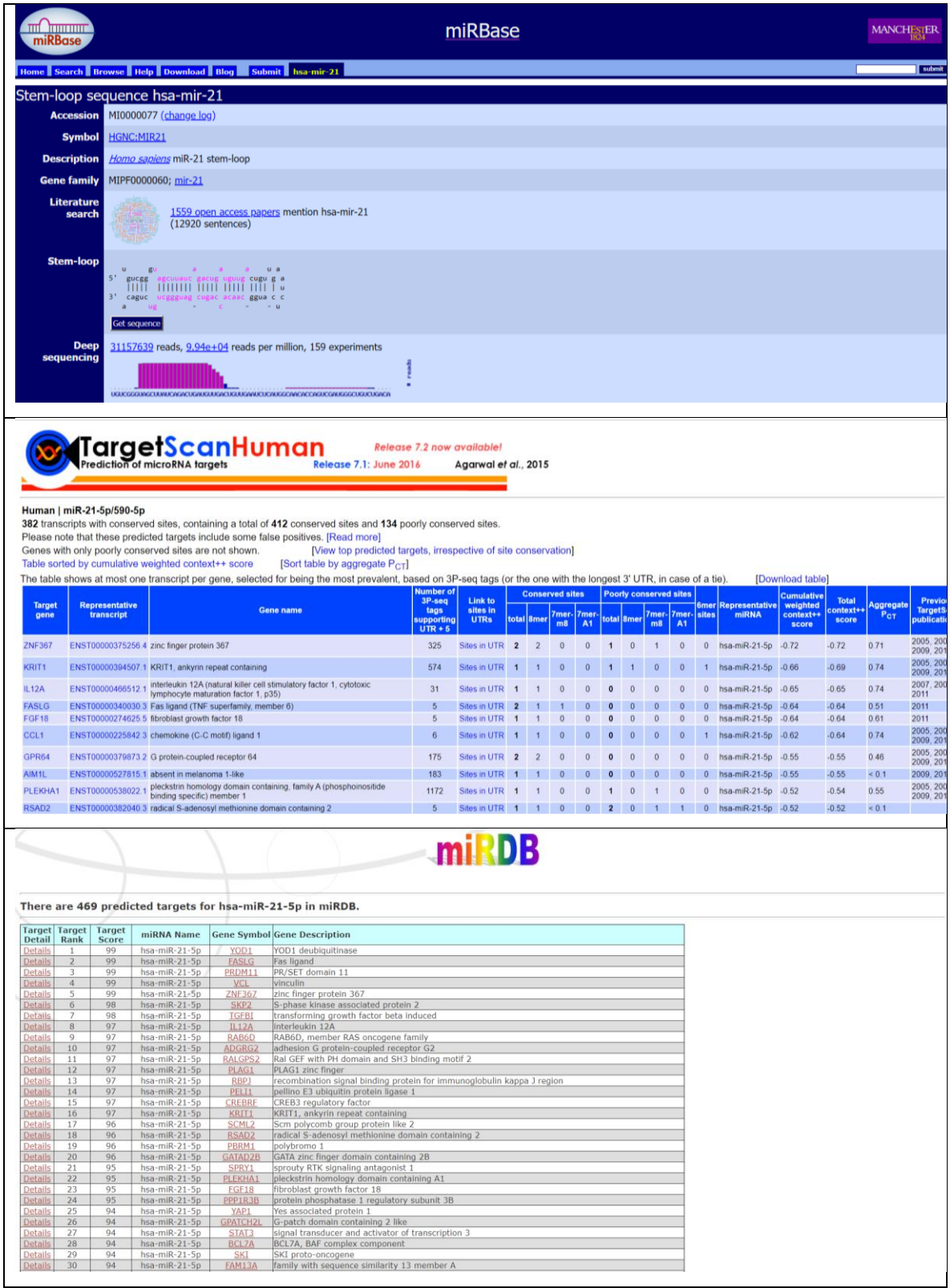


Figure 35. The screens returned for each database related to hsa-miR-21-5p.

Figure 36 screens of each database interrogated for hsa-miR-205-5p.

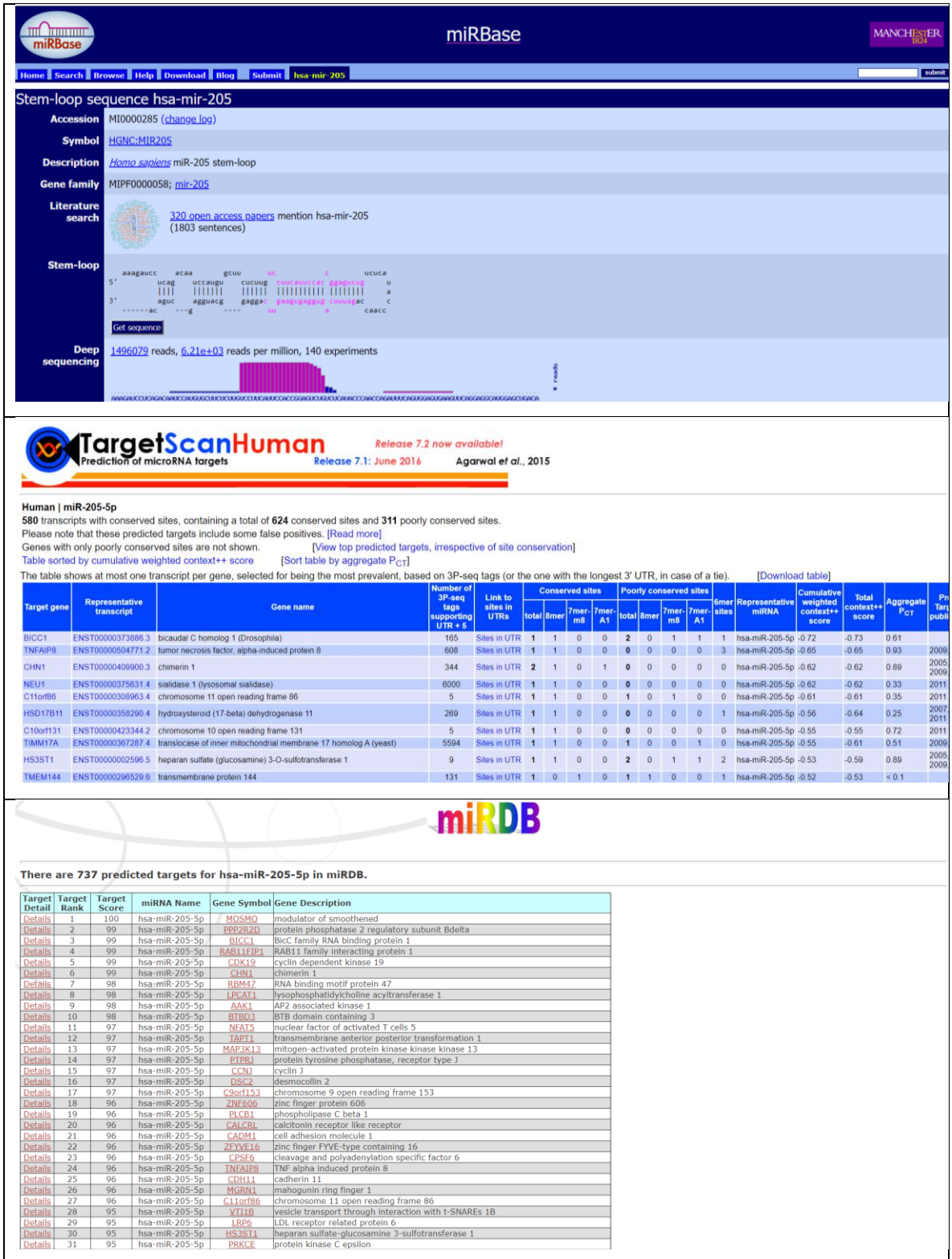


Figure 36. The screens returned for each database related to hsa-miR-205-5p.

On liver tissue, the following miRNAs were tested: hsa-miR-21-5p, hsa-miR-122-5p, hsa-miR-132-3p, hsa-miR-155-3p. The screens of each database questioned for the hsa-miR-21-5p are reported in figure 35.

Figure 37 screens of each database interrogated for hsa-miR-122-5p.

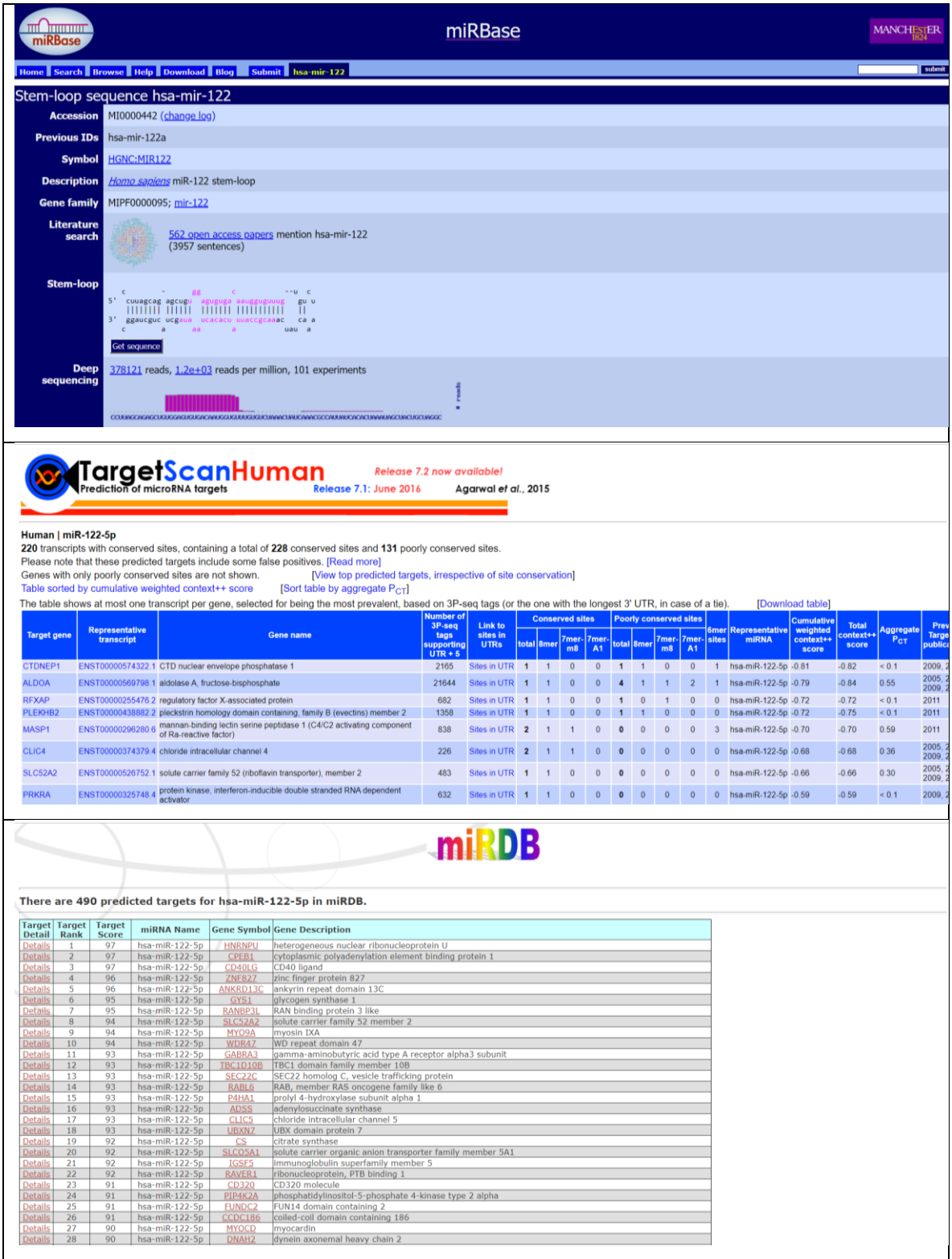


Figure 37. The screens returned for each database related to hsa-miR-122-5p.

Figure 38 screens of each database interrogated for hsa-miR-132-3p.

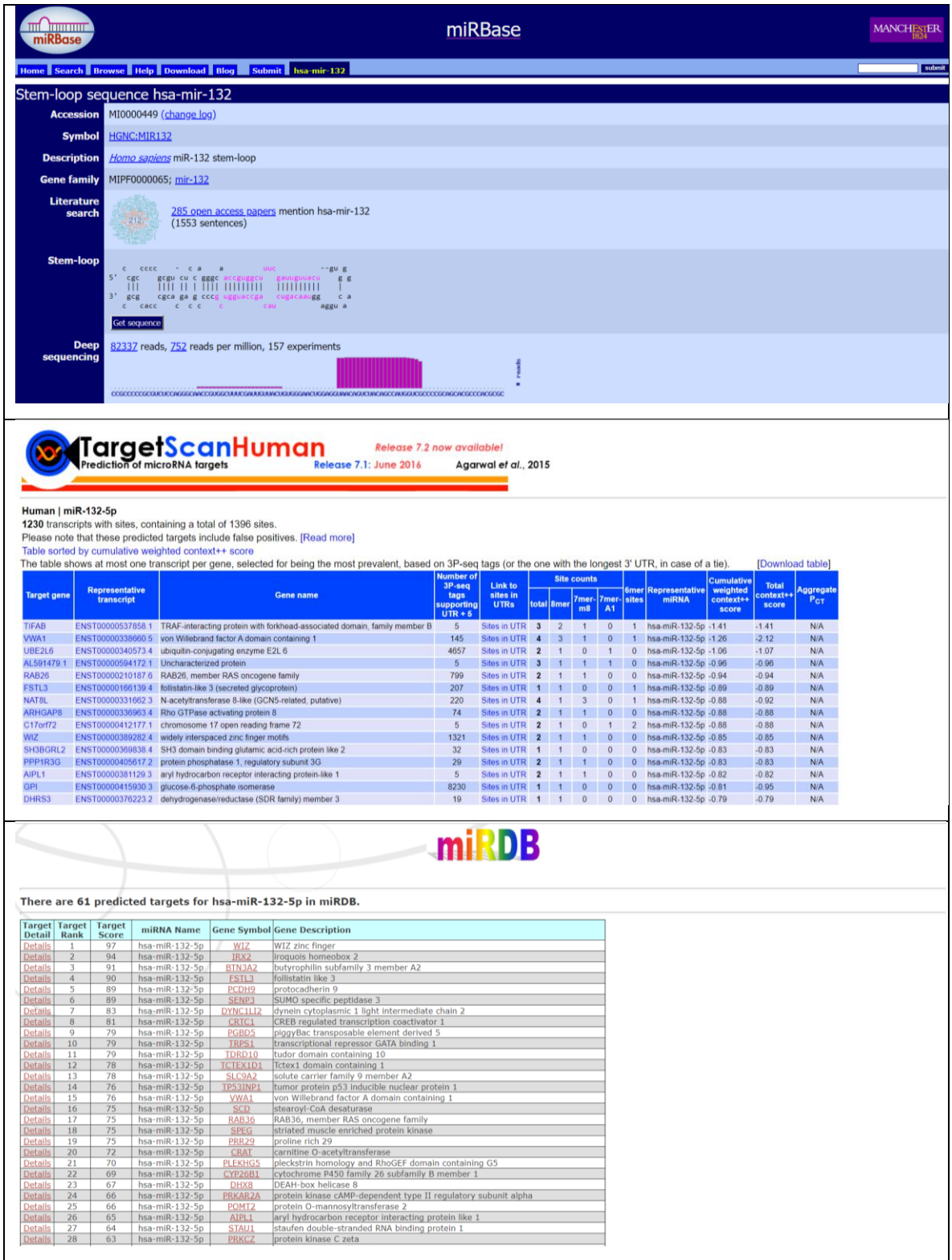


Figure 38. The screens returned for each database related to hsa-miR-132-3p.

Figure 39 screens of each database interrogated for hsa-miR-155-3p.

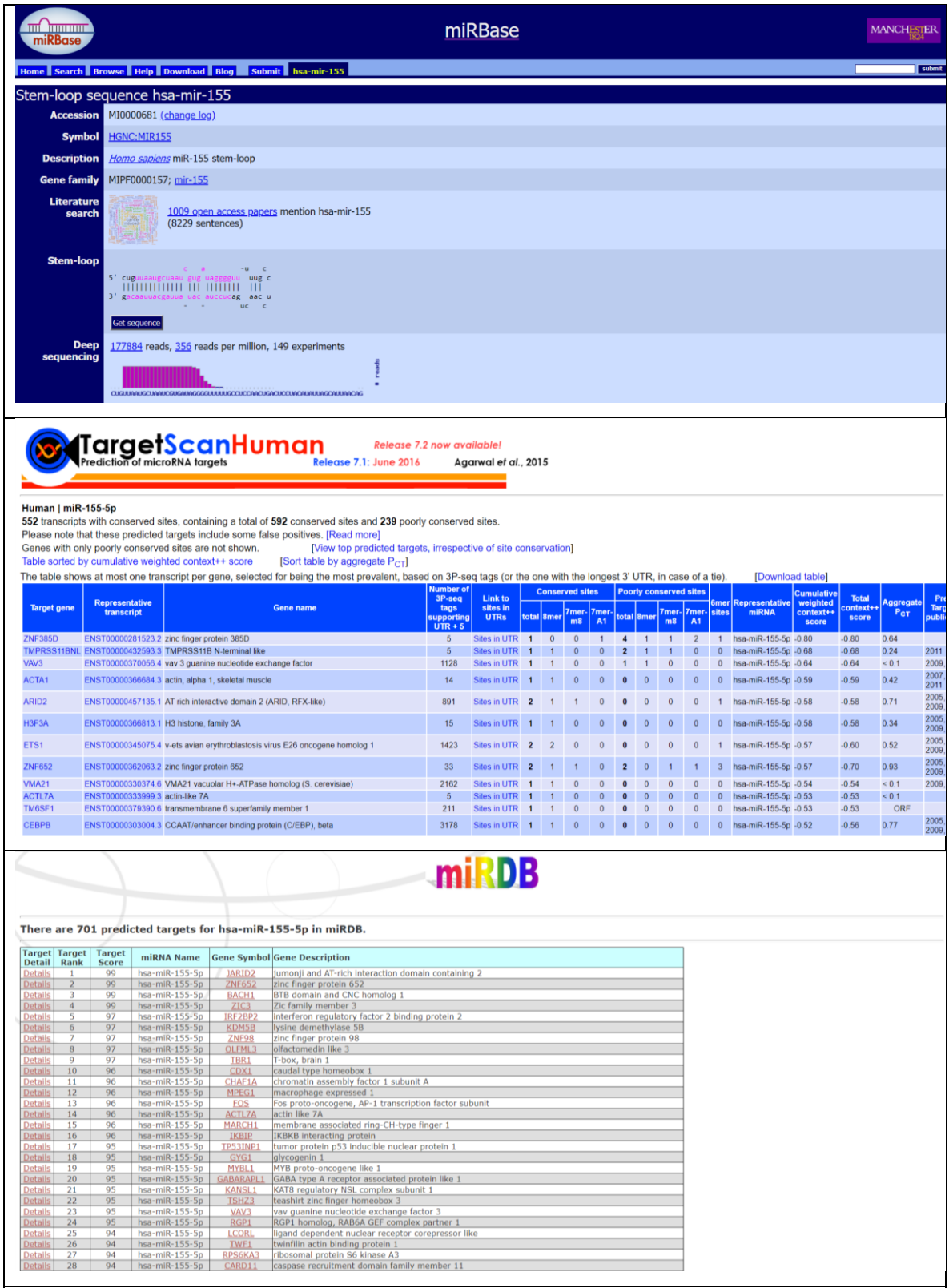
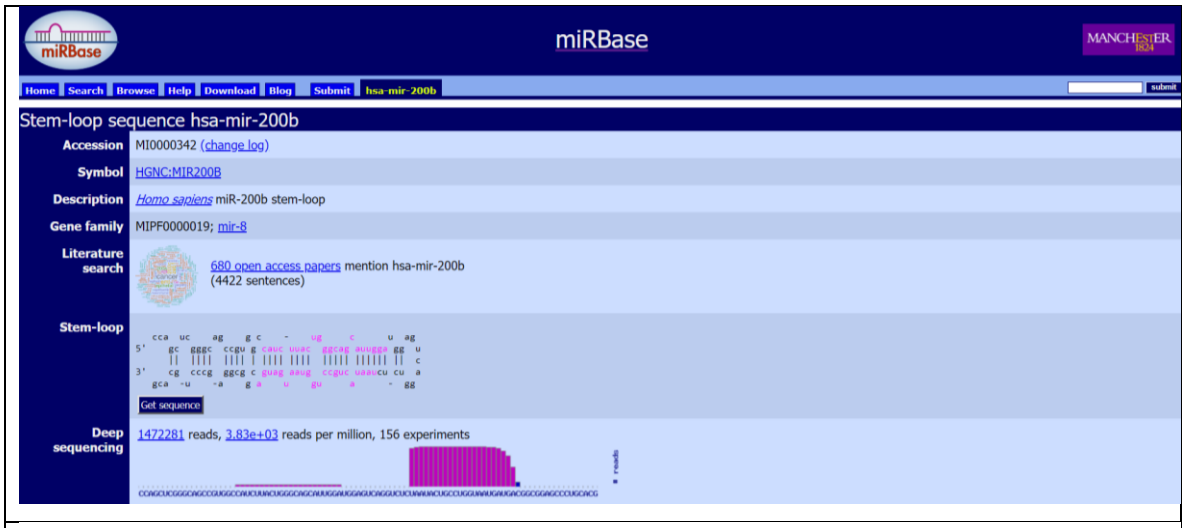


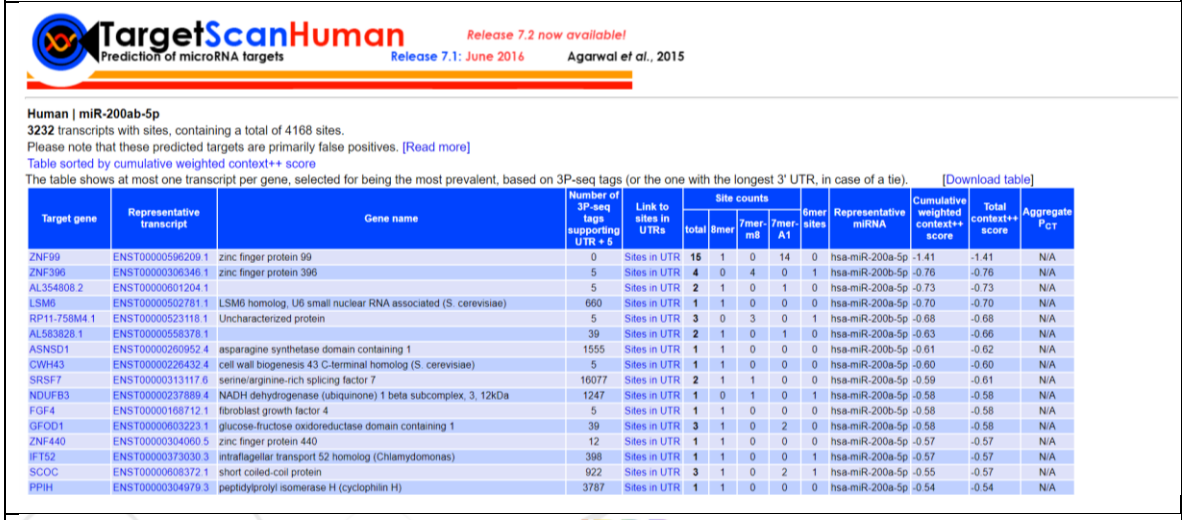
Figure 39. The screens returned for each database related to hsa-miR-155-3p.

On brain tissue, the following miRNAs were tested: hsa-miR-21-5p, hsa-miR-132-3p, hsa-miR-200b-3p, hsa-miR-34a-5p, hsa-miR-124-5p. The screens of each database questioned

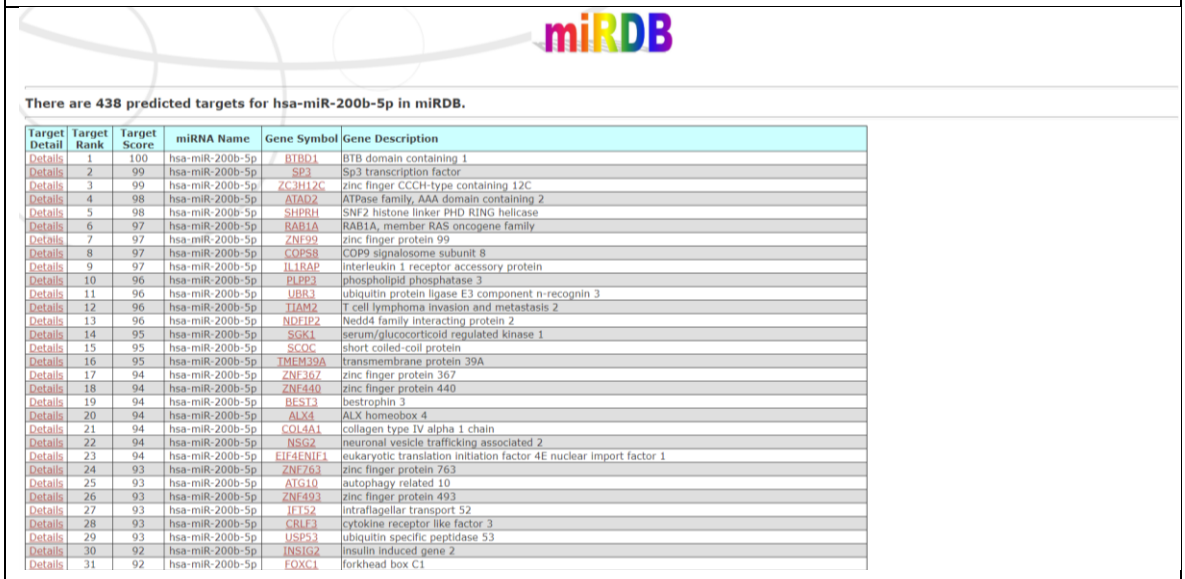
for hsa-miR-21-5p are reported in figure 35. The screens of each database questioned for hsa-miR-132-3p are reported in figure 38.

Figure 40 screens of each database interrogated for hsa-miR-200b-3p.





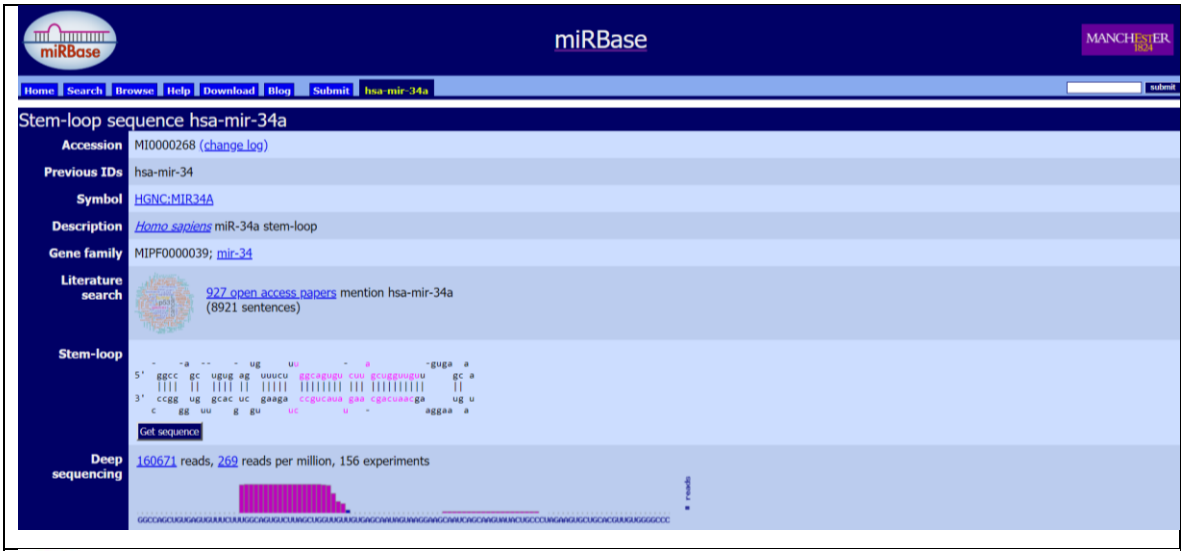
Target gene	Representative transcript	Gene name	Number of 3P-seq tags supporting UTR + 5	Link to sites in UTRs	Site counts				6mer sites	Representative miRNA	Cumulative weighted context++ score	Total context++ score	Aggregate P_{CT}
					total	8mer	7mer-m8	7mer-A1					
ZNF99	ENST00000596209.1	zinc finger protein 99	0	Sites in UTR	15	1	0	14	0	hsa-miR-200a-5p	-1.41	-1.41	N/A
ZNF396	ENST00000306346.1	zinc finger protein 396	5	Sites in UTR	4	0	4	0	1	hsa-miR-200b-5p	-0.76	-0.76	N/A
AL354808.2	ENST00000601204.1		5	Sites in UTR	2	1	0	1	0	hsa-miR-200a-5p	-0.73	-0.73	N/A
LSM6	ENST00000502781.1	LSM6 homolog, U6 small nuclear RNA associated (S. cerevisiae)	660	Sites in UTR	1	1	0	0	0	hsa-miR-200a-5p	-0.70	-0.70	N/A
RP11-758M4.1	ENST00000523118.1	Uncharacterized protein	5	Sites in UTR	3	0	3	0	1	hsa-miR-200b-5p	-0.68	-0.68	N/A
AL583828.1	ENST00000558378.1		39	Sites in UTR	2	1	0	1	0	hsa-miR-200a-5p	-0.63	-0.66	N/A
ASNSD1	ENST00000260952.4	asparagine synthetase domain containing 1	1555	Sites in UTR	1	1	0	0	0	hsa-miR-200b-5p	-0.61	-0.62	N/A
CWH43	ENST00000226432.4	cell wall biogenesis 43 C-terminal homolog (S. cerevisiae)	5	Sites in UTR	1	1	0	0	0	hsa-miR-200a-5p	-0.60	-0.60	N/A
SRSF7	ENST00000313117.6	serine/arginine-rich splicing factor 7	16077	Sites in UTR	2	1	1	0	0	hsa-miR-200a-5p	-0.59	-0.61	N/A
NDUFB3	ENST00000237889.4	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa	1247	Sites in UTR	1	0	1	0	1	hsa-miR-200a-5p	-0.58	-0.58	N/A
FGF4	ENST00000168712.1	fibroblast growth factor 4	5	Sites in UTR	1	1	0	0	0	hsa-miR-200b-5p	-0.58	-0.58	N/A
GFOD1	ENST00000603223.1	glucose-fructose oxidoreductase domain containing 1	39	Sites in UTR	3	1	0	2	0	hsa-miR-200a-5p	-0.58	-0.58	N/A
ZNF440	ENST00000304060.5	zinc finger protein 440	12	Sites in UTR	1	1	0	0	0	hsa-miR-200a-5p	-0.57	-0.57	N/A
IFT52	ENST00000373030.3	intraflagellar transport 52 homolog (Chlamydomonas)	398	Sites in UTR	1	1	0	0	1	hsa-miR-200a-5p	-0.57	-0.57	N/A
SCOC	ENST00000608372.1	short coiled-coil protein	922	Sites in UTR	3	1	0	2	1	hsa-miR-200a-5p	-0.55	-0.57	N/A
PPIH	ENST00000304979.3	peptidylprolyl isomerase H (cyclophilin H)	3787	Sites in UTR	1	1	0	0	0	hsa-miR-200a-5p	-0.54	-0.54	N/A

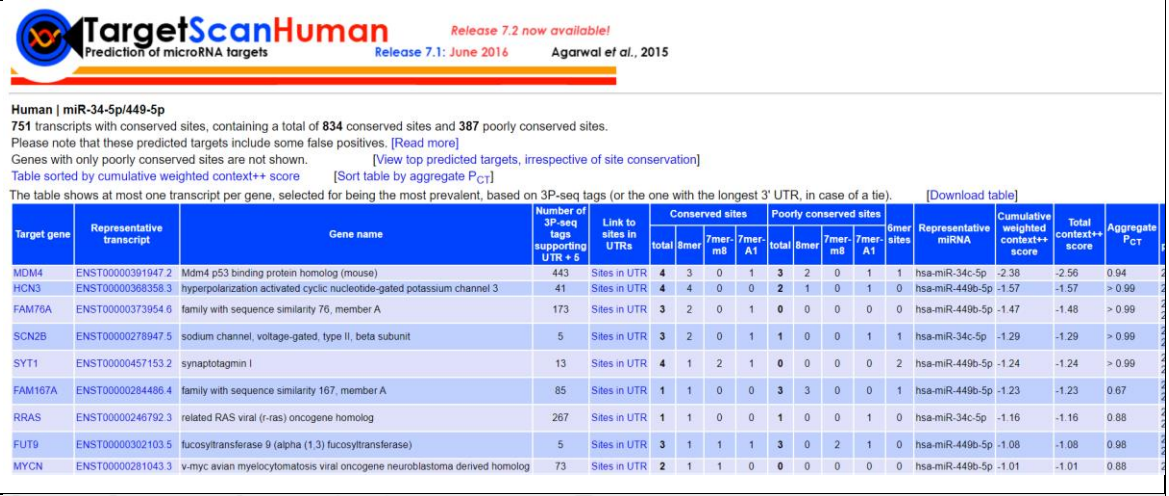


Target Detail	Target Rank	Target Score	miRNA Name	Gene Symbol	Gene Description
Details	1	100	hsa-miR-200b-5p	BTBD1	BTB domain containing 1
Details	2	99	hsa-miR-200b-5p	SP3	Sp3 transcription factor
Details	3	99	hsa-miR-200b-5p	ZC3H12C	zinc finger CCCH-type containing 12C
Details	4	98	hsa-miR-200b-5p	ATAD2	ATPase family, AAA domain containing 2
Details	5	98	hsa-miR-200b-5p	SHPRH	SNF2 histone linker PHD RING helicase
Details	6	97	hsa-miR-200b-5p	RAB1A	RAB1A, member RAS oncogene family
Details	7	97	hsa-miR-200b-5p	ZNF99	zinc finger protein 99
Details	8	97	hsa-miR-200b-5p	COPS8	COP9 signalosome subunit 8
Details	9	97	hsa-miR-200b-5p	IL1RAP	interleukin 1 receptor accessory protein
Details	10	96	hsa-miR-200b-5p	PLPP3	phospholipid phosphatase 3
Details	11	96	hsa-miR-200b-5p	UBR3	ubiquitin protein ligase E3 component n-recognin 3
Details	12	96	hsa-miR-200b-5p	TIAM2	T cell lymphoma invasion and metastasis 2
Details	13	96	hsa-miR-200b-5p	NDFIP2	Nedd4 family interacting protein 2
Details	14	95	hsa-miR-200b-5p	SGK1	serum/glucocorticoid regulated kinase 1
Details	15	95	hsa-miR-200b-5p	SCOC	short coiled-coil protein
Details	16	95	hsa-miR-200b-5p	TMEM39A	transmembrane protein 39A
Details	17	94	hsa-miR-200b-5p	ZNF367	zinc finger protein 367
Details	18	94	hsa-miR-200b-5p	ZNF440	zinc finger protein 440
Details	19	94	hsa-miR-200b-5p	BEST3	bestrophin 3
Details	20	94	hsa-miR-200b-5p	ALX4	ALX homeobox 4
Details	21	94	hsa-miR-200b-5p	COL4A1	collagen type IV alpha 1 chain
Details	22	94	hsa-miR-200b-5p	NSG2	neuronal vesicle trafficking associated 2
Details	23	94	hsa-miR-200b-5p	EIF4ENIF1	eukaryotic translation initiation factor 4E nuclear import factor 1
Details	24	93	hsa-miR-200b-5p	ZNF763	zinc finger protein 763
Details	25	93	hsa-miR-200b-5p	ATG10	autophagy related 10
Details	26	93	hsa-miR-200b-5p	ZNF493	zinc finger protein 493
Details	27	93	hsa-miR-200b-5p	IFT52	intraflagellar transport 52
Details	28	93	hsa-miR-200b-5p	CRLF3	cytokine receptor like factor 3
Details	29	93	hsa-miR-200b-5p	USP53	ubiquitin specific peptidase 53
Details	30	92	hsa-miR-200b-5p	INSIG2	insulin induced gene 2
Details	31	92	hsa-miR-200b-5p	FOXC1	forkhead box C1

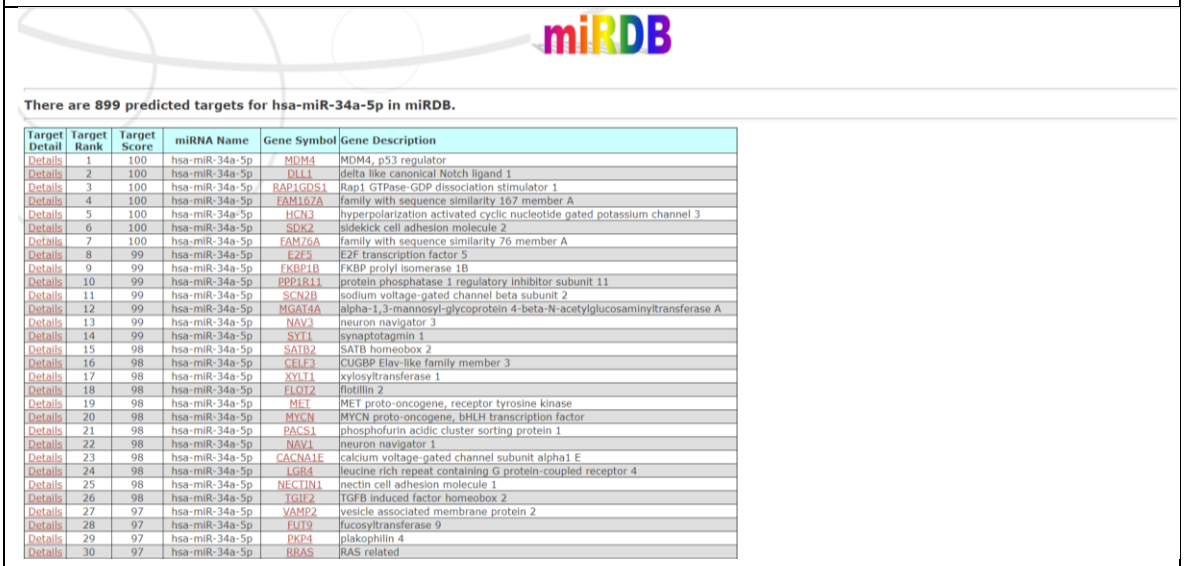
Figure 40. The screens returned for each database related to hsa-miR-200b-3p.

Figure 41 screens of each database interrogated for hsa-miR-34a-5p.





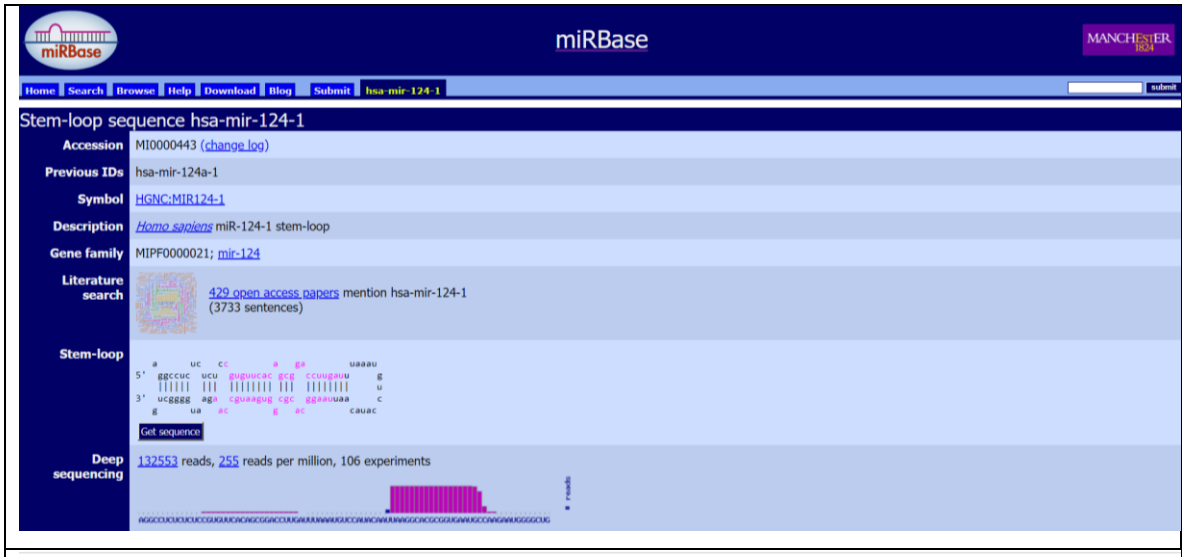
Target gene	Representative transcript	Gene name	Number of 3P-seq tags supporting UTR + 5	Link to sites in UTRs	Conserved sites				Poorly conserved sites				6mer sites	Representative miRNA	Cumulative weighted context++ score	Total context++ score	Aggregate P_{CT}
					total	8mer	7mer-m8	7mer-A1	total	8mer	7mer-m8	7mer-A1					
MDM4	ENST00000391947.2	Mdm4 p53 binding protein homolog (mouse)	443	Sites in UTR	4	3	0	1	3	2	0	1	1	hsa-miR-34c-5p	-2.38	-2.56	0.94
HCN3	ENST00000368358.3	hyperpolarization activated cyclic nucleotide-gated potassium channel 3	41	Sites in UTR	4	4	0	0	2	1	0	1	0	hsa-miR-449b-5p	-1.57	-1.57	> 0.99
FAM76A	ENST00000373954.6	family with sequence similarity 76, member A	173	Sites in UTR	3	2	0	1	0	0	0	0	0	hsa-miR-449b-5p	-1.47	-1.48	> 0.99
SCN2B	ENST00000278947.5	sodium channel, voltage-gated, type II, beta subunit	5	Sites in UTR	3	2	0	1	1	0	0	1	1	hsa-miR-34c-5p	-1.29	-1.29	> 0.99
SYT1	ENST00000457153.2	synaptotagmin I	13	Sites in UTR	4	1	2	1	0	0	0	0	2	hsa-miR-449b-5p	-1.24	-1.24	> 0.99
FAM167A	ENST00000284486.4	family with sequence similarity 167, member A	85	Sites in UTR	1	1	0	0	3	3	0	0	1	hsa-miR-449b-5p	-1.23	-1.23	0.67
RRAS	ENST00000246792.3	related RAS viral (r-ras) oncogene homolog	267	Sites in UTR	1	1	0	0	1	0	0	1	0	hsa-miR-34c-5p	-1.16	-1.16	0.88
FUT9	ENST00000302103.5	fucosyltransferase 9 (alpha (1,3) fucosyltransferase)	5	Sites in UTR	3	1	1	1	3	0	2	1	0	hsa-miR-449b-5p	-1.08	-1.08	0.98
MYCN	ENST00000281043.3	v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog	73	Sites in UTR	2	1	1	0	0	0	0	0	0	hsa-miR-449b-5p	-1.01	-1.01	0.88

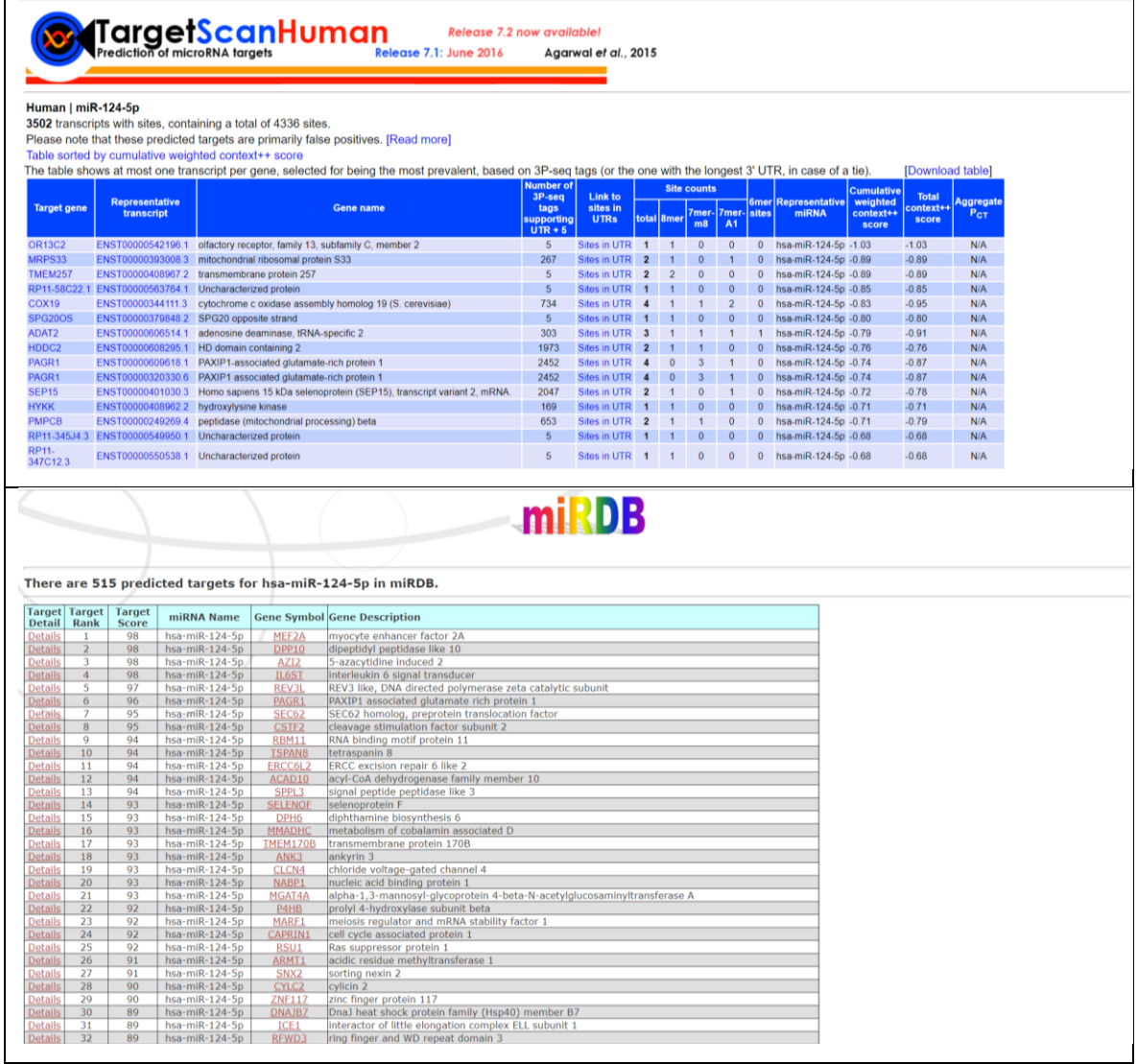


Target Detail	Target Rank	Target Score	miRNA Name	Gene Symbol	Gene Description
Details	1	100	hsa-miR-34a-5p	MDM4	MDM4, p53 regulator
Details	2	100	hsa-miR-34a-5p	DLL1	delta like canonical Notch ligand 1
Details	3	100	hsa-miR-34a-5p	RAP1GDS1	Rap1 GTPase-GDP dissociation stimulator 1
Details	4	100	hsa-miR-34a-5p	FAM167A	family with sequence similarity 167 member A
Details	5	100	hsa-miR-34a-5p	HCN3	hyperpolarization activated cyclic nucleotide gated potassium channel 3
Details	6	100	hsa-miR-34a-5p	SDK2	sidekick cell adhesion molecule 2
Details	7	100	hsa-miR-34a-5p	FAM76A	family with sequence similarity 76 member A
Details	8	99	hsa-miR-34a-5p	E2F5	E2F transcription factor 5
Details	9	99	hsa-miR-34a-5p	FKBP1B	FKBP prolyl isomerase 1B
Details	10	99	hsa-miR-34a-5p	PPP1R11	protein phosphatase 1 regulatory inhibitor subunit 11
Details	11	99	hsa-miR-34a-5p	SCN2B	sodium voltage-gated channel beta subunit 2
Details	12	99	hsa-miR-34a-5p	MGAT4A	alpha-1,3-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyltransferase A
Details	13	99	hsa-miR-34a-5p	NAV3	neuron navigator 3
Details	14	99	hsa-miR-34a-5p	SYT1	synaptotagmin 1
Details	15	98	hsa-miR-34a-5p	SATB2	SATB homeobox 2
Details	16	98	hsa-miR-34a-5p	CELF3	CUGBP Elav-like family member 3
Details	17	98	hsa-miR-34a-5p	XYLT1	xylosyltransferase 1
Details	18	98	hsa-miR-34a-5p	FLOT2	flotillin 2
Details	19	98	hsa-miR-34a-5p	MET	MET proto-oncogene, receptor tyrosine kinase
Details	20	98	hsa-miR-34a-5p	MYCN	MYCN proto-oncogene, bHLH transcription factor
Details	21	98	hsa-miR-34a-5p	PACS1	phosphofurin acidic cluster sorting protein 1
Details	22	98	hsa-miR-34a-5p	NAV1	neuron navigator 1
Details	23	98	hsa-miR-34a-5p	CACNA1E	calcium voltage-gated channel subunit alpha1 E
Details	24	98	hsa-miR-34a-5p	LGR4	leucine rich repeat containing G protein-coupled receptor 4
Details	25	98	hsa-miR-34a-5p	NECTIN1	nectin cell adhesion molecule 1
Details	26	98	hsa-miR-34a-5p	TGIF2	TGFB induced factor homeobox 2
Details	27	97	hsa-miR-34a-5p	VAMP2	vesicle associated membrane protein 2
Details	28	97	hsa-miR-34a-5p	FUT9	fucosyltransferase 9
Details	29	97	hsa-miR-34a-5p	PKP4	plakophilin 4
Details	30	97	hsa-miR-34a-5p	RRAS	RAS related

Figure 41. The screens returned for each database related to hsa-miR-34a-5p.

Figure 42 screens of each database interrogated for hsa-miR-124-5p.





Target gene	Representative transcript	Gene name	Number of 3P-seq tags supporting UTR + 5	Link to sites in UTRs	Site counts				6mer sites	Representative miRNA	Cumulative weighted context++ score	Total context++ score	Aggregate P_{CT}
					total	8mer	7mer-m8	7mer-A1					
OR13C2	ENST00000542196.1	olfactory receptor, family 13, subfamily C, member 2	5	Sites in UTR	1	1	0	0	0	hsa-miR-124-5p	-1.03	-1.03	N/A
MRPS33	ENST00000393008.3	mitochondrial ribosomal protein S33	267	Sites in UTR	2	1	0	1	0	hsa-miR-124-5p	-0.89	-0.89	N/A
TMEM257	ENST00000408967.2	transmembrane protein 257	5	Sites in UTR	2	2	0	0	0	hsa-miR-124-5p	-0.89	-0.89	N/A
RP11-58C22.1	ENST00000563764.1	Uncharacterized protein	5	Sites in UTR	1	1	0	0	0	hsa-miR-124-5p	-0.85	-0.85	N/A
COX19	ENST00000344111.3	cytochrome c oxidase assembly homolog 19 (S. cerevisiae)	734	Sites in UTR	4	1	1	2	0	hsa-miR-124-5p	-0.83	-0.95	N/A
SPG20OS	ENST00000379848.2	SPG20 opposite strand	5	Sites in UTR	1	1	0	0	0	hsa-miR-124-5p	-0.80	-0.80	N/A
ADAT2	ENST00000606514.1	adenosine deaminase, tRNA-specific 2	303	Sites in UTR	3	1	1	1	1	hsa-miR-124-5p	-0.79	-0.91	N/A
HDDC2	ENST00000608295.1	HD domain containing 2	1973	Sites in UTR	2	1	1	0	0	hsa-miR-124-5p	-0.76	-0.76	N/A
PAGR1	ENST00000609618.1	PAXIP1-associated glutamate-rich protein 1	2452	Sites in UTR	4	0	3	1	0	hsa-miR-124-5p	-0.74	-0.87	N/A
PAGR1	ENST00000320330.6	PAXIP1 associated glutamate-rich protein 1	2452	Sites in UTR	4	0	3	1	0	hsa-miR-124-5p	-0.74	-0.87	N/A
SEP15	ENST00000401030.3	Homo sapiens 15 kDa selenoprotein (SEP15), transcript variant 2, mRNA.	2047	Sites in UTR	2	1	0	1	0	hsa-miR-124-5p	-0.72	-0.78	N/A
HYKK	ENST00000408962.2	hydroxylysine kinase	169	Sites in UTR	1	1	0	0	0	hsa-miR-124-5p	-0.71	-0.71	N/A
PMPCB	ENST00000249269.4	peptidase (mitochondrial processing) beta	653	Sites in UTR	2	1	1	0	0	hsa-miR-124-5p	-0.71	-0.79	N/A
RP11-345J4.3	ENST00000549950.1	Uncharacterized protein	5	Sites in UTR	1	1	0	0	0	hsa-miR-124-5p	-0.68	-0.68	N/A
RP11-347C12.3	ENST00000550538.1	Uncharacterized protein	5	Sites in UTR	1	1	0	0	0	hsa-miR-124-5p	-0.68	-0.68	N/A

Target Detail	Target Rank	Target Score	miRNA Name	Gene Symbol	Gene Description
Details	1	98	hsa-miR-124-5p	MEF2A	myocyte enhancer factor 2A
Details	2	98	hsa-miR-124-5p	DPP10	dipeptidyl peptidase like 10
Details	3	98	hsa-miR-124-5p	AZI2	5-azacytidine induced 2
Details	4	98	hsa-miR-124-5p	IL6ST	interleukin 6 signal transducer
Details	5	97	hsa-miR-124-5p	REV3L	REV3 like, DNA directed polymerase zeta catalytic subunit
Details	6	96	hsa-miR-124-5p	PAGR1	PAXIP1 associated glutamate rich protein 1
Details	7	95	hsa-miR-124-5p	SEC62	SEC62 homolog, preprotein translocation factor
Details	8	95	hsa-miR-124-5p	CSTF2	cleavage stimulation factor subunit 2
Details	9	94	hsa-miR-124-5p	RBM11	RNA binding motif protein 11
Details	10	94	hsa-miR-124-5p	TSPAN8	tetraspanin 8
Details	11	94	hsa-miR-124-5p	ERCC6L2	ERCC excision repair 6 like 2
Details	12	94	hsa-miR-124-5p	ACAD10	acyl-CoA dehydrogenase family member 10
Details	13	94	hsa-miR-124-5p	SPPL3	signal peptide peptidase like 3
Details	14	93	hsa-miR-124-5p	SELENOF	selenoprotein F
Details	15	93	hsa-miR-124-5p	DPH6	diphthamine biosynthesis 6
Details	16	93	hsa-miR-124-5p	MMADHC	metabolism of cobalamin associated D
Details	17	93	hsa-miR-124-5p	TMEM170B	transmembrane protein 170B
Details	18	93	hsa-miR-124-5p	ANK3	ankyrin 3
Details	19	93	hsa-miR-124-5p	CLCN4	chloride voltage-gated channel 4
Details	20	93	hsa-miR-124-5p	NABP1	nucleic acid binding protein 1
Details	21	93	hsa-miR-124-5p	MGAT4A	alpha-1,3-mannosyl-glycoprotein 4-beta-N-acetylglucosaminyltransferase A
Details	22	92	hsa-miR-124-5p	P4HB	prolyl 4-hydroxylase subunit beta
Details	23	92	hsa-miR-124-5p	MARF1	meiosis regulator and mRNA stability factor 1
Details	24	92	hsa-miR-124-5p	CAPRIN1	cell cycle associated protein 1
Details	25	92	hsa-miR-124-5p	RSU1	Ras suppressor protein 1
Details	26	91	hsa-miR-124-5p	ARMT1	acidic residue methyltransferase 1
Details	27	91	hsa-miR-124-5p	SNX2	sorting nexin 2
Details	28	90	hsa-miR-124-5p	CYLC2	cylicin 2
Details	29	90	hsa-miR-124-5p	ZNF117	zinc finger protein 117
Details	30	89	hsa-miR-124-5p	DNAJB7	DnaJ heat shock protein family (Hsp40) member B7
Details	31	89	hsa-miR-124-5p	ICE1	interactor of little elongation complex ELL subunit 1
Details	32	89	hsa-miR-124-5p	RFWD3	ring finger and WD repeat domain 3

Figure 42. The screens returned for each database related to hsa-miR-124-5p.

4.5 Questionnaire results

Two hundred forty-seven subjects correctly filled in the questionnaire (mean age 29.58 ± 9.72 years). 47.35 % were male (mean age 29.74 ± 10.07 years), while 52.6% were female (mean age 29.44 ± 9.43 years). All subjects enrolled were Italian and resident in the South of Italy. The main characteristics are summarized in table 16:

GENERAL DATA					
	SUBJECTS	**AGE (MEAN)**	**WEIGHT (MEAN - Kg)**	**HEIGHT (MEAN - m)**	**BMI (MEAN)**
MALE	117	29,74±10,07	77,75±11,19	1,78±0,06	24,38±2,92
FEMALE	130	29,44±9,43	59,64±10,76	1,64±0,06	21,91±3,57
TOT	247	29,58±9,72	68,22±14,20	1,71±0,09	23,08±3,5

Table 16. Summary of the main data of the interviewed people.

The questionnaire results demonstrated that 8 subjects (6 females and 2 males) admitted using AASs (about 3.23%). The main characteristics of the AAS users/abusers are summarized in table 17.

ABUSER DATA					
	SUBJECTS	**AGE (MEAN)**	**WEIGHT (MEAN - Kg)**	**HEIGHT (MEAN - m)**	**BMI (MEAN)**
MALE	2	33,5±7,77	83±18,38	1,85±0,14	24,04±1,68
FEMALE	6	30,16±9,19	56,16±4,70	1,62±0,05	21,26±1,4
TOT	8	31±8,45	62,87±14,77	1,68±0,12	21,95±1,86

Table 17. Summary of the main data of the interviewed AAS users/abusers.

Figure 43 summarizes the data under the sex criteria, subdividing users from non-users.

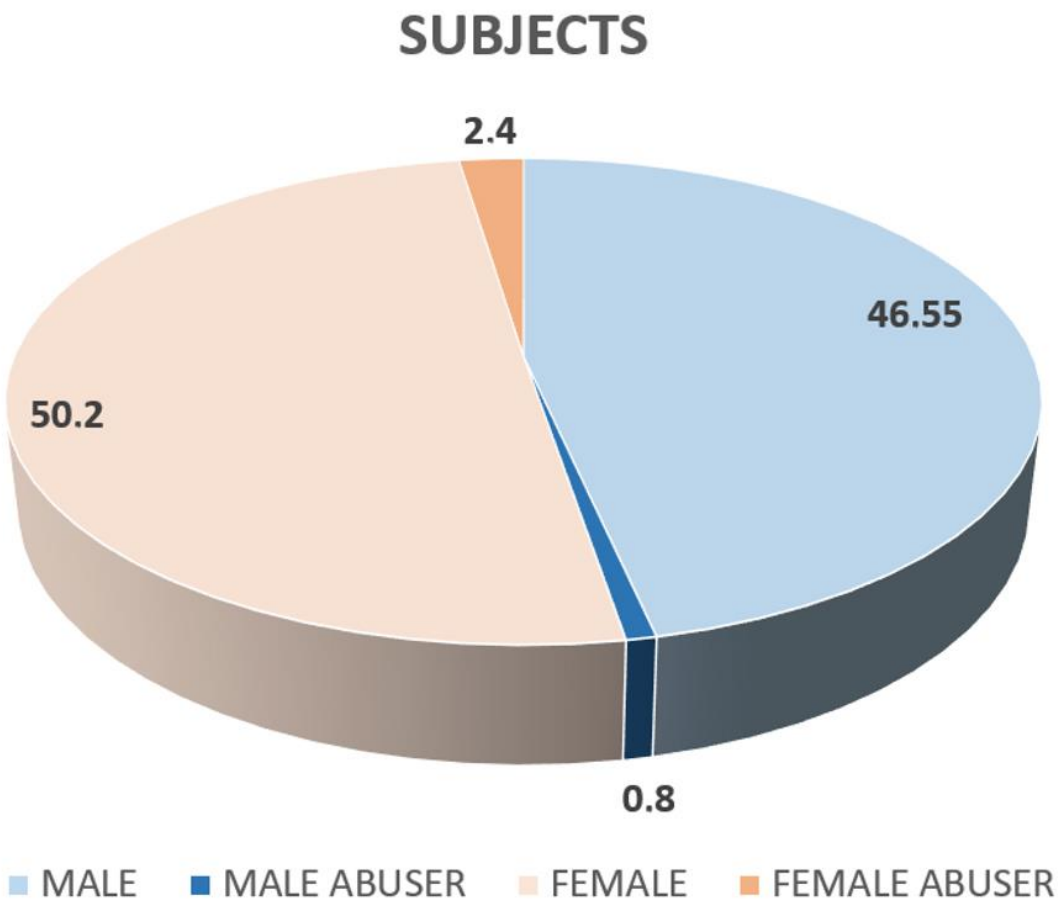


Figure 43. Percentages of males and females of the interviewed people, highlighting the differences between users/abusers under sex evaluation.

The data of the MBSR questionnaire were used to obtain the score related to "appearance evaluation" and "appearance orientation" in the AAS users vs non-users. Through the box plot analysis, the results demonstrated that the values of both items evaluated were higher in the AAS users than non-users (figure 44).

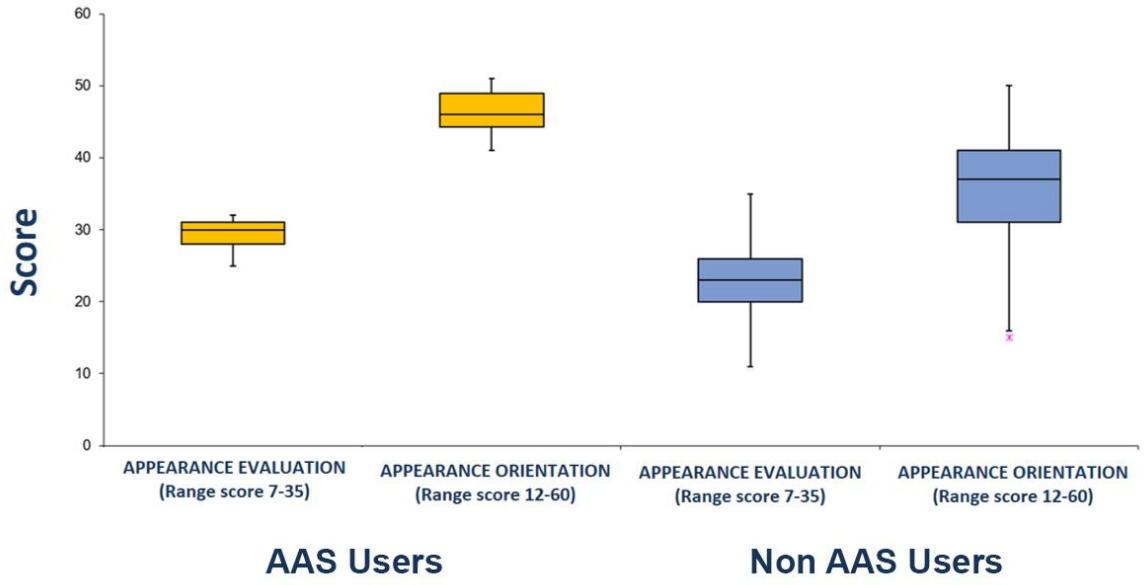


Figure 44. Box plot analysis showing that the score values of the two items analyzed ("appearance evaluation" and "appearance orientation") were higher in AAS users.

These differences were statistically significant for both analyzed items ($p<0.05$).

Moreover, analyzing the kind of self-administrated AASs during use/abuse, in 7 out of 8 cases, the subjects injected the anabolic agents. Only one subject used AASs in pill form (figure 45).

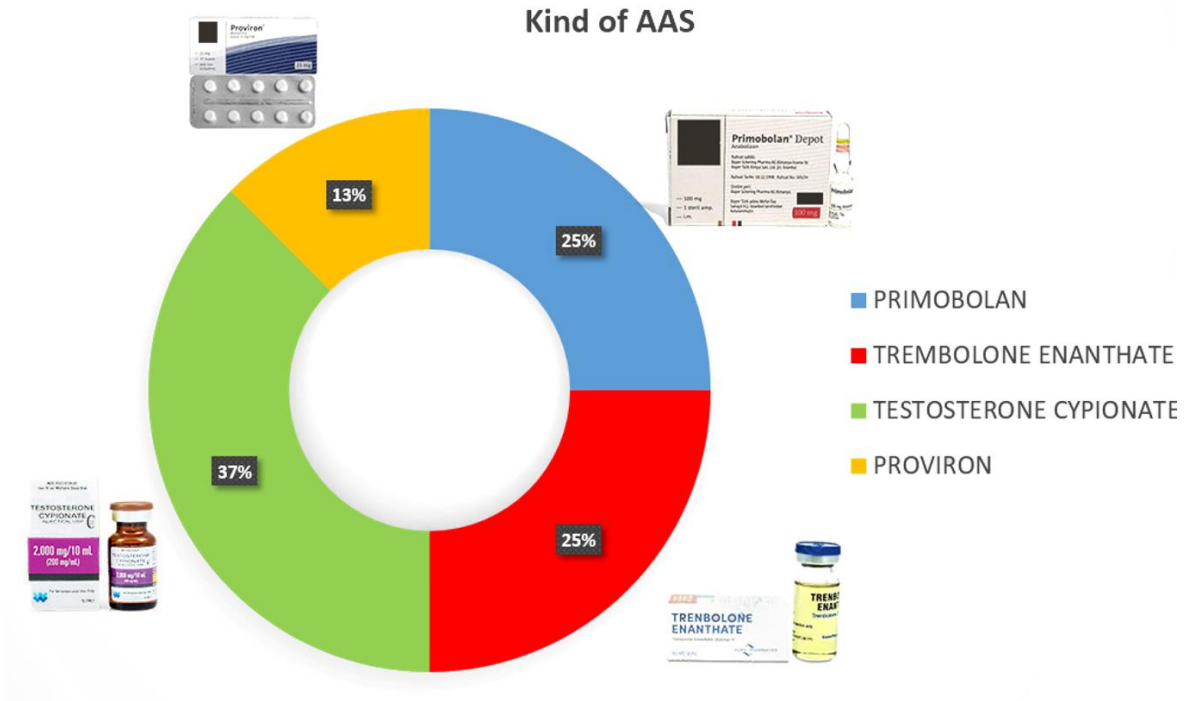


Figure 45. Type of the anabolic agents used by the users/abusers interviewed.

Moreover, energy drink use was evaluated in the interviewed people. The results are

summarized in figure 46. The most used energy drink was the coffee, followed by classic Coca Cola, Pepsi, Red Bull, tea and Monster drink.

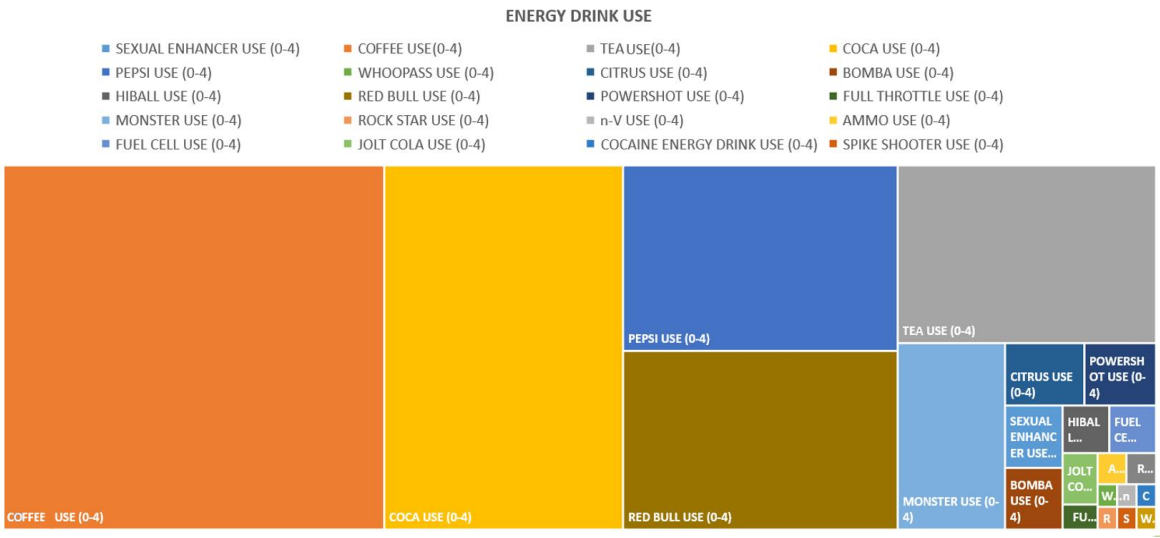


Figure 46. Type of energy drinks used by the interviewed people.

Comparing the data through box plot analysis, the score values related to energy drink use is higher in the AAS users compared to other people, even if it was no significant, p=0.09 (figure 47).

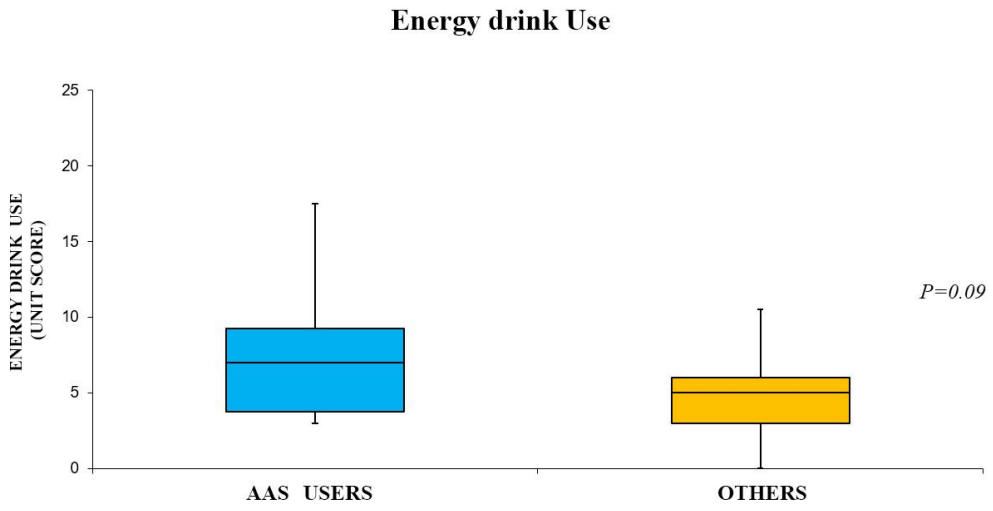


Figure 47. The box plot summarizes the data about the use of energy drinks in AAS users vs other interviewed people.

Finally, the use of supplements was evaluated in the interviewed people. The results are summarized in figure 48. The most used was Vitamin C, followed by B Complex, Organic and liquid magnesium, creatin monohydrate and soy protein.

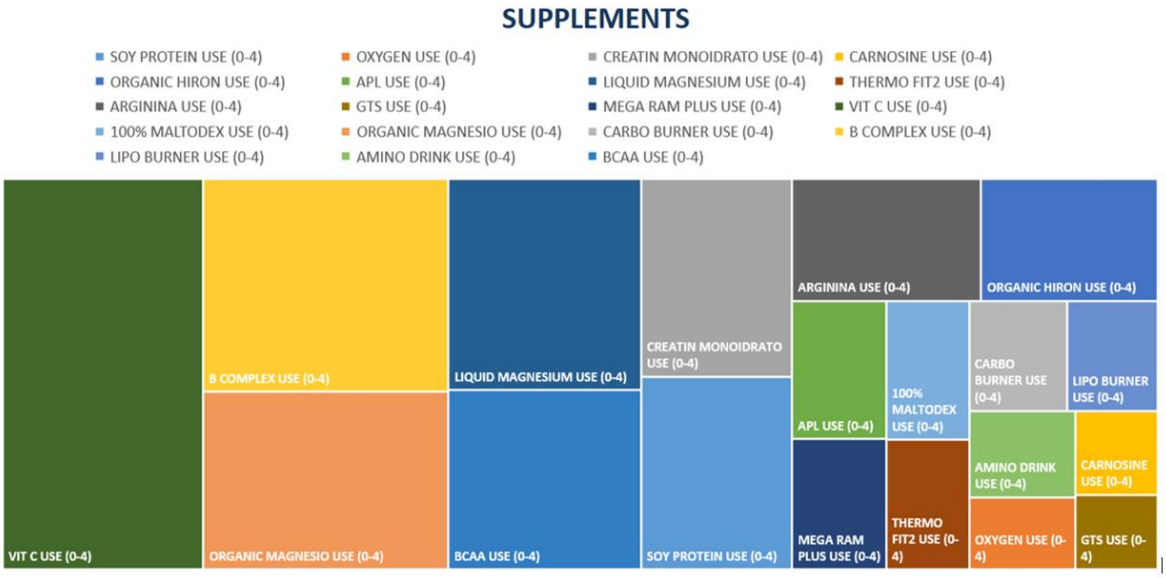


Figure 48. Type of the supplements used by the interviewed people.

Comparing the data through box plot analysis, the score values related to supplement use is significantly higher in the AAS users compared to other people, $p<0.05$ (figure 49).

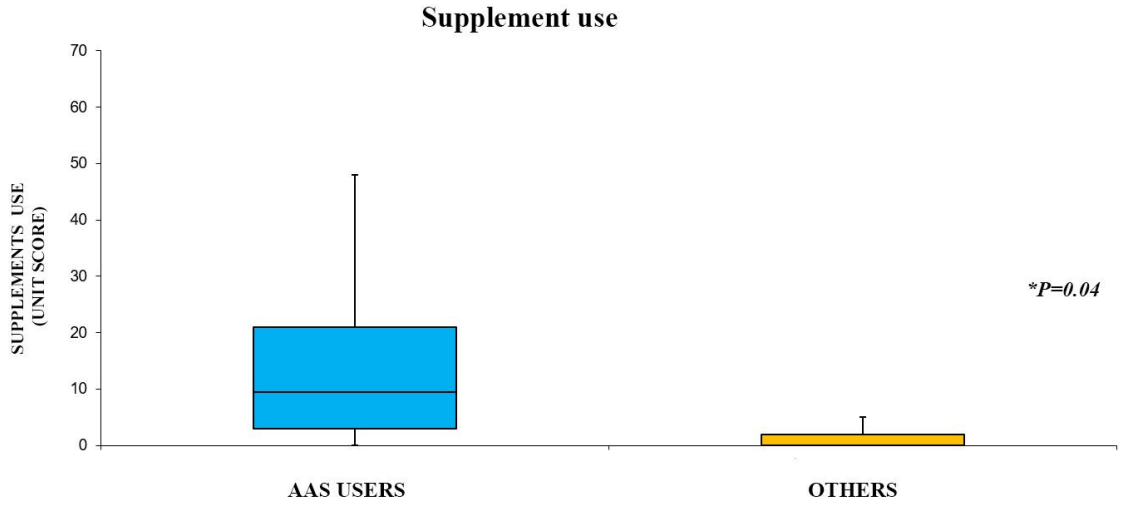


Figure 49. The box plot summarizes the data about the use of supplements in AAS users vs other interviewed people.

Chapter 5. DISCUSSION AND CONCLUSIONS

5.1. AASs adverse effects and miRNAs applications: a literature review

It may be expected that the use of AASs through self-administration combined with physiological production, both by men and women, yields circulating androgen levels that are orders of magnitude higher than normal physiological levels. AAS effects are strictly linked to the age of the abuser. Several studies in humans suggest that adolescents may be more sensitive than adults (O'Connor et al., 2004) and females may be more sensitive than males (Franke and Berendonk, 1997).

The main adverse effect described in AAS users is a significant and often permanent change in sexual behavior and their reproductive system (Frati et al., 2014). The adverse effects of AAS use/abuse on the reproductive system are suppression of gonadal steroidogenesis, amenorrhoea, clitoral hypertrophy, testicular atrophy, disproportionate growth of the inner prostate, and masculinization of female fetuses (Barone et al., 2017; Pomara et al., 2016).

Other organs that are involved in the adverse effects after AAS assumption are the liver and cardiovascular systems. Several studies have described liver tissue damage, such as impaired function, hepatic cholestasis (bile canal obstruction) causing jaundice, peliosis hepatitis (blood-filled sacs in the liver), and liver tumors (increased risk) (Pope et al., 2014b, 2014a).

Moreover, the cardiovascular system can be considered as one of the most critical systems involved in the evaluation of AAS abuse damage. Indeed, several diseases were previously described in AAS users: increased risk of thrombotic events such as myocardial infarction or stroke (raised LDL, lowered HDL and apolipoprotein-1, raised hematocrit (due to polycythemia), and lowered plasma fibrinogen, cardiac damage (left ventricular hypertrophy, fibrosis, and heart failure), and sudden cardiac death (Frati et al., 2015; Kanayama et al., 2008b; Pope et al., 2014b, 2014a).

In this PhD project, 5 cases of subjects who had died after AAS use/abuse were selected. Based on the autoptic evidence, in 4 cases the subject had died of heart failure after steroid assumption. It is very interesting to note that in the last selected case, the cause of death was related to immunodeficiency. Particularly, the subject died of septic shock subsequent to necrotizing myofasciitis. This pathological condition may develop in the site of any injury, such as surgical incision, skin biopsy, lacerations, abrasions, cuts, insect bites, surgical wounds, skin abscesses, ulcers, burns, blunt trauma, and injection site, as in the analyzed

case (Puvanendran et al., 2009; Wong et al., 2003). Over the past few years, there have only been reported a few cases of athletes who have developed necrotizing fasciitis or pyomyositis or any other soft tissue infection, often in association with AAS use, by intramuscular injection (Grant et al., 2010; Hughes and Ahmed, 2011; Souza et al., 2011). Both testosterone and AAS adversely influence immune response, affecting leucocyte growth or activity, antibody and cytokine production, above all at supraphysiological doses mimicking a condition of secondary immunodeficiency (Choi and Lee, 2015; Hughes et al., 1995; W. Brenu et al., 2011). Secondary immunodeficiency, much more common than primary immunodeficiency (that is to say those caused by genetic defects affecting the cells of the immune system), includes various causes that affect a normal immune system, including infectious, iatrogenic, metabolic and environmental causes (Chinen and Shearer, 2010). These immune deficiencies are manifested clinically with an increased frequency or unusual complications of common infections and occasionally with the onset of opportunistic pathogen infections. Depending on the etiopathogenesis, secondary immunodeficiency will manifest with different clinical conditions, on which act, moreover, the magnitude of the triggering cause and the susceptibility of the host. For example, immunodeficiency induced by the use of corticosteroids depends on the dose used (Tan et al., 2006).

Considering the adverse effects caused by AAS use/abuse, the identification of new tools for AAS use/abuse represents an important challenge for the scientific community. In the last few years, several studies have highlighted the role of miRNAs as a highly accurate diagnostic tool. To date, the limits of traditional techniques for the diagnosis of numerous diseases (such as cardiac imaging) are costs and not being quantitative. The detection of circulating miRNAs could go beyond these limitations as diagnostic or prognostic tools of several diseases, both because miRNAs are very sensitive, and their detection requires minimal peripheral blood. Several advantages are linked to the use of circulating miRNAs as anti-doping methods: high stability during transport and storage, the long period for detection, no sensitivity to unregulated room-temperature storage, and the stability in plasma subjected to multiple freeze-thaw cycles (Leuenberger et al., 2013a).

At the light of this PhD work, a pivotal role could be played by miRNAs that seem dysregulated in cardiovascular or liver diseases, considering that these organs are involved in adverse effects caused by AAS use.

Knowledge regarding miRNAs in human diseases related to AAS use/abuse may eventually lead to identify serum or tissue biomarkers with anti-doping potential. In this regard, the

need for careful validation of diagnostic miRNA candidates in well-annotated toxicological studies is mandatory. The rapid progress in anti-doping technologies using miRNA based strategies for the discovery of drug-abuse, such as AAS use/abuse, could optimize new approaches based on existing and emerging knowledge.

5.2. Abuser characteristics

In all selected cases, the AAS users were males with a BMI > 25. For all cases, at the external examination, it was not possible to clarify the exact cause of death. After autopsy, combining all findings (histological and toxicological examination), the cause of death was clarified: it was always strictly related to the adverse effects of AAS use, both direct and indirect ways. In the reported cases, the blood and/or urine and/or hair analysis revealed the presence of AASs and their principal metabolite (19-norandrosterone). In all cases, the high testosterone/epitestosterone ratio (1:6) suggested the use of androgens, such as dihydrotestosterone (DHT) and dehydroepiandrosterone (DHEA) or, more likely, testosterone. Inhibition of natural androgens occurs secondary to inappropriate and prolonged use of anabolic hormones. Chronic AAS abuse results in different patterns of pathologic alterations that depend on the dose, frequency, and mode of use: for example, in case 5, death was caused by infective risk post AAS assumption. This event is uncommon in AAS users as discussed in the previous paragraph.

Contrariwise, as well described in the literature, the cardiovascular adverse effects generated by AAS use/abuse could be considered very important in the determination of the cause of death. In 4 out of the 5 tested cases, the use of AAS damaged the cardiovascular system, probably through the alteration of the lipoprotein metabolism that may be strictly linked to myocardial infarction and/or other adverse effects.

Cardiovascular effects of AASs are frequently reported in published case reports: indeed, acute myocardial infarction can be linked to premature atherosclerosis. Acute non-fatal myocardial infarction was first reported in 1988 (Büttner and Thieme, 2010) and fatal myocardial infarction in 1990 (D'Andrea et al., 2007). Myocardial infarction without significant coronary atherosclerotic disease has also been reported (Ferenchick and Adelman, 1992; Hoffman et al., 2009; McNutt et al., 1988; Montisci et al., 2012). Other adverse cardiovascular effects such as left ventricular hypertrophy, impaired left ventricular function, arterial thrombosis, and pulmonary embolism have been described (Büttner and Thieme, 2010; D'Andrea et al., 2007; Fineschi et al., 2001, 2007; Montisci et al., 2012; Parkinson and Evans, 2006; Payne et al., 2004; Thiblin et al., 2009). The most typical myocardial abnormality in AAS abusers is left ventricular hypertrophy, associated with fibrosis and myocytolysis (Frati et al., 2014; Montisci et al., 2012).

Until today, 19 fatal cases (89.5% males), age range 18–37 years, have been reported in medical literature (Di Paolo et al., 2007; Dickerman et al., 1995; Ferenchick, 1991; Fineschi

et al., 2001, 2007; Hausmann et al., 1998; Kennedy and Lawrence, 1993; Luke et al., 1990; Montisci et al., 2012; Payne et al., 2004; Thiblin et al., 2009), but with only one case of occlusive thrombus in the left coronary artery (Ferenchick, 1991).

Melchert and Welder (Melchert and Welder, 1995) suggested that there are at least four hypothetical models of ASS-induced adverse cardiovascular effects: atherogenic, thrombosis, vasospasm, and direct myocardial injury.

Cardiovascular responses to AASs are due to specific myocardial receptors, which have transcriptional regulatory functions. The cardiac hypertrophy induced by AASs appears to be generated by a direct action on cardiac androgen receptors, whose effects are directly proportional to the dose, time and duration of drug administration (Ferrera et al., 1997; Marsh et al., 1998; Montisci et al., 2012; Wu and Von Eckardstein, 2003).

The sympathetic nervous system involved in the neurological control of the cardiovascular system may be influenced by AASs when combined with exercise and confer an increased risk of life-threatening arrhythmias (Thiblin and Petersson, 2005; van Amsterdam et al., 2010).

According to Achar et al.(Achar et al., 2010), AASs are often consumed concomitantly with GH, erythropoietin, and other agents. This is important because GH may lead to cardiomyopathy, abnormal lipoprotein profiles (Saugy et al., 2006; Thomas et al., 2013), and left ventricular hypertrophy (Karila et al., 2003). Erythropoietin abuse is linked to hypertension and increased risk of thromboembolic events (Baron et al., 2013). These effects may be difficult to separate from the results of AAS abuse alone and motivate the need for more rigorous clinical and forensic screening.

5.3 miRNA quantification

miRNAs have been considered to play a pivotal role in precision medicine. Apparently, this depends not only on differences at the interindividual level but also at the intercellular level (Collins and Varmus, 2015).

Indeed, miRNAs are exquisite regulators of gene expression that inhibit translation and/or promote mRNA degradation by base pairing to precise complementary sequences within the 3′-untranslated region.

miRNAs are expressed in a cell-specific manner and, for these reasons, they are used as selective treatments, targeting bad cells and preserving good cells. The major applications studied by the scientific community concern the fight against cancer, even if scientific efforts are also applied to several other disorders (Edwards et al., 2010; Santulli, 2015).

Moreover, in the near future, clinicians will have to deal with miRNAs, not just as diagnostic biomarkers but also as potential tools to design selective treatments, alongside their emerging important role in prognostic signatures and prediction models (Santulli, 2015).

In this scenario, one of the significant challenges in the anti-doping field is the identification of specific and sensitive non-invasive biomarkers that can be routinely measured in easily accessible samples.

In the same way that specific biomarkers are used to detect the biological fingerprint of disease, other biomarkers can be used to identify the biological fingerprint of doping.

The potential use of circulating miRNAs as specific biomarkers in the antidoping field has been demonstrated by several groups (Gasparello et al., 2019; Gunnarsdottir et al., 2019; Leuenberger et al., 2013a).

Based on this evidence, the research hypothesis of this project was the detection of the misexpression of several miRNAs at the different organs that were identified as target organs linked to AAS use. In the light of the results of the first year of this project, different miRNAs were tested on the following tissues: heart, kidney, liver, and brain.

5.3.1 Heart and musculoskeletal systems

Four miRNAs (miR-133a-3p, miR-208a-3p, miR-499a-3p, and miR-1-3p) were tested in the two groups: HF group and AAS group.

Cardiovascular disease is the leading cause of morbidity and mortality in developed countries. Cardiovascular remodeling represents an important aspect of disease progression in heart failure (HF), regardless of cause. It manifests clinically by changes in cardiac size,

shape, and function in response to aging, cardiac injury, or increased load. The importance of remodeling as a pathogenic mechanism is not completely understood because the factors leading to remodeling have not been fully investigated. Generally, pathological processes of the heart are associated with an altered expression profile of genes that are important for cardiac function (Kumarswamy and Thum, 2013; Williams et al., 2009; Zampetaki et al., 2013).

The cardiac genes involved in the remodeling are very numerous, and each gene is individually controlled by multiple transcription factors. Moreover, epigenetic and post-transcriptional regulations can be very important in gene expression. Cardiovascular remodeling involves many pathologies including myocardial ischemia/myocardial infarction (MI), cardiac hypertrophy, cardiac fibrosis, arrhythmia, and vascular diseases (Olson, 2006; Williams and Carroll, 2009).

Tissue-specific expression of miRNAs was first reported in 2002 (Lagos-Quintana et al., 2002). It is known that there is a family of so-called myomiRs that are encoded within the introns of the separate myosin heavy chain genes. miR-208a, miR-208b, and miR-499 are located within the Myh6, Myh7, and Myh7b genes, respectively. It was reported that miR-208 −/− mice show reduced cardiac hypertrophy in response to pressure overload (Van Rooij et al., 2007). Targets of miR-208a include thyroid hormone receptor-associated protein 1 (Callis et al., 2009; Van Rooij et al., 2007), suggesting that miR-208a initiates cardiomyocyte hypertrophy by regulating triiodothyronine-dependent repression of β-MHC expression. Overexpression of miR-208a was sufficient to upregulate Myh7 and to elicit cardiac hypertrophy, resulting in systolic dysfunction (Callis et al., 2009). Although miR-208a is required for cardiac hypertrophy, the role of miR-208b in these pathologic conditions remains to be elucidated. The therapeutic inhibition of miR-208a using the antimiR-208a during hypertension-induced heart failure in animal model improving cardiac function, overall health, and survival (Montgomery et al., 2011). Moreover, it was described that miR-208a is sufficient to induce arrhythmias, cardiac remodeling, and to regulate the expression of hypertrophy pathway components and the cardiac conduction system. The emerging role of miR-208a in the heart suggested that it is involved in the regulation of the myosin heavy chain isoform switch during development and in pathophysiological conditions (Oliveira-Carvalho et al., 2013). The results of this research project confirmed a pivotal role for the miR-208 considering that it has been detected overexpressed both in HF group and in AASs group. Indeed, these results suggest that the expression levels of this miRNA could be related to heart tissue damage in a direct way: when more remodeling on

the heart tissue was detected, the miRNA level was higher. No modification on the expression levels of this miRNA on the musculoskeletal tissue was reported.

In a recent study a pivotal role for the miR-1 and miR-499 in discriminating sudden cardiac death (SCD) from acute myocardial infarction (AMI) was reported (Pinchi et al., 2019): both miRNAs were overexpressed compared to the control group for SCD and AMI even if significant differences were described among them.

miR-1 is also a cardiac and skeletal muscle-specific miRNA, and it is probably one of the most abundant miRNAs in the heart. It was reported to target a cytoskeletal regulatory protein, Twinfilin-1 (Twf1), which binds to actin monomers and prevents their assembly into filaments (Li et al., 2010b). The role of this miRNA was investigated in heart diseases. Tang et al. reported a pro-apoptotic effect on cardiomyocytes by targeting Bcl-2 when this miRNA was overexpressed. They reported that Bcl-2 was silenced by miR-1 at the protein and mRNA levels (Tang et al., 2009). The mechanisms of apoptosis in ischemic heart disease (IHD) and dilated cardiomyopathy (DCM) have yet to be clearly elucidated. Apoptosis and necrosis in heart failure may be induced by the same agents, with the type of cell death being dependent on the severity of the insult. Post-transcriptional repression of Bcl-2 by miR-1 is probably one of the mechanisms underlying their regulation of apoptosis versus survival (Latif et al., 2000). In agreement with a previous study (Pinchi et al., 2019), the results of the present study suggest that this miRNA is overexpressed compared to controls in both the AAS and HF groups, although no significant differences were reported among groups. Contrariwise, this miRNA has been detected significantly higher in the AAS group in the musculoskeletal tissue compared to the other groups. miR-1 and miR-133 are specifically expressed in adult cardiac and skeletal muscle tissues, but not in other tissues tested (Sempere et al., 2004). miR-1 and miR-133 modulate muscle proliferation and differentiation, in part, by targeting HDAC4 and Serum Response Factor (SRF), respectively. Particularly, overexpression of miR-1 led to the downregulation of endogenous HDAC4 protein, while overexpression of miR-133 repressed the expression of endogenous SRF proteins (Chen et al., 2006). Moreover, both miR-1 and miR-133 are involved in cardiac repolarization acting on cardiac ion channel genes such as GJA1/Cx43/IJ (Yang et al., 2007), KCNJ2/Kir2.1/IK1 (Yang et al., 2007), potassium voltage-gated channel, subfamily H (eag-related) member 2 (KCNH2)/human ether-à-go-go-related gene (HERG)/IKr (Xiao et al., 2007), potassium voltagegated channel, KQT-like subfamily, member 1 (KCNQ1)/KvLQT1/IKs, and potassium voltage-gated channel, Isk-related family, member 1 (KCNE1)/mink/IKs (Luo et al., 2007). The aberrant expression of miR-

133 was usually accompanied with cardiac hypertrophy, arrhythmogenesis and heart failure (Lau et al., 2001). Although miR-133 has been demonstrated to be involved in pathogenesis and progression of heart failure (HF), its effect remains controversial (Liu et al., 2017). Nevertheless, miR-133 has been described as one of the most promising biomarkers for understanding the biological response to physical activity and for the potential use for diagnosing muscle injury as well as in anti-doping testing (Danese et al., 2017). In the present study, the expression levels of this miRNA were higher in all tested groups compared to controls, suggesting that anabolic agent use is related to higher expression levels, similarly to those of the HF group.

miR-499 is encoded in an intron of the myh7 gene and is considered likely to play a role in myosin gene regulation (Bell et al., 2010; van Rooij et al., 2009). Similarly, miR-499 and miR-30 family members diminished apoptosis in injured hearts by attenuating activation of dynamin-related protein-1 and thus inhibiting mitochondrial fission (Li et al., 2010a; Wang et al., 2011). In the present PhD study, miR-499 resulted overexpressed in the HF group, while, in the AAS group, it had about the same expression values as the control. Even if this miRNA was expressed similarly to the control in the AAS group, the results related to the HF group suggested that miR-499 could be used as a prognostic marker for clinicians and pathologists for heart diseases, even if further studies are needed.

The results of the present study highlight a pivot role in the cardiovascular remodeling both for miR-499 and miR-208. The data obtained concerning the expression levels of miR-1 and miR-133 suggest that their role remains not completely known: the scientific community should consider to improve their effort in this field of research in order to better clarify all aspects.

5.3.2 Kidney tissue

Analyzing the results about the kidney tissue, the two tested miRNAs (mir-21 and miR-205) were improved in both tested groups (AASs vs CKD), even if significant differences were described only for miR-21. This miRNA is widely expressed in all tissues and is expressed quite highly in the normal kidney, heart, spleen, liver, and lung as well. Indeed, the expression levels were higher in the CKD groups compared to AAS group.

As previously described, miR-21 was upregulated in injury with fibrosis both in mouse and human models (Chau et al., 2012). In addition to its involvement in kidney fibrosis (Zarjou et al., 2011; Zhong et al., 2011), several studies indicated that miR-21 may play an important role in stimulating fibrosis in other tissues after injury, such as cardiac and pulmonary tissues

(Liu et al., 2010; Thum et al., 2008). Moreover, it has been described that high levels of this miRNA promote kidney injury and fibrosis, while the inhibition of miR-21 in animal models ameliorates the effects (Chau et al., 2012).

The way to regulate these mechanisms has been investigated in heart tissue: miR-21 regulates the ERK–MAP kinase signaling pathway in cardiac fibroblasts, impacting global cardiac structure and function. miR-21 levels are increased selectively in fibroblasts of the failing heart, augmenting ERK–MAP kinase activity through inhibition of sprouty homologue 1 (Spry1). This mechanism regulates fibroblast survival and growth factor secretion, apparently controlling the extent of interstitial fibrosis and cardiac hypertrophy (Thum et al., 2008). It is commonly thought that the same pathway was followed for kidney tissue. Moreover, the action of this miRNA is exerted on PPARα as a major upstream regulator of lipid metabolism. The major miR-21 targets in the kidney are epithelial metabolic pathways, particularly the PPARα-regulated lipid metabolic signaling pathway (Lefebvre et al., 2006). For these reasons, miR-21 represents a post-transcriptional regulator in kidney tissue that amplifies injury responses, resulting in increased fibrosis.

In the present study, the levels of miR-205 were overexpressed in both groups compared to controls. Even if the expression values were higher in the CKD groups compared to the AAS group, no significant differences were reported. These data were in agreement with previous studies. Wang et al. reported that the level of intra-renal of miR-205, similarly to other miRNA investigated, resulted significantly higher in patients with hypertensive nephrosclerosis than controls (Wang et al., 2010b). Moreover, high intra-renal expression of miR-205 was also found in renal biopsies of patients with hypertensive glomerulosclerosis: these expression values were correlated with disease severity (Schena et al., 2014). It is very important to note that the overexpression of this miRNA is related to no cancer diseases: indeed, miR-205 was markedly upregulated in non-tumor tissues (Ying et al., 2018).

Finally, the results of the present study suggest that kidney damage in AAS abusers is similar to subjects suffering from chronic kidney disease, remarking that these miRNAs could be considered candidate markers for the identification of AAS abusers.

5.3.3 Liver tissue

As previously described, miR-21 is expressed in liver tissue similarly to other tissues. Its role has been largely discussed by the scientific community, resulting in controversy. Using an animal model, a recent study reported that MiR-21 expression was increased in the liver

of mice fed a high-fat diet (HFD) compared with controls (Loyer et al., 2016). This study demonstrated that miR-21 inhibition or suppression decreased liver injury, inflammation and fibrosis, in different murine models of non-alcoholic steatohepatitis (NASH), by restoring PPARα expression. MiR-21 has been reported to be upregulated in many inflamed states: this feature might explain the upregulation of miR-21 in NASH since plasma lipopolysaccharide levels are higher in patients and mice with NAFLD than controls (Mouzaki et al., 2013; Thuy et al., 2008). Analyzing the expression data from the liver tissue in the two tested groups (AASs and NAFLD), a pivotal role for miR-21 in fibrosis in liver tissue clearly appears. Particularly, the expression values of this miRNA are significantly higher in the AAS group, suggesting its possible use as a molecular biomarker both in anti-doping and in diagnostic usage.

MicroRNA-122 (miR-122) is involved in various physiological processes in hepatic function as well as in liver pathology; moreover, it is the most abundant liver-specific microRNA (Hu et al., 2012). The molecular mechanisms underlying regulation of lipid homeostasis by miR-122 are still unclear, both AMP-activated protein kinase (APK) and circadian metabolic regulators of the peroxisome proliferator-activated receptor (PPAR) family have been suggested to be putative effectors of miR-122-mediated metabolic controls (Bandiera et al., 2015). The results of the present study show that the expression levels of miR-122 were significant higher in the NAFLD group compared to the AAS group. Moreover, this miRNA was overexpressed in both groups, compared to controls. Recent studies were performed on miRNAs involved in the control of lipid metabolism (such as MiR-122, MiR-16, miR-33, miR-34, miR-103, miR-104, and miR-370), with the aim of identifying new tools for modern anti-doping. For example, Salamin et al. (2016) identified three potential candidate miRNAs for testosterone use, even if one of these showed a response related to dose-effect: in fact, levels of miR-122 increased 3.5-fold after 1 day of drug intake. These results suggest that miR-122 could be used as a reliable fingerprint of testosterone misuse (Salamin et al., 2016).

miR-132 was investigated because it plays an important role in liver tissue: it targets mRNAs that regulate key biological processes including metabolism (Purushotham et al., 2009; Stiles et al., 2004), cell proliferation (Anand et al., 2010), epigenetic regulation (Lagos et al., 2010) and inflammation (via suppression of acetylcholinesterase) (Shaked et al., 2009), as well as nervous system-related cholinergic functions (Hanin et al., 2018). miR-132 targets such as Pten and Sirt1, associated with hepatic steatosis, hyperlipidaemia and glucose regulation; moreover, it was elevated in a mouse model of alcoholic liver disease

(Hanin et al., 2018). The results of the present study show that the expression levels of miR-132 were overexpressed in both groups compared to controls, resulting higher in the AAS group even if no significant differences were reported. These findings could be considered very important because the adverse effects generated by AAS assumption may cause the same organ damage as NAFLD.

Finally, miR-155 was tested on selected samples. In recent years, the role of miR-155 in several cellular processes such as fatty acid metabolism and fibrogenic events has also been investigated (Bala et al., 2016). miR-155 targets genes involved in lipid metabolism (Fab4, Cpt1a) and early fibrosis (C/EBPβ, Smad3) (Csak et al., 2015). A recent study conducted on animal models reported that alcohol-induced liver injury, steatosis and inflammation were significantly reduced in miR-155 knockout (KO) mice compared to controls. The same study demonstrated that the inhibition of miR-155 using a specific miR-155 inhibitor resulted in an increase in PPARγ gene expression, with a subsequent benefit in treatment. Moreover, considering that alcohol use/abuse reduces PPARα (Kersten et al., 2010), steatogenic, inflammatory and fibrogenic changes observed in miR-155 KO mice after ethanol diet are likely due to the effect of miR-155 on PPAR signaling. (Bala et al., 2016). The results of the present study show that the expression levels of miR-155 were overexpressed in both groups compared to controls, resulting higher in the AAS group even if no significant differences were reported. These findings could be linked to the organ damage generated by AAS use/abuse.

5.3.4 Brain tissue

The data obtained from brain tissue in the tested group are very interesting. Brain damage is a complex dysfunction that involves a variety of conditions whose pathogenesis includes a number of mediators that lead to clinical sequelae. For this reason, the identification of specific circulating and/or tissue biomarkers that could indicate brain injury is challenging (Zaninotto et al., 2016). Moreover, this is true whether diagnosing early in order to benefit patients by starting appropriate treatment early or whether investigating the cause of death, except for cases in which the circumstances of the event are strongly evocative.

In this context, miRNAs can play an important role in modulating a variety of brain conditions and can serve as new biomarkers. The miRNAs selected in this study (miR-21, miR-34, miR-124, miR-132, and miR-200b), have been predicted to control important target genes involved in neuronal apoptosis and neuronal stress-induced adaptation.

In detail, as well as being a strong indicator of widespread axonal damage (Pinchi et al.,

2018), miR-21 could be a diagnostic biomarker of cerebral ischemia (Liu et al., 2016). The miR-21 pathway of expression seems to be related to the activity of Akt signaling by suppression of phosphatase and tensin homolog (PTEN)-gene expression, resulting in further suppression of caspase-3 expression (Han et al., 2014). This cascade prevents apoptosis of cortical neurons. In addition, patients with stroke and atherosclerosis show significantly higher plasmatic levels of miR-21, which has been interpreted as a strong anti-apoptotic prevention measure (Tsai et al., 2013). Furthermore, the study conducted by Buller et al., employing in situ hybridization, demonstrated that miR-21 expression was upregulated in neurons in the area adjacent to the ischemic area as an expression of protection from ischemic neuronal death (Buller et al., 2010). Finally, miR-21 acts as an anti-inflammatory marker during ischemic stroke (Gaudet et al., 2018). This evidence is in line with our results with miR-21 expression levels being significantly higher in the AAS, SG and AG groups compared to the control trauma group and the DG group.

Analyzing the data about miR-34, it was found to be overexpressed in the AAS and DG groups. The overexpression of miR-34 was related to an increased rate of apoptosis associated with a decrease in Bcl-2 expression in mouse models and in human cell lines (Khanna et al., 2011). In contrast, an induced down-regulation of miR-34 blocked the inhibition of the expression of the target gene, Bcl-2. In addition, the high levels of the post-transcriptional protein that were induced, were accompanied by concomitant low levels of Bax expression and low cleavage by caspases. This mechanism was also observed in the brains of mice on a low-calorie diet. Furthermore, in an experimental study on the hippocampus of rats, in order to evaluate the evolution of neurodegenerative diseases, such as Alzheimer's disease, miR-34 levels were found to be elevated (Zovoilis et al., 2011). Therefore, in our analysis, the expression values of miR-34 were higher in all four groups compared to the control group, probably interpretable as an important index of neurodegeneration being statistically significant in the DG and AAS groups.

This trend is confirmed analyzing the data about the expression values of miR-124. Concerning the literature, similar considerations have been made about miR-124. In fact, Jeyaseelan et al. demonstrated that miR-124 was increased in a murine model following cerebral artery occlusion and 24 h reperfusion (Jeyaseelan et al., 2008). Moreover, miR-124 is significantly increased, as well as miR-21, in the area surrounding an ischemic zone. In the same animal model, the nervous overexpression of miR-124 correlated with a decreased infarct size (Sun et al., 2013). The probable explanation would result in the anti-apoptotic genes targeted by miR-124 being Bcl-2 and Bcl-xL (Graham et al., 2000). According to this

data, miR-124 expression showed significant differences, resulting in a higher expression in the SG and AAS groups with respect to the other two groups. A further target of miR-124 is the REST (RE1 Silencing Transcription Factor) gene that determines a reduction of the expression of neuronal plasticity genes (synaptophysin, BDNF, and Brain Derived Neurotrophic Factor). Cocaine induces REST and suppresses miR-124, influencing tolerance, sensitization, and addiction (Chandrasekar and Dreyer, 2009). In our experimental setting, the expression values of this miRNA were significantly higher in the DG group with respect to the AG group. Moreover, this neuroprotective function of miR-124 could also be extended to other neurodegenerative diseases. In fact, the miR-124 level in the brain of patients with Alzheimer's disease is down-regulated, in parallel to the increase in expression of beta-site expression APP-cleaving enzyme 1 (BACE1) (Sun et al., 2015). In addition to this mechanism of neurodegeneration, miR-124 is involved in long-term plasticity affecting the transcription factor CREB (cAMP response element-binding protein) (Rajasethupathy et al., 2009). The experimental findings of this PhD project showed that miR-124 expression in the AG group was similar to that in the control group, showing the lowest statistical significance in inter-group variation. The expression was statistically higher in the SG and in AAS groups, leading us to hypothesize its role as a diagnostic tool in the diagnosis of ischemic stroke, or for brain damages linked to AASs use/abuse. However, cognitive decline can be attributed to complex interactions involving cellular dysfunction, cumulative over time, and life habits that cause the reduction of plasticity in the elderly.

Moreover, for miR-132 the expression values in the AAS group were about the same of the DG group. In the SG group, which had expression values about the same as the controls, an important role was played by miR-132, whose function is to regulate the glutamate receptor expression level as well as post-stroke excitotoxicity (Karr et al., 2009). In fact, some recent studies have shown that the use of miR-132 antagomir during cerebral ischemic attacks may have neuroprotective effects through the suppression of glutamate receptor expression, acting on CREB as stated for miR-124 (Luikart et al., 2011; Nudelman et al., 2010). Moreover, higher serum miR-132 levels were recently suggested as biomarkers for mild cognitive impairment, a stage often preceding Alzheimer's disease (Hadar et al., 2018). Considering this effect on memorization processes, recent studies have proved that miR-132 is induced in culture by neurotrophins and neuronal activity and is able to modulate dendritic morphology via the suppression of p250 GTPase-activating protein (p250GAP) (Vo et al., 2005; Wayman et al., 2008). Acting between the neural and immune system, miR-132 has

recently been discovered to reduce brain inflammation and to increase the level of acetylcholine (O'Neill, 2009; Shaked et al., 2009; Wanet et al., 2012). In our experimental setting, the expression levels of miR-132 were higher in all four groups. Therefore, the DG and AAS groups presented significantly higher levels than the SG and AG groups.

The reason for this could be found in some studies demonstrating the upregulation of mature miR-132 expression in rats following cocaine self-administration (SA). Furthermore, recent observations from Hollander et al. confirmed that 6 h (but not 1 h) access to cocaine SA for 7 days increased miR-132 expression in the rat striatum (Hollander et al., 2010). The significant increase in miR-132 levels was long-lasting and remained high in rats that had been withdrawn from cocaine SA. Changes in dopamine and glutamate neurotransmission, alterations in specific signaling pathways, and/or epigenetic regulation have been advocated as a possible explanation. As chronic cocaine exposure alters dopamine and glutamate signaling by stimulating the dopamine D1 and glutamate NMDA receptors, these targets trigger the activation of the corresponding downstream signaling pathways and lead to CREB-dependent gene expression (Sadakierska-Chudy et al., 2017).

Finally, the data about mir-200b showed higher values in the AAS group: this result suggests a positive response in order to reduce brain damage in a subject who used AASs. miR-200b is expressed in brain tissue, particularly in microglia, and involved in inflammatory response. Oligodendrocytes (OL) are myelin-forming cells of the CNS that are vulnerable to cerebral ischemia. The loss of OL and myelin impairs axonal function and is detrimental to functional recovery. Another study by Buller et al. proved that ischemic stroke causes an up-regulation of Serum response factor (SRF) and a down-regulation of miR-200b in OL white matter (Buller et al., 2012), indicating that miR-200b plays an important role in stroke-induced SRF up-regulation, which ultimately affects OL progenitor cell differentiation. Analogously, microglia treated with miR-200b-inhibitor cause neuronal apoptosis in cell culture that is due to an excessive release of inflammatory cytokines and NO in the conditioned medium via increased c-Jun activity (Jadhav et al., 2014). In addition, impaired microglial migration has been shown to contribute to the pathogenesis of several brain diseases such as Prion disease (Ciesielski-Treska et al., 2004), Parkinson's disease (Park et al., 2008), and Alzheimer's disease (Mizuno, 2012), and it inhibits axonal regeneration during acute CNS injury (Vargas and Barres, 2007). However, further experiments are required to ascertain the possible neuroprotective role of microglial miR-200b. These literature data are in line with the statistical results of our experimental setting: the expression levels of miRNA-21 and miRNA-200b were higher in the AAS, SG and AG

groups compared to the control group, while in the DG group, the expression was similar to that in the control group.

The main limitation of this step is related to the characteristics of the enrolled subjects in the AAS group. Indeed, even if the androgen use was ascertained through the toxicological examination, the data about the utilization time is unknown. For this reason, it is impossible to establish if the adverse effects can be considered as a consequence of chronic or short term use.

Moreover, to achieve the goal of identification of new molecular biomarkers, the levels of the tested miRNAs should be evaluated in the blood, as for miR-122 by Salamin et al. (Salamin et al., 2016). However, in this PhD project, several difficulties were encountered in enrolling AAS users, making impossible the *"in vivo"* studies. Indeed, the Italian regulation in this field discourages the people from declaring the use of androgens, even only for research purposes. Contrariwise, as reported in the next paragraph, the use was declared anonymously. To solve this critical problem, in the upcoming future, collaborations with universities of other countries should be activated.

5.4 Questionnaire results

Anabolic-androgenic steroid(s) (AAS) refer to testosterone and its synthetic derivatives mainly used non-medically for enhancing muscle growth and strength, boosting physical activity or sports performance, and for aesthetic purposes as well as for enhancing psychological well-being (Sagoe et al., 2014a). During assumption, users sometimes combine different injectable and oral AASs. This phenomenon is referred to as 'steroid stacking' or simply 'stacking' (McVeigh et al., 2012). Moreover, many users complement AAS use or stacking with the use of other substances. In this respect, AAS use has been found to be associated with the use of both licit and illicit substances in systematic reviews of predominantly quantitative literature (Sagoe et al., 2014b, 2015).

It has been noted that one of the major drawbacks to successful AAS interventions is public health officials' failure to recognize AAS users' extensive pharmacological regimen (Sagoe et al., 2015). A synthesis of the qualitative or descriptive literature on polypharmacy by AAS users is, both from a clinical and research perspective, important in order to increase the understanding of the polypharmacy often associated with AAS use.

The analysis of the questionnaire data demonstrated that the use of AASs in a young Italian population is 3.23 %. Analyzing the data obtained through the questionnaires in the USA reported in a recent study, the percentage of users among the interviewed people was between 1-2% (Pope et al., 2014a). The AAS users reported a significantly higher appearance evaluation and orientation compared to no-users.

It is singular to note that the kind of the anabolic agents used has been self-administrated through injection in 87.5% of cases. Considering the sex differentiation in AAS users, in a study performed in the USA, it has been reported that the rarity of female AAS use and dependence is hardly surprising (McCabe et al., 2007), in this report the prevalence of users was female (6 on 8).

Energy drinks are non-alcoholic beverages that typically contain high levels of caffeine (>150 mg/L) and sugar in combination with other ingredients known to have stimulant properties. They are marketed explicitly as a way to relieve fatigue and improve mental alertness, in contrast with sports or isotonic drinks which are intended to help athletes rehydrate after exercise (Ballard et al., 2010). In a recent report about the consumption data for energy drinks in 16 European countries it was reported that young people aged 10–18 years old had the highest reported consumption prevalence (68%), compared with adults over 18 years old (30%) and children under 10 years old (18%) (Zucconi et al., 2017). In the report, 105 subjects (42.5%) declared that they used Energy drinks. In agreement with

the scientific literature, the users declared that the consumption was related to the effects of the energy drink, increasing cognitive performance, enhanced mood, more physical energy and promotion of wakefulness (Ganio et al., 2009; Goldstein et al., 2010; Spriet, 2014). However, evidence is emerging on the harmful physiological and psychological effects of these drinks, and it is possible that prolonged use may affect physical and mental well-being (Ishak et al., 2012). In the present study, about 42% declare coffee assumption, and 10.5% of the interviewed people declared consuming more than 3 coffees/day, while 36% declared that they used Red Bull and 17% used Monster. Based on the known effects of caffeine, consumption of energy drinks may lead to the following: caffeine intoxication and withdrawal; sleep disruption and insomnia; and disruptive, hyper-active and risky behavior (Mahoney et al., 2019; Terry-McElrath et al., 2014). Furthermore, these drinks are also likely to be negative in terms of health implications associated with excessive sugar intake, such as dental erosion, obesity and type 2 diabetes (Greenwood et al., 2014; Nissinen et al., 2009). Moreover, new developments in marketing are also aimed at increasing the perceived health safety of energy drinks in order to gain acceptance in an increasingly health-driven society. As energy drink sales are rarely regulated by age, like alcohol and tobacco, and there is a proven negative effect of caffeine on children, there is the potential for a significant public health problem in the future. To date, policy development has been limited. Where policies exist, they are yet to be systematically evaluated in terms of their impact on heavy energy drink consumption, particularly among children and young adults. From a cautionary viewpoint, further research and policy action is necessary to minimize the risk of harm from heavy and long-term energy drink consumption. Moreover, it is very important to note that energy drink consumption is frequently combined with supplements. Previous independent reviews on energy drinks highlighted a number of implications for users' health and well-being (Ali et al., 2015; Alsunni, 2015). Finally, based on the result of the present study, energy drink use is higher in subjects who reported AAS consumption: this event could be considered in the evaluation of the adverse effects linked to the use of these kinds of substances.

Another important aspect analyzed through the questionnaire is related to supplement use. What it is known is that the majority of these products can be bought from internet web sites anonymously, with the risk of buying something different, counterfeit or completely fake (Pacifici et al., 2016); caffeinated energy drinks are often mixed with alcoholic beverages with an increased risk of binge drinking, impaired driving, risky sexual behavior, and decreased perceived intoxication (Marczinski and Fillmore, 2014); and male sexual

enhancement products (e.g. sildenafil) have been used concomitantly with cocaine with severe cardiovascular effects (Famularo et al., 2001; Megalla et al., 2011).

Another sizable emerging consumption is that of physical performance enhancers, which is recognized as unsafe by half of the responders and are consumed to replace "perceived" nutritional deficiency and to improve performance (Fernandez and Hosey, 2009; Yager and O'Dea, 2014).

The reason of performance increase is shared with AASs, taken by a minority of responders and with drugs for sexual enhancement, used exclusively by males with a prevalence of older males (Kanayama et al., 2009; Pantalone et al., 2008). Surprisingly, physical performance enhancers that are dietary supplements and both steroids and drugs for sexual enhancement, are medicinal and similarly perceived as harmful to health. In addition, steroid consumers are more likely to also use alcohol, smart-drugs and energy drinks. Whereas recreational substances such as energy drinks and smart-drugs were mainly consumed for fun, related to nightlife and associated to influence by peers and internet use, compounds enhancing physical performance were associated with training (Breda et al., 2014; Polak et al., 2016).

In the present study, the combined use of AASs with supplements is significantly higher compared to non-users, demonstrating the idea of polypharmacy for AAS users.

Although the present study has an important limitation of participants who cannot be perfectly representative of the Italian population, the sample size and its diffusion throughout the national territory support the validity of the study. In addition, it has to be remembered that results are based on self-reported answers to the questionnaire with no objective assessment (i.e. consumption biomarkers in biological fluids) of personal statements.

There was a significant association ($p < 0.05$) between the intake of anabolic steroids and consumption of other substances, such as energy drinks and smart-drugs.

A typical trend of the 21st century is that these substances can be anonymously supplied through the internet, where they are sold with alleged effects on mental, physical and sexual performance without any real evidence-based research. Moreover, considering that in Italy the sale of the AASs is not allowed, it could be related to the online/black market. This is true for energy drinks, smart-drugs and physical performance enhancers whose advantage/disadvantage ratio is not systematically established and for androgenic anabolic steroids and drugs for sexual enhancement administered without any real hormonal or sexual dysfunction (Yager and O'Dea, 2014).

The potential health risks related to heavy consumption of these products have largely gone unaddressed. For these reasons, the efforts of the scientific community, both to better understand and to better communicate the risks linked to the use of these substances, should be improved.

Significance and impact of PhD research

In the light of the results discussed in this project, it would appear that concerns in the scientific community and among the public regarding the potential adverse health effects of the increased consumption of energy drinks, supplements and AASs are broadly valid.

As extensively discussed, AAS use is not limited to athletes, but concerns young people who use these kinds of substances for esthetical purposes. For these reasons, this field of ongoing research is very challenging for the scientific community, in particular for the forensic field, involving a large number of people. Although AAS use is strictly linked to the adverse effects on several organs, the use of these substances is constantly increasing. As demonstrated in this research project, the main organ involved is the heart; in several cases, the pathologies could be fatal for the abusers as described in the selected cases. Moreover, other organs are damaged during AAS use/abuse, such as reproductive systems, liver, kidney and brain. The identification of new molecular biomarkers can be considered of interest for the scientific community, not only for anti-doping purposes but for public health too.

In this project, the evaluation of several miRNAs was performed in different selected tissues (heart, liver, kidney, brain). At the end of this project, several miRNAs were identified as candidate miRNAs that could be used for anti-doping. Moreover, the identification of new molecular biomarkers for different pathologies could be very useful in clinical practice to quickly orient diagnosis and determine the most suitable treatment in the shortest time. For these reasons the results obtained with this study are very promising to encompass both new anti-doping methods and new biomarkers linked to organ damage.

Finally, evaluating all the data of the performed questionnaire, a complete scenario could be obtained on the voluptuary practices linked to AAS or smart drug use. Considering the results obtained through the questionnaires, the previous suggestions of associations between AAS use and the use of a wide range of other licit and illicit substances have been corroborated. AAS-related polypharmacy has potential serious harmful effects for people who engage in such behavior, which should be of serious public health concern. Clinicians, policymakers, researchers, and public health workers dealing with AAS users must be made aware of these issues. Importantly, efforts must be intensified to fight the debilitating effects of AAS concomitant polypharmacy. Furthermore, there needs to be ongoing research to investigate trends in AAS use and polypharmacy.

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