Alma Mater Studiorum – Università di Bologna
Dipartimento di Medicina Specialistica, Diagnostica e Sperimentale

Dottorato di Ricerca in Scienza Chirurgiche

Coordinatore Prof. Annalisa Patrizi

35° Ciclo

Tesi di Dottorato

Dottorando: Dott. Massimo Milani

Supervisore: Prof. Emi Dika

Titolo del progetto di ricerca:

Identification of potential miRNAs related to classification and treatment response of actinic keratosis

Settore Scientifico Disciplinare: Malattie Cutanee e veneree
Settore Concorsuale: Malattie Cutanee e Malattie Veneree

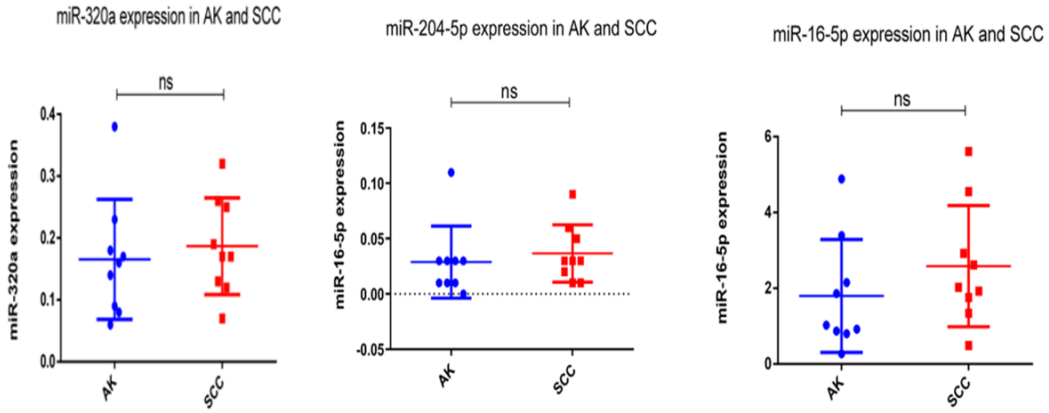
Sedi di lavoro: Policlinico sant'Orsola Bologna

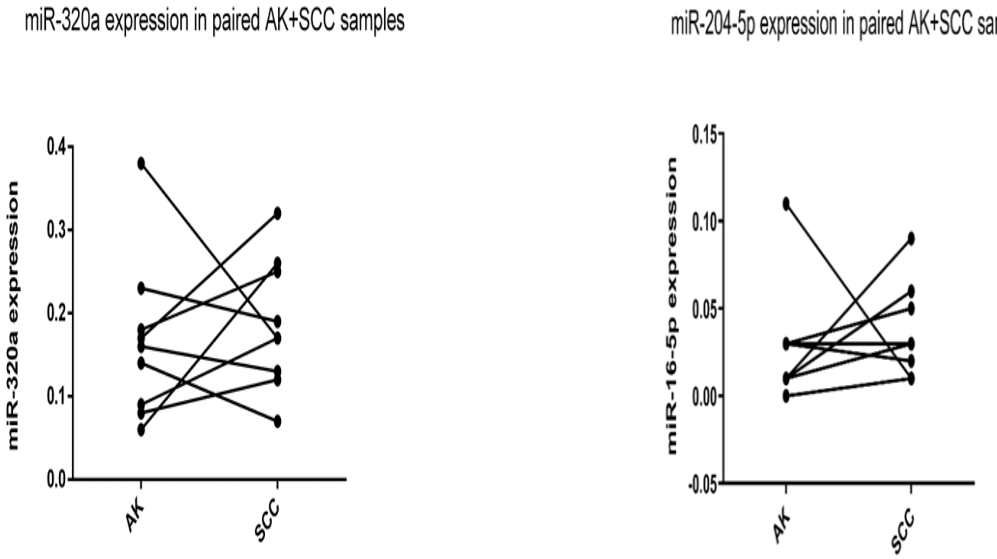
Abstract

Introduction: Actinic Keratosis (AK), the most common cancerous skin lesion, is considered a pre-neoplastic condition originated from keratinocytes (Non-Melanoma Skin Cancer: NMSC) that arises in sun-damaged skin. AK has a risk of progression to invasive SCC ranging from 0.025% to 16% per year. Twenty-seven percent of SCC arise from an AK lesion and furthermore 56% of SCC have a AK lesion within close proximity. The «cancerization field» concept is pivotal to understand the biological and clinical implications behaviour of NMSCC. The microRNAs (miRNA) are short, 22-nt, non-coding RNA molecules involved in post-transcriptional control of gene expression, able to edit RNA, modulating chromatin modification or silencing RNA. Some miRNA are involved in apoptosis processes or in cell proliferation. Differential expression profiles of miRNA have been reported in a variety of different cancers, including skin cancers. Mizrahi et al (2018) have demonstrated that AK and SCC histological samples could express in different manner some miRNAs: i.e., mir-320 a, mir-16-5p and mir-204-5p.mir-130a, a tumor suppressor microRNA, is downregulated in SCC (Lohcharoenkal 2021). The title of our project is: ***Analysis of some miRNAs expression (miRNA320, miRNA-16-5p, and miRNA-204) of AK and SCC samples of a defined canceritazion field.*** **Methods**: The Workflow activity was the following: Preliminary phase: Identification of 18 Formalin-fixed paraffin embedded (FFPE) samples (9 patients) («matched» 9 AK lesions and 9 SCC lesions). Working on biopsies samples we perform an extraction and RNA analysis with droplet Digital PCR (ddPCR) and we perform the data analysis. Second and final step phase: Evaluation of additional 39 subjects (36 men and 3 women).

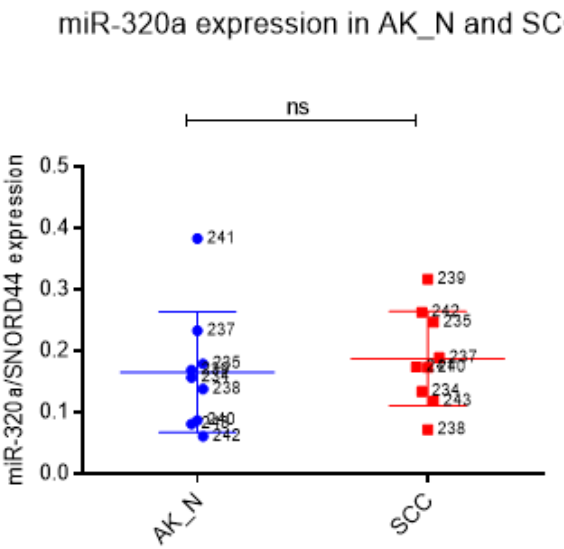
Results: We perform an evaluation and comparison of the following miRNA: miR-320 (a miRNA involved in apoptosis and cell proliferation control; miR-204, a miRNA involved in cell proliferation in and miRNA-16-5p, a miRNA involved in apoptosis).

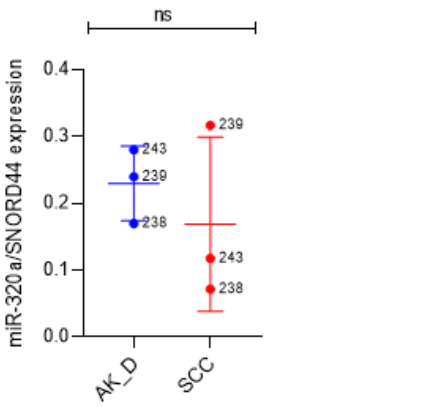
Preliminary data (n=9 subjects)



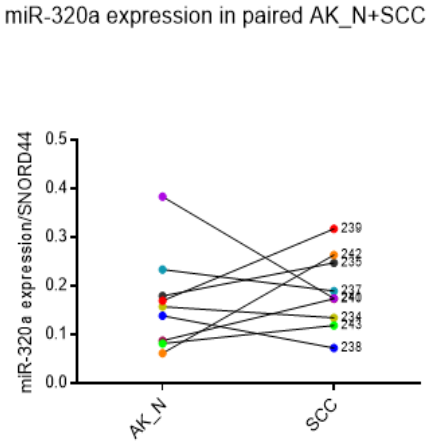


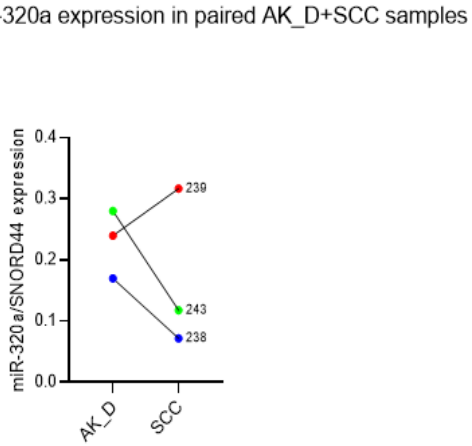
Second step data (n=39)





AK_N: Ak lesions located in the proximity of SCC; AK_D: AK lesions located distant from SCC





Conclusion: Our data suggest that there is no significant variation in the expression of the three tested microRNAs between adjacent AK lesions and squamous-cell carcinoma. However, a relevant trend has been observed Furthermore, by evaluating the miRNA expression trend between keratosis and carcinoma of the same patient, it is observed that there is no "uniform trend": for some samples the expression rises for the transition from AK to SCC and viceversa.

Introduction

Aim of the project

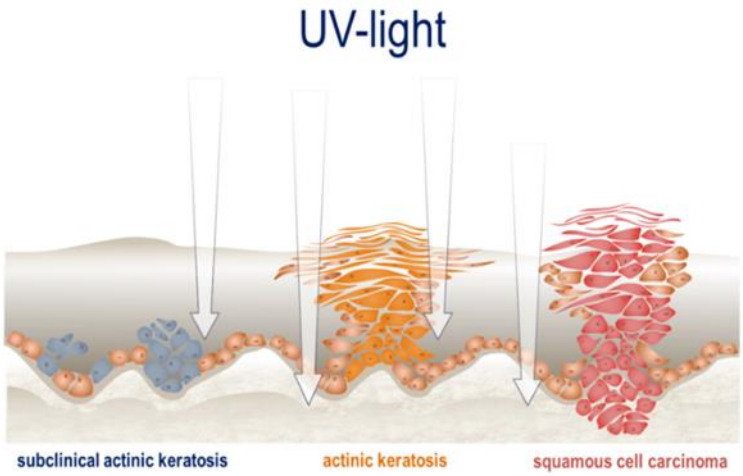
Actinic Keratosis (AK) and cutaneous squamous cell carcinoma (cSCC) have some similarities in genetic profile and histopathological features[1]. Importantly, NMSC represents one of the most common types of cancer, especially among Caucasians, with 1 million new cases per year in the United States, and cSSC also represents the second most common skin malignancy (20% of all nonmelanoma skin cancers, NMSCs), although most of statistical data seem to be underestimated[2]. All NMSCs are associated with a substantial morbidity but in contrast to basal cell carcinoma, cSCC is characterized by much higher risk of metastases[3],[4]. The vast majority of cSSC arise from AK, and the prevalence of AK was estimated at 11–25% with significant increase during chronological age[5]. All those observations indicate AK as one of the most common dermatological conditions and a very important health care concern especially for growing elderly populations. The assessment of miRNA (both circulating or at tissue level) could be an interesting strategy in order to find possible markers of evolution of AK lesions[6]. One study in particular (Mizrahi et al., 2018)[7] in which deep sequencing was performed on 55 samples (FFPE from 41 patients) deriving from: healthy tissue (n = 9), tissue with solar elastosis (n = 15), keratosis stage 1-2 actinic keratosis (n = 6), stage 3 actinic keratosis (n = 6), squamous cell carcinoma (n = 19), highlights some miRNAs that were differentially expressed between the different stages: miR-320a , miR-16-5p and miR-204-5p. The latter miR-204-5p resulted down-regulated in carcinoma compared to keratosis, as also mentioned by another study (Toll et al. 2016)[8] in which 30 OCT biopsies from 20 patients with carcinoma were

analysed by microarray, 5 patients with stage 3 keratosis and 5 controls. The data was confirmed by RT-qPCR by comparing the expression of miRNA in the 20 biopsy samples of carcinoma with respect to 5 of keratosis and in another independent cohort of 45 biopsies (of which 15 cSCC, 15 AK and 15 controls). The aim of our project was to assess at tissue level the expression of a panel of miRNA in AK lesions and SCC lesions in the surrounding area with the aim to find if some miRNA expression modification could be predictive of evolution of AK into SCC.

Background

Actinic Keratosis and Non Melanoma (NMSC) skin cancer

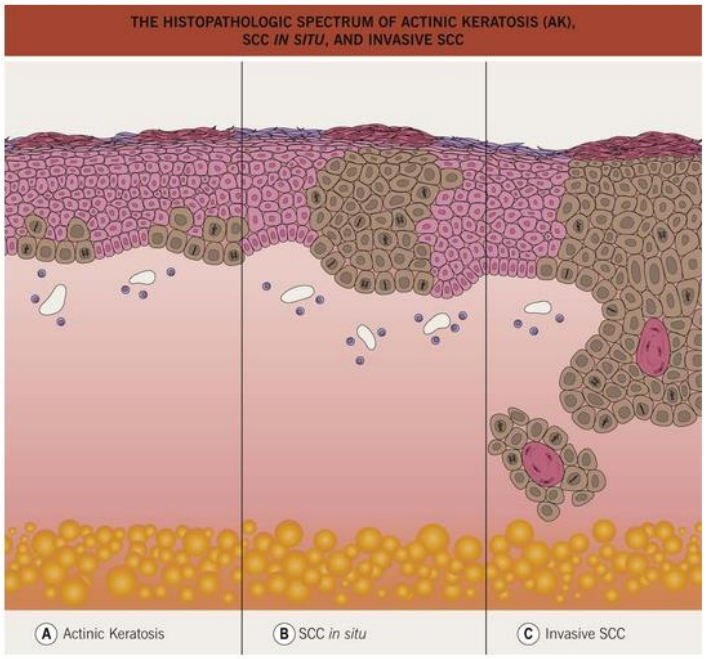
Actinic (also called solar) keratoses are keratotic lesions mainly located on chronically light-exposed adult skin[9]. They represent focal areas of abnormal keratinocyte proliferation and differentiation that carry a low risk of progression to invasive squamous cell carcinoma (SCC)[10].



A spectrum of histology is seen but the cardinal feature of an AK is epithelial dysplasia[11]. This may be restricted to the basal layer or may extend to full-

thickness atypia at which point differentiation from Bowen's disease can be difficult. There is disorderly arrangement and maturation of epithelial cells. Multiple sprouts of epithelial cells may occur at the membrane zone, but no invasion is seen. Histological variants of AK have been described, including hypertrophic, Bowenoid, lichenoid, acantholytic and pigmented[12]. AKs are commonly considered to be premalignant lesions with low individual potential for invasive malignancy and higher potential for spontaneous regression[13]. They present as discrete, sometimes confluent, patches of erythema and scaling on predominantly sun-exposed skin, usually in middle-aged and elderly individuals[14]. They are often asymptomatic but may occasionally be sore or itch[15]. Lesions may be single or multiple. The epidemiology, risk factors, disease associations and demographics of the 'at-risk' population are all pertinent to patient management. Studies indicate a high spontaneous regression rate in the order of 15–25% for AKs over a 1-year period and a low rate of malignant transformation, less than one in 1000 per annum[16]. None the less, mathematical models derived from this study predict that for an individual with an average of 7 AKs, the probability of at least one transforming within a 10-year period is approximately 10%. When 918 adults (mean age 61 years) with AKs but no previous history of skin cancer were followed prospectively for 5 years, the incidence rate for basal cell carcinoma (BCC) and SCC was estimated at 4106 and 3198 per 100 000 person/years, respectively, representing a substantial excess incidence compared with the general population[17]. These data suggest that even though the risk of malignant transformation for any given AK is very low, the probability of an

individual with AKs presenting subsequently with skin cancer is none the less high compared with the population at large.



Skin cancer is the most common cancer and comprises both non-melanoma and melanoma skin cancers, whose incidence has been increasing constantly over the past decades[18]. Skin cancers that are detected at early, non-metastatic stages are curable by surgical resection with a 5-year relative survival of 99.4% for localized melanoma and basal cell carcinoma[19]. Nevertheless, for metastatic melanoma disease, the 5-year survival rate drops to about 29.8%[20]. Therefore, there is a pressing need to identify biomarkers for the early detection of skin cancers at the earliest stages. Other biomarkers of the outmost interest for skin cancer patients include the ones able to identify patients at higher risk of recurrence or whose levels are associated with response to therapies.

MicroRNA

Cells contain a variety of noncoding RNAs, including components of the machinery of gene expression, such as tRNAs and rRNAs, and regulatory RNAs that influence the expression of other genes[21]. One class of small noncoding RNAs has recently been recognized to be quite numerous and phylogenetically extensive. MicroRNAs are short non-coding RNAs of about 22–24nt in length that regulate the expression of mRNAs by binding the complementary sequences in the 3' untranslated regions (3'UTR) of target mRNAs, thus inducing their degradation or repressing their transcription[22,23]. The cellular process of miRNA biogenesis involves both nuclear and cytoplasmic processes. MiRNAs originate from large primary (pri) and precursor (pre) transcripts[24]. All miRNAs are transcribed by RNA polymerase II into primary transcripts known as pri-miRNAs. A microprocessor complex constituted by Drosha and Microprocessor Complex Subunit DGCR8 (DGCR8) processes the resulting pri-miRNAs into a double stranded stem-loop structure called precursor miRNA (pre-miRNA)[25]. Pre-miRNAs are then transported to the cytoplasm and further cleaved into miRNA/miRNA duplexes.

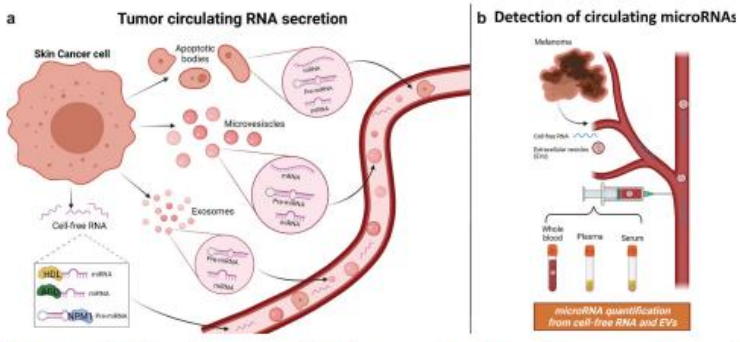


Figure 1. **RNA release from tumor cells. A)** microRNAs are released from the cancer cell into the extracellular microenvironment packaged and transported in extracellular vesicles, including exosomes, microvesicles and apoptotic bodies; or associated with RNA-binding proteins, such as Argonaute (AGO) and nucleophosmin1 (NPM1), or with lipoproteins, including high-density lipoprotein (HDL). **B)** Melanoma cells release RNAs in the extracellular microenvironment and in the bloodstream. RNA molecules are either complexed with RNA-binding proteins or lipoproteins or packaged in extracellular vesicles (EVs). microRNAs can be detected in whole blood, plasma, and serum samples. Circulating miRNA levels are different in melanoma *vs.* normal *vs.* other tumors and can be used as diagnostic biomarkers.

Finally, miRNA/miRNA duplexes separate from each other, and one strand incorporated with Argonaute (AGO) protein resulting in the formation of miRNAInduced Silencing Complex (miRISC) complex[26]. Several studies have demonstrated the release of extracellular RNAs in blood and biological fluids including urine, saliva, seminal, ascites, and cerebrospinal fluid. In addition, the expression profile of circulating miRNAs is different if considering biological fluids' origin and different pathophysiological conditions, thus indicating that extracellular miRNAs may be selectively released from cells and not only passively released from necrotic or injured cells[27],[28]. Studies suggest two major ways for miRNA release into the extracellular microenvironment: miRNAs can be packaged and transported in extracellular vescicles (EVs) or associated with RNA binding proteins or lipoproteins[29]. EVs transport in their cargo or in their surface several biomolecules including RNA, which is a key mediator of EV function in normal and cancer cells. EVs include different vesicles, such as exosomes, microvescicles (MVs), and apoptotic bodies (ABs). Recently, the International Society for Extracellular Vesicles (ISEV) has provided the guidelines 'Minimal Information for studies of EVs 2018 (MISEV2018)[30], where EVs are classified in two groups based on their size: small EVs (<200 nm) and large EVs (>200 nm). The expression profile of mRNA and miRNAs associated with EVs differs from that of their parent cells, thus suggesting their active and selective enrichment. Several studies demonstrated that most circulating miRNAs are not derived from EVs; in fact, miRNAs are released from the cells associated primarily with AGO, but also with other proteins, such as nucleophosmin1 (NPM1), or with lipoproteins, including

high-density lipoprotein (HDL). The association with these proteins stabilized miRNAs and protected them from degradation by RNAse in the extracellular environment. It was hypothesized that circulating miRNAs bound by AGO protein result from passive secretion by apoptotic or necrotic cells. Vickers and colleagues demonstrated that human HDL and low-density lipoprotein (LDL) can bound miRNAs using Scavenger Receptor Class B Member 1 (SRB1), which mediates the uptake of cholesteryl ester from HDL. Moreover, it has been demonstrated that miRNAs complexed with lipoproteins can be released and delivered to distant cells with functional implications.

miRNA in non melanoma skin cancer

As stated previously MicroRNAs (miRNAs) are 17- to 23-nucleotide (nt), short, non-coding RNA molecules that are capable of regulating gene expression at a post-transcriptional level[31]. Encoded within both exons and introns, they play a pivotal role in a variety of physiologic cellular functions and diseases, including cancer. Approximately 30%–60% of all human genes are affected by miRNA regulation, and our understanding of their role as both tumor suppressors and oncogenes in a variety of different cancers is gradually evolving[32]. miRNA maturation begins in the nucleus where RNA polymerase II transcribes the primary-miRNA (pri-miRNA) transcript. Drosha, an intranuclear RNase III endonuclease, and its co-factor, DiGeorge syndrome critical region gene 8 (DGCR8 or Pasha (Partner of Drosha), form the microprocessor complex that cleaves the pri-miRNA transcript into several 70–90-nt precursor-miRNAs (pre-miRNAs) that share a characteristic stem loop structure[33].

miRNA in Basal Cell Carcinoma

Basal cell carcinoma (BCC) is the most common form of human cancer. Although it rarely metastasizes (1:50,000), it has a huge socioeconomic impact on healthcare systems worldwide because of its high incidence. The potential role of miRNA dysregulation in BCC development has recently started to develop as a new path to enlighten the pathology of BCC[34]. At least 37 miRNA dysregulations have been described in the literature (Dika et al)

Table 1. MiRNAs dysregulations reported in basal cell carcinoma.

miRNA (Published Name)	miRNA (Current Name)	Expression in BCC	Ref.
miR-203	miR-203a-3p	Downregulated	[49]
miR-17	miR-17-5p	Upregulated	[50]
miR-18a	miR-18a-5p	Upregulated	[50]
miR-18b	miR-18b-5p	Upregulated	[50]
miR-19b	miR-19b-3p	Upregulated	[50]
miR-19b-1*	miR-19b-1-5p	Upregulated	[50]
miR-93	miR-93-5p	Upregulated	[50]
miR-106b	miR-106b-5p	Upregulated	[50]
miR-125a-5p	miR-125a-5p	Upregulated	[50]
miR-130a	miR-130a-3p	Upregulated	[50]
miR-181c	miR-181c-5p	Upregulated	[50]
miR-181c*	miR-181c-3p	Upregulated	[50]
miR-181d	miR-181d-5p	Upregulated	[50]
miR-182	miR-182-5p	Upregulated	[50]
miR-455-3p	miR-455-3p	Upregulated	[50]
miR-455-5p	miR-455-5p	Upregulated	[50]
miR-542-5p	miR-542-5p	Upregulated	[50]
miR-29c	miR-29c-3p	Downregulated	[50]
miR-29c *	miR-29c-5p	Downregulated	[50]
miR-139-5p	miR-139-5p	Downregulated	[50]
miR-140-3p	miR-140-3p	Downregulated	[50]
miR-145	miR-145-5p	Downregulated	[50]
miR-378	miR-378a-5p	Downregulated	[50]
miR-572	miR-572	Downregulated	[50]
miR-638	miR-638	Downregulated	[50]
miR-2861	miR-2861	Downregulated	[50]
miR-3196	miR-3196	Downregulated	[50]
miR-21	miR-21-5p	Upregulated in sclerosing BBC	[52]
miR-99a	miR-99a-5p	Upregulated in sclerosing BBC	[52]
miR-26a-2	miR-26a-2-3p	Upregulated in sclerosing BBC	[52]
miR-let-7f	let-7f-5p	Upregulated in sclerosing BBC	[52]
miR-let-7g	let-7g-5p	Upregulated in sclerosing BBC	[52]
miR-let-7i	let-7i-5p	Upregulated in sclerosing BBC	[52]
miR-100	miR-100-5p	Upregulated in sclerosing BBC	[52]
miR-205	miR-205-5p	Upregulated in sclerosing BBC	[52]
miR-451a	miR-451a	Downregulated	[53]
miR-34a	miR-34a-5p	Downregulated (serum)	[54]

The miRNA machinery components including the microprocessor complex consisting of Drosha, DGCR8, Dicer, and the RISC components argonaute-1, argonaute-2, PACT, TARBP1 and TARBP2 have recently been investigated regarding their expression in both BCC and cSCC. The Drosha, DGCR8, AGO1, AGO2, PACT, and TARBP1 expression levels have been shown to be significantly higher in BCC and cutaneous squamous cell carcinoma (cSCC)

when compared to healthy controls[35]. While this initial screening of the miRNA machinery in BCC was the first study that searched for the possible role of miRNA involvement in BCC pathogenesis, the focus has now shifted toward more specific miRNA profiling studies. A microarray-based miRNA profiling studies of BCC has recently identified sixteen significantly up-regulated (hsa-miR-17, miR-18a, hsa-miR18b, hsa-miR-19b, hsa-miR-19b-1*, hsa-miR-93, hsamiR-106b, hsa-miR-125a-5p, hsa-miR-130a, hsa-miR181c, hsa-miR-181c*, hsa-miR-181d, hsa-miR-182, hsa-miR-455-3p, hsa-miR-455-5p and hsa-miR-542- 5p) and ten significantly down-regulated (hsa-miR-29c, hsa-miR-29c*, hsa-miR-139-5p, hsa-miR-140-3p, hsamiR-145, hsa-miR-378, hsa-miR-572, hsa-miR-638, hsa-miR-2861 and hsa-miR-3196) miRNAs in BCC compared with non-lesional skin. Data mining revealed connections to tumor-promoting pathways, such as the hedgehog and the MAPK/ERK signalling cascades which have previously been connected to BCC.

miRNA in Squamous Cell Carcinoma

cSCC is an epithelial skin tumor that is the second most common form of human cancer[36]. Depending on the depth of the lesions, it can result in metastasis with fatal consequences accounting for 20% of all skin cancer-related deaths. Some cSCCs become locally invasive and show an aggressive course. The rate of metastasis has been shown to be 0.3–3.7% with an overall 5-year survival rate < 30% in cases in which it spreads systemically[37]. The role of miRNAs in SCC of different origins has been investigated in cervical, lung, esophageal, oral, pharyngeal, and tongue tissue[38],[39]. Investigations of the involvement of miRNA dysregulation in cSCC have begun and published data on this topic is increasing[40],[41]. Similar to BCC, the expression levels of the

miRNA machinery, namely Drosha, DGCR8, AGO1, AGO2, PACT, and TARBP1, were significantly higher compared to healthy controls and Dicer levels were significantly higher compared to intra-individual controls. Furthermore, Dziunycz et al[42]. have investigated a distinct set of miRNAs (hsamiR-21, hsa-miR-203, hsa-miR-205, and hsa-miR184) in cSCC modulated by UV radiation. They described the significantly increased expression of hsamiR-21 and hsa-miR-184 and the decreased expression of hsa-miR-203 in cSCC. Interestingly, they found that UVA increased the expression of hsa-miR-21, hsamiR-203, and hsa-miR-205, whereas UVB increased hsa-miR-203 and decreased hsa-miR-205. Yamane et al[43]. showed that hsa-miR-214 is the regulator of extracellular-signal-regulated kinase 1 (ERK1 or – mitogen-activated protein kinase 3 MAPK3) and hsa-miR-124 and hsa-miR-214 are both regulators of ERK2 (mitogen-activated protein kinase 1 or MAPK1). Both, hsa-miR-124 and hsa-miR-214, were shown to be significantly down-regulated in SCC in vitro and in vivo. Other scientific data show that at least 20 miRNAs (such asvhsa-miR-1, hsa-miR-124, and hsa-miR-125b) were found to be associated with cSCC[44].

miRNA in Actinic Keratosis

Dańczak-Pazdrowska in 2022[45], working on circulating miRNA, has showed that in AK subjects 2 regulated miRNAs for AK cohort and 12 miRNAs for cSCC patients were identified, while there were 26 miRNAs differentially regulated between cSCC and AK patients. In the same study, there was also one commonly regulated miRNA between AK and cSCC patients and ten miRNAs that were regulated in cSCC when compared with both control and AK patients. The authors did not observe any differences between the AK

groups. In conclusion, this study analysis detected in circulation some miRNA that were previously recognized as important in AK, cSCC, and other type of skin cancer supporting this approach as potential non-invasive diagnosis of AK and cSCC. As described by Sand et al (2012)[46] several miRNAs could be involved in the pathogenesis of AK and NMSC. In particular some miRNAs seem to be over-expressed and other down-regulated:

Listing selected microRNAs (miRNAs) and their molecular impact relevant to non-melanoma skin cancer (n.a. = not available)

miRNA	Regulation	Tumor	Molecular impact	Author miRNA	Author molecular impact
let-7	+	BCC	involved in regulating cell proliferation	Heffelfinger et al. [12]	Heffelfinger et al. [12]
hsa-miR-17	+	BCC	pro-growth miRNA regulated in vitro by MAPK/ERK-induced phosphorylation of TRBP	Sand et al. [13]	Parroo et al. [29]
hsa-miR-18a	+	BCC	member of the hsa-miR-17-92 cluster, also known as Oncomir-1; responsible for enhanced cell proliferation and the suppression of apoptosis	Sand et al. [13]	He et al. [30] Al-Nakhle et al. [31]
hsa-miR-18b	+	BCC	same seed sequence as hsa-miR-18a	Sand et al. [13]	
hsa-miR-19b	+	BCC	member of the hsa-miR-17-92 cluster, also known as Oncomir-1; responsible for enhanced cell proliferation and the suppression of apoptosis	Sand et al. [13]	He et al. [30] Al-Nakhle et al. [31]
hsa-miR-19b-1*	+	BCC	member of the hsa-miR-17-92 cluster, also known as Oncomir-1; responsible for enhanced cell proliferation and the suppression of apoptosis	Sand et al. [13]	He et al. [30] Al-Nakhle et al. [31]
hsa-miR-21	+ +	BCC SCC	UVA radiation results in increased expression; oncogene that represses a variety of tumor suppressors such as PTEN and PCDC4	Heffelfinger et al. [12] Dziunycz et al. [23]	Heffelfinger et al. [12]
hsa-miR-29c	−	BCC	associated with hypermethylation of tumor-related genes and disease outcome in cutaneous melanoma; downregulates DNA methyltransferases DNMT3A and DNMT3B	Sand et al. [13]	Nguyen et al. [32]
hsa-miR-29c*	−	BCC	n.a.	Sand et al. [13]	
hsa-miR-93	+	BCC	part of the hsa-miR-106b-25 cluster; transcription factor *E2F1* is a target gene of hsa-miR-93	Sand et al. [13]	Li et al. [33]
hsa-miR-106b	+	BCC	part of the hsa-miR-106b-25; transcription factor *E2F1* is a target gene of hsa-miR-106b	Sand et al. [13]	Li et al. [33]
hsa-miR-124	−	SCC	correlates inversely with tumor progression, regulates ERK2 together with hsa-miR-214	Yamane et al. [24]	Yamane et al. [24]
hsa-miR-125a-5p	+	BCC	induces apoptosis via a p53-dependent pathway (shown in human lung cancer cells)	Sand et al. [13]	Jiang et al. [34]
hsa-miR-130a	+	BCC	predicted regulatory effect on the apoptosis regulator *BCL-2*	Sand et al. [13]	Sand et al. [13]
hsa-miR-139-5p	−	BCC	n.a.	Sand et al. [13]	
hsa-miR-140-3p	−	BCC	n.a.	Sand et al. [13]	
hsa-miR-143	+	BCC	n.a.	Heffelfinger et al. [12]	
hsa-miR-145	−	BCC	targets epidermal growth factor receptor (EGFR) and nucleoside diphosphate linked moiety X-type motif 1 (NUDT1) (shown in lung adenocarcinoma)	Sand et al. [13]	Cho et al. [35]
hsa-miR-148a	+	BCC	n.a.	Heffelfinger et al. [12]	
hsa-miR-181c	+	BCC	targets *NOTCH4* and *KRAS* which have both been implicated in the pathogenesis of BCC	Sand et al. [13]	Proweller at al. [36] van der Schroeff et al. [37]
hsa-miR-181c*	+	BCC	n.a.	Sand et al. [13]	
hsa-miR-181d	+	BCC	n.a.	Sand et al. [13]	
hsa-miR-182	+	BCC	described to negatively regulate human Forkhead-box O1 (*FOXO1*) and linked to oncogenic transformation	Heffelfinger et al. [12] Sand et al. [13]	Guttilla et al. [38]
hsa-miR-184	+	SCC		Dziunycz et al. [23]	
hsa-miR-214	−	SCC	correlates inversely with tumor progression, regulates ERK1	Yamane et al. [24]	Yamane et al. [24]
hsa-miR-378	+	BCC	n.a.	Heffelfinger et al. [12]	

Activities of the Project

First Years

In the first year we organized an outpatient clinic for actinic keratoses and squamous cell carcinomas at the U.O. of Dermatology of S. Orsola, for patients treated at the skin cancer center. These patients will be appropriately selected by the multidisciplinary group that involves colleagues from the Oncology of the S. Orsola Polyclinic, colleagues from Radiotherapy, Plastic Surgery from the S. Orsola Polyclinic. It will be necessary to establish a database for the collection of personal and clinical data of the selected patients), which will be periodically repeated at the end of the different treatments, according to the following table:

Activities Planning Table

Activities	Identification of Cases (6 months)	6 months	12 months	24 months after PDT	FU every years
Immunohistochemical evaluation of actinic keratoses at baseline time	X	X	X		
Evaluation of miRNA panel expression levels in actinic keratoses	X	X	X	X	X
Evaluation of miRNA panel expression levels in squamous cell carcinomas		X	X	X	X
Evaluation of miRNA pannel expression after topical treatment			X		
Comparisons of immuno histochemistry and miRNA expression data between the two groups of actinic keratosis and squamous cell carcinoma preparations and between treated and untreated patients			X	X	X
Preparation of an analysis panel that can be used in the evaluation and differentiation of tumors with characteristics of biological aggressiveness					X

Second Year

According to the plannning Table, in the second year we performed the following activities:

- Evaluation of miRNA panel expression levels in actinic keratoses
- Evaluation of miRNA panel expression levels in squamous cell carcinomas

Third Year

According to the planning Table, in the third year we performed the following activities:

- Evaluation of miRNA panel expression levels in actinic keratoses
- Evaluation of miRNA panel expression levels in squamous cell carcinomas
- Comparisons of immune histochemistry and miRNA expression data between the two groups of actinic keratosis and squamous cell carcinoma preparations and between treated and untreated patients*

*Due to logistic problems related with COVID pandemic these last activities have been delayed. Final data will be available end 2022

In the present project we utilised the **Droplet Digital Polymerase Chain Reaction** (DDPCR).

DDPCR is a bio technological refinement of conventional polymerase chain reaction methods that can be used to directly quantify and clonally amplify nucleic acids strands including DNA, cDNA, or RNA. The key difference between dPCR and traditional PCR lies in the method of measuring nucleic acids amounts, with the former being a more precise method than PCR, though also more prone to error in the hands of inexperienced users. A "digital" measurement quantitatively and discretely measures a certain variable,

whereas an "analog" measurement extrapolates certain measurements based on measured patterns. PCR carries out one reaction per single sample. dPCR also carries out a single reaction within a sample, however the sample is separated into many partitions and the reaction is carried out in each partition individually. This separation allows a more reliable collection and sensitive measurement of nucleic acid amounts. The method has been demonstrated as useful for studying variations in gene sequences — such as copy number variants and point mutations — and it is routinely used for clonal amplification of samples for next-generation sequencing.

Results

The Workflow activity of the present project was the following: First step: Identification 18 Formalin-fixed paraffin embedded (FFPE) samples (9 patients) («matched» 9 AK lesions and 9 SCC lesions). Working on biopsies samples we perform an extraction and RNA analysis with droplet Digital PCR (ddPCR) and we perform the data analysis. Results: We perform an evaluation and comparison of the following miRNA: miR-320 (a miRNA involved in cell proliferation; miR-204, a miRNA involved in apoptosis regulation, and miRNA-16-5p, a miRNA involved in in the control of cellular replication and apoptosis. The figures show the main results:

Figure 1: Comparison of miR-320, miR204 and miR-16-5P expression in AK FFEP samples of AK and SCC lesions

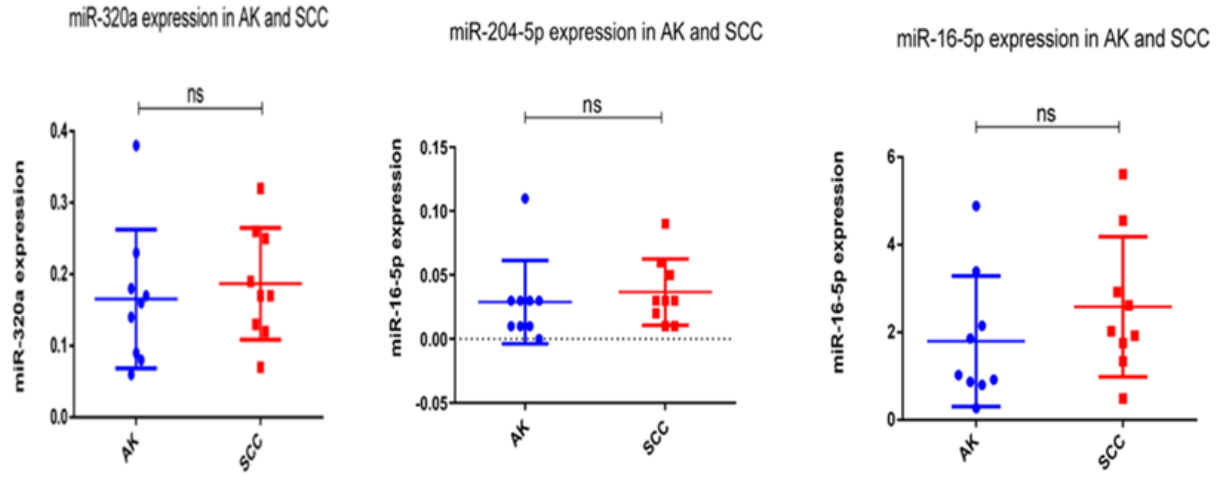
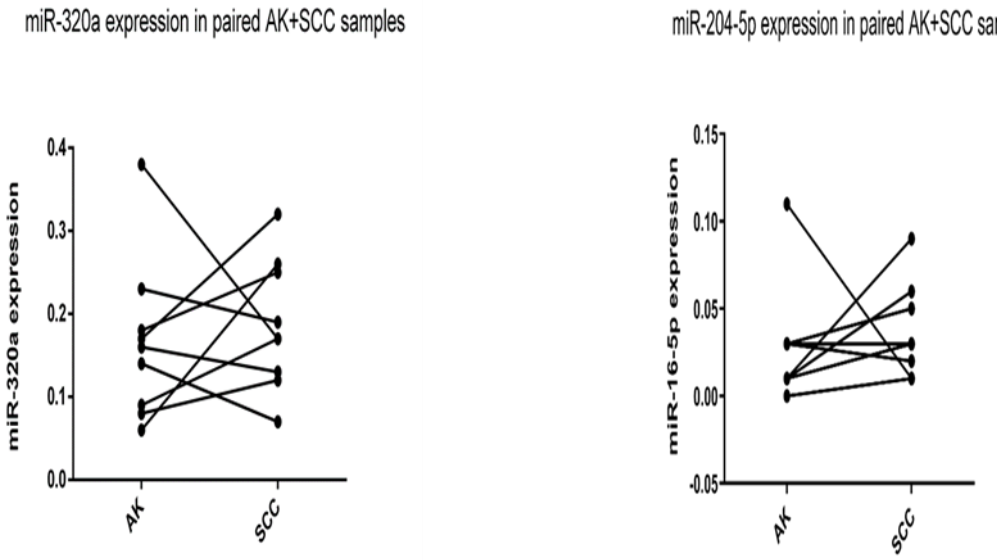
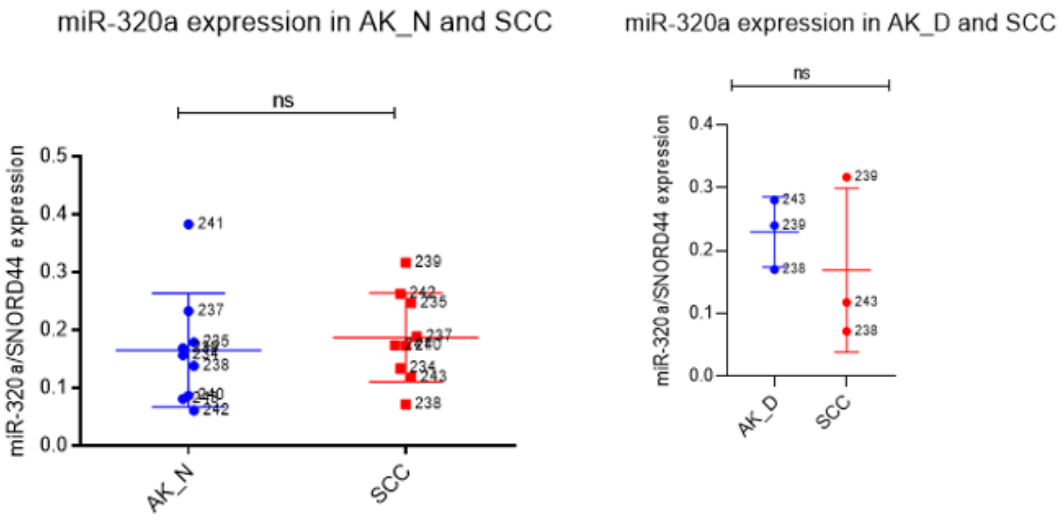


Figure 2: Comparison of miR320 and miR204 expressions with paired comparison

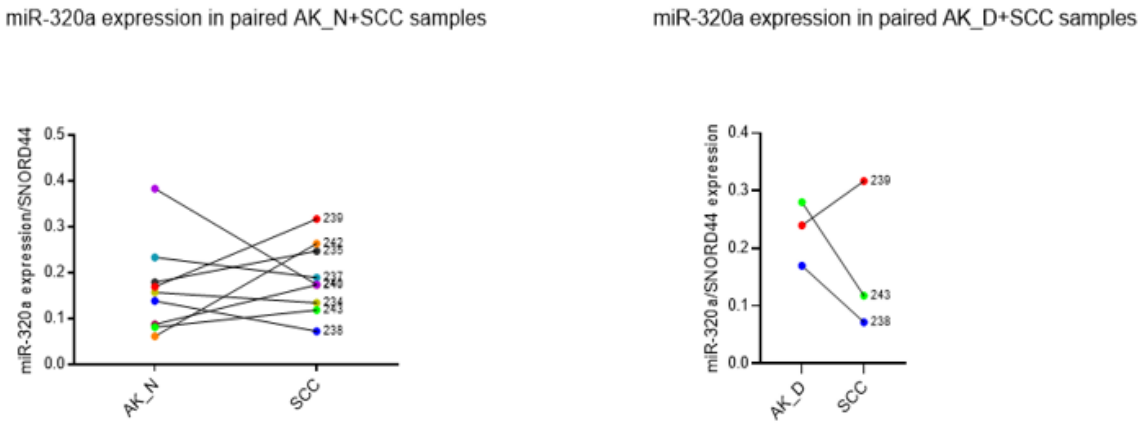


These data were confirmed in the final data set of 39 subjects as show in these figures:

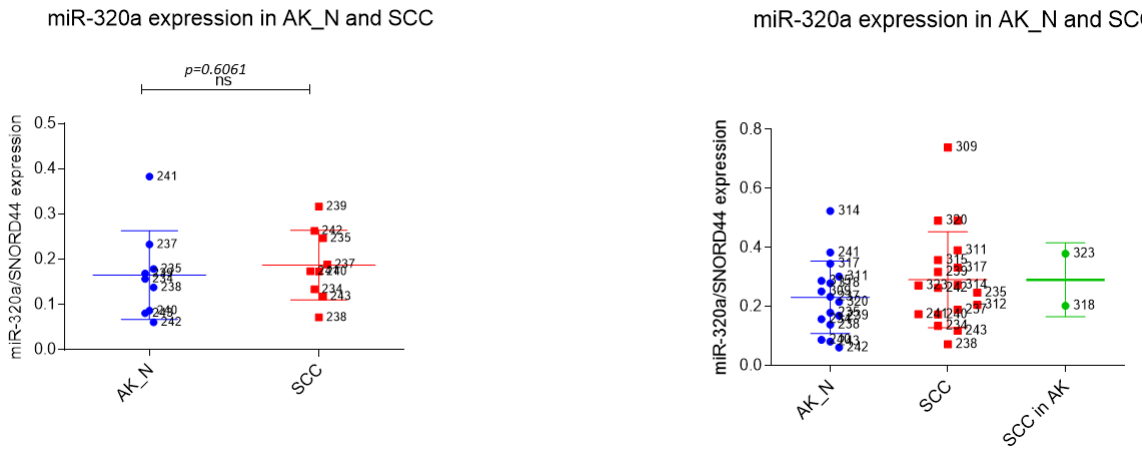
Figure 3 and Figure 4



AK_N: Ak lesions located in the proximity of SCC; AK_D: AK lesions located distant from SCC

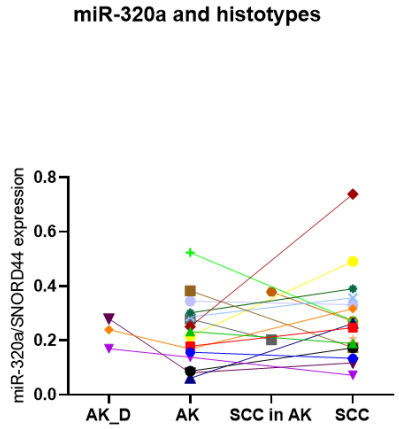


For the mRNA we did not found different levels of expression when AK samples, SCC sample distant from AK lesions and SCC samples located near AK were compared. **(Figure 5**)



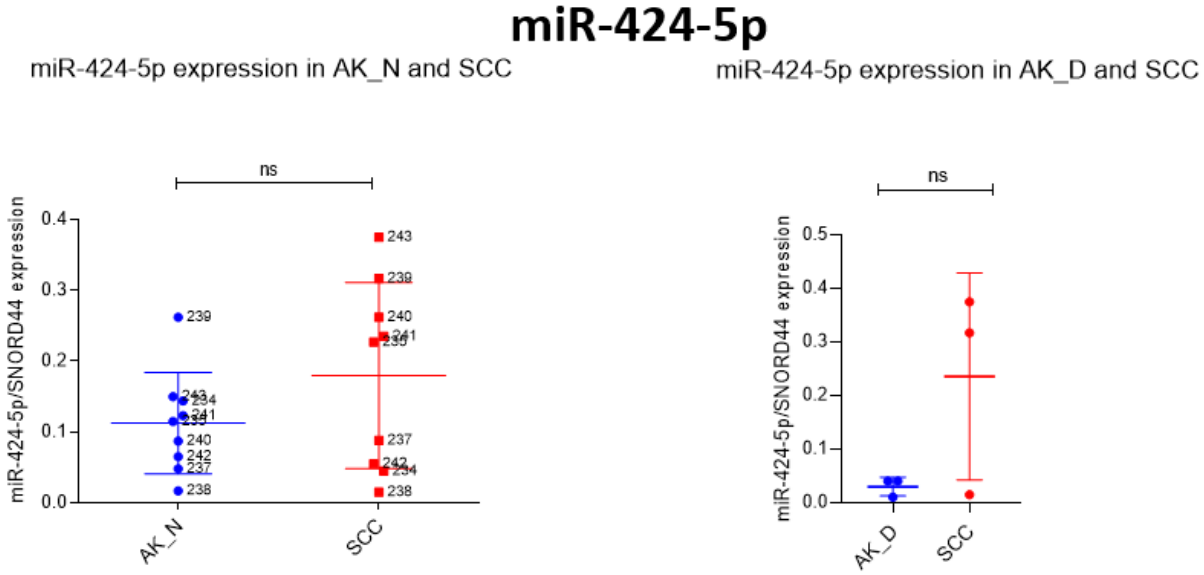
This figure clearly shows that miRNA 320 expressions in AK distant or near SCC lesions, SCC lesions and SCC lesions near AK are not different:

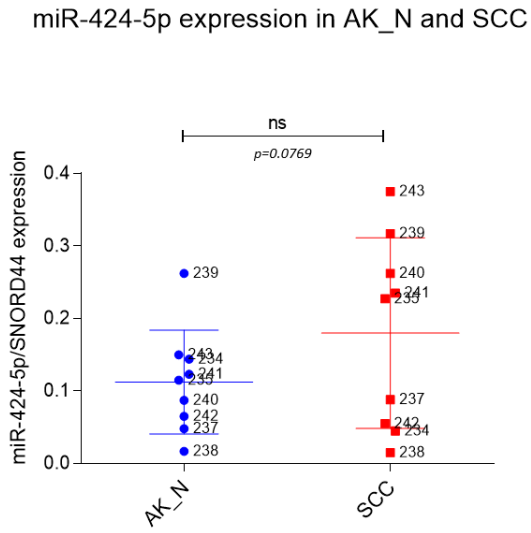
Figure 6

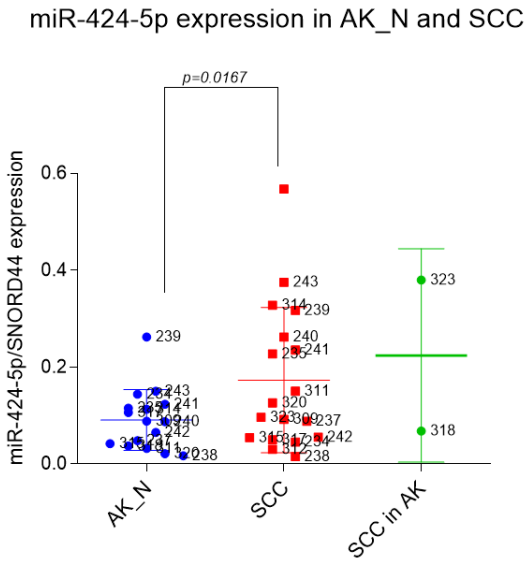


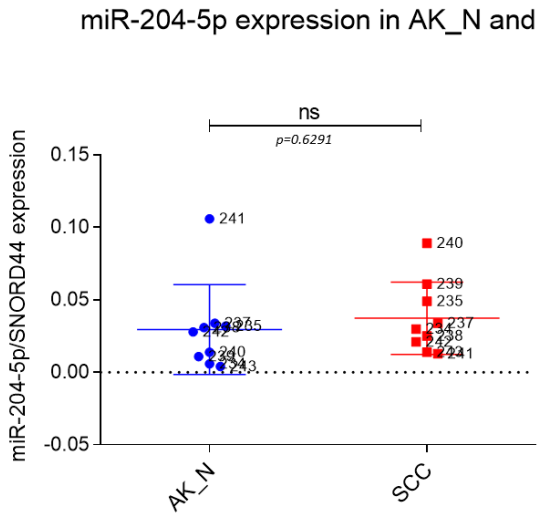
The same data were observed when miR-16-5p, miR-424-5p, miR-204-5p were evaluated.

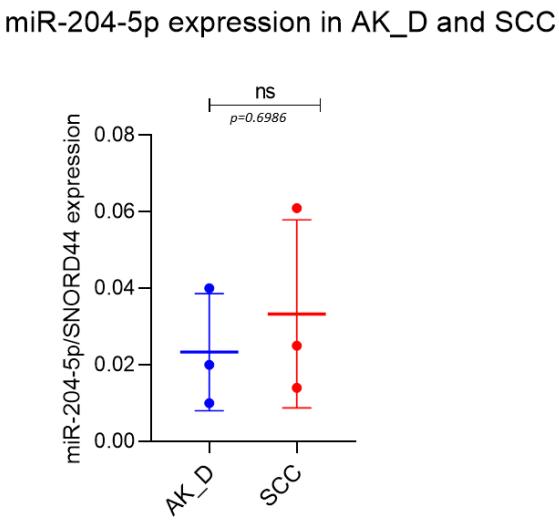
Figure 7











Conclusion:

Our data suggest that there is no significant variation in the expression of the three tested microRNAs between adjacent AK lesions and squamous-cell carcinoma. However, a relevant trend has been observed Furthermore, by evaluating the miRNA expression trend between keratosis and carcinoma of the same patient, it is observed that there is no "uniform trend": for some samples the expression rises for the transition from AK to SCC and viceversa.

Appendix

Appendix 1: Learning formation followed by Massimo Milani during the Doctorate course

Attività formative (Insegnamenti/Seminari) (Presso l'ateneo di Bologna) 2019-2022

Carcinoma a cellule basali :novità in tema di terapia	Dika	29 novembre 2019 15:00 – 18:00	Aemilia Hotel
Ruolo dei miRNA nella progressione tumorale del melanoma	Dika Ferracin	Da definre gennaio 2019	Pol.S.Orsola Pad 29 - Aula Giada Dermatologia
Immunobiologia dei tumori			DIMES-Sede di Cancerologia
Microbiota e neoplasie	Dika	Gennaio 2019	Osp S. Orsola- Pad 29 – auletta seragnoli
Istopatologia e biologia molecolare dei tumori I	Dika	Gennaio 2019	Pol.S.Orsola Pad 29 - Aula Giada Dermatologia
Istopatologia e biologia molecolare dei tumori II	Dika	Gennaio 2019	
Epidemiologia e prevenzione dei tumori	Dika	Aprile 2019	DIMES-Sede di Cancerologia
non-coding RNAs as cancer biomarkers and therapeutic targets	Ferracin	18/06/2020 14.30-16.30	DIMES-Sede di Patologia Generale

Appendix 2: Publications List done during the Doctorate Years (2019-2022): Total 9 articles.

- **Propylene glycol free 5% minoxidil lotion formulation: cosmetic acceptability, local tolerability, clinical efficacy and in-vitro skin absorption evaluations**. Mauro BARBARE SCHI 1, Veronica VESCOVI 2, Michela STARAtaraCE 3, Bianca M. PIRACCINI 3, Massimo MILANI 4. Giornale Italiano di Dermatologia e Venereologia 2020 June;155(3):341-5
- **Efficacy of lidocaine 7 %, tetracaine 7 % self-occlusive cream in reducing MAL-cPDT-associated pain in subjects with actinic keratosis: A randomized, single-blind, vehicle-controlled trial (The "3P-Trial")**. Marta Benedetta Brumanaa, Massimo Milanib,*, Mario Puviani. Photodiagnosis and Photodynamic Therapy 30 (2020) 101758.
- **Efficacy of Single Day-Light Photodynamic Therapy Session with Aminolevulinic Acid 5% Thermosetting Gel with a Penetration-Empowering Facial Mask in the Treatment of Severe Actinic Damage of the Face and Scalp**. Mario Puviani1*, Massimo Milani2. J Clin Exp Dermatol Res, Vol.10 Iss.4 No:1000502.
- **Generalized verrucosis: A therapeutic challenge: Efficacy of topical sinecatechins (Veregen) 10%.**.Francesca Satolli MD1 Marco Gandolfi MD1 Miriam Rovesti MD1Alfredo Zucchi MD1 Massimo Milani MD2 Claudio Feliciani MD, PhD1. Dermatologic Therapy 2019. DOI: 10.1111/dth.12986
- **Treatment of brittle nail with a hydroxypropyl chitosan-based lacquer, alone or in combination with oral biotin: A randomized, assessor-blinded trial.** Agatina Chiavetta1 | Sabrina Mazzurco1 | Maria P. Secolo1 | Gisella Tomarchio1 | Massimo Milani2. Dermatologic Therapy2019;32:e13028. https://doi.org/10.1111/dth.13028
- **A Serum Containing Deschampsia antartica Extract, Ferulic Acid and Vitamin C has Anti-Pollutant Effects on Skin Exposed to High Tropospheric Ozone Levels: A Controlled Single-Blind, Prospective Clinical Trial in Women Living in Urbanized, High Air Pollution Area during the Summer Season**. Massimo Milani1*, Marco Piacentini2, Leonardo Celleno2. J Clin Exp Dermatol Res, Vol.10 Iss.6 No:1000510.
- **Efficacy and Tolerability of a Tretinoin 0.02%, Clindamycin 0.8% and Glycolic Acid 4% Gel in Acne: A Multicenter, Prospective, Pragmatic, Assessor-Blinded, 12-Week**

Trial on 159 Subjects. Massimo Milani*, Chiara Cortelazzi (Parma)**, Paolo Chieco** (Bari), Anna Ferrazzi** (Feltre), Nicola Gargano** (Cosenza), Elisa Maiani** (Rome), Amanda Mazzi** (Livorno), Maria Grazia Mannino** (Carini), Daniela Marciani** (Rome), Sergio Pastena** (Nola), Silvia Pugliarello** (Verona), Valentina Salamone** (Trieste), Teresa Sisto** (Rome), Roberta Scarselli** (Florence), Carmen Solaroli** (Turin), Gustavo Spanò** (Naples). J Clin Exp Dermatol Res, Vol.10 Iss.4 No:1000503.

- **Effects of a cream containing 5% hyaluronic acid mixed with a bacterial-wall-derived glycoprotein, glycyrretinic acid, piroctone olamine and climbazole on signs, symptoms and skin bacterial microbiota in subjects with seborrheic dermatitis of the face.** Mario Puviani1. Elena Campione2 Anna Maria Offidani3 Roberta De Grandi4 Luca Bianchi2 Ivan Bobyr3 Melania Giannoni3 Anna Campanati3 Marta Bottagisio4 Alessandro Bidossi4 Elena De Vecchi4 Klaus Eisendle5 Massimo Milani5,6. Cosmetic and Investigational Dermatology 2019:12 285–293.
- Durante, G., Broseghini, E., Comito, F., Naddeo, M., Milani, M., Salamon, I., & Ferracin, M. (2022). **Circulating microRNA biomarkers in melanoma and non-melanoma skin cancer.** Expert Review of Molecular Diagnostics, 22(3), 305-318.

Appendix3: Participation at scientific congresses a.a. 2019/2022

- Congress EADV 2019
- Congress EADV 2020
- Congress EADV 2021
- Congress EADV 2022
- Congress SIME 2019 (Oral presentation)
- Congresso AGORA 2019 (Oral Presentation)
- Congress SIDEMAST 2019
- XIX RIUNIONE (1983-2020) GIORNATE DI TERAPIA IN DERMOVENEREOLOGIA. Presidente: Prof. Giuseppe Micali. (Oral presentation)

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