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Dottorando: **Orazio Fortunato**

The diabetic foot: relevance of endothelial progenitor cells as a prognostic marker of mortality and disease progression.

Direttore della Scuola: Ch.mo Prof. Mario Clerici

Tutore: Prof. Cristina Barlassina

Correlatore: Prof. Gianluigi Condorelli

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ABSTRACT

The World Health Organization estimated that the incidence of the diabetes is projected to rise from 120 million in 1996 to 366 million in 2030. Diabetes mellitus refers to a metabolic disorder of multiple etiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The effects of diabetes mellitus include long–term damage, dysfunction and failure of various organs. Nowadays, the main issues for diabetic patients are no longer those associated with survival but those associated with the chronic complications of the diabetes. In this context, diabetic foot ulcers represent a serious medical and socio-economic issue worldwide. To date, the best predictors of clinical outcomes are the presence or the absence of ischemia, infection, footwear, pressure relief, and overall glycaemic control. None of them, however, reflects the activity of endogenous repair mechanisms. Endothelial progenitor cell (EPCs) availability and functionality are relevant to vascular repair. It was demonstrated in patients with diabetes, the availability of EPCs is reduced. Different mechanisms may potentially be involved, including impaired mobilization, proliferation, and apoptosis..

Based on this consideration, the goal of my thesis is to determine the additive value of circulating EPCs in predicting major end-points such as mortality, amputation and post-angioplasty restenosis in a cohort of 120 type 2 diabetic patients with Critical Limb Ischemia (CLI). Moreover, we aimed to obtain mechanistic insights into diabetic progenitor cells impairment and to provide the first characterization of the EPC-associated miRNA with special respect to those potentially involved in the control of angiogenesis.

To perform functional and molecular analysis normal criteria were not available so we decided to include in the study two different control groups: a groups of not diabetic CLI subjects and a group of healthy volunteer.

To assess the first objective of this project we have to wait the end of 12 months follow-up (FU) so we reported an ad interim analysis of the data. To date, about 60% of patients have reached one year FU visit and we have registered 37 restenosis, 10 major amputations and 7 death events. We did not observe any statistical difference in the number of antigenically defined EPCs between complicated (with at least one event) and not complicated (no event) diabetic patients. Also the migration capacity analysis did not show difference between the two groups. Interestingly, our preliminary data showed a significant increase in the basal migration of EPC in patients with event compared with patients with no event, so we propose that the unspecific motility associated with altered directed migration may be indicative of the incapacity of the cells to find their way to injured tissues thereby compromising the healing process. This preliminary finding need to be confirmed after the end of the study.

Second, to assess the other objective of the study we analyzed the diabetic EPC characteristics and functionality. We first measured by flow cytometry the percentage of antigenically-selected MNCs subpopulations with pro-angiogenic potential in all of the three groups of enrolled subject. We observed a decrease in $CD34^{pos}$ and EPCs ($CD45^{dim}$,$CD34^{pos}$,$CXCR4^{pos}$,KDR^{pos}) in ischemic patients as compared to healthy volunteer. Unexpectedly however, diabetic patients showed a higher percentage of progenitor cells compared with non diabetic controls.

Functionally, migration assay showed an impairment of cell motility in chronic limb ischemic patients with or without diabetes compared to healthy subjects. Migration assay results showed an enrichment of EPCs in the controls only; these data are in line with those previously published on an altered migratory function of EPCs from diabetic patients. Mechanisms underlying the reduction of EPCs in diabetes are largely unknown. Weak bone marrow mobilization, impaired peripheral differentiation, and short survival in peripheral blood are all candidates and we therefore attempted to investigate the mechanisms involved in this impairment.

Recently, miRNAs have been shown to regulate EC functions, including proliferation, migration and assembling in branched networks. To this aim, we performed a preliminary screening on EPCs from the patients enrolled and demonstrated that diabetes induces specific microRNAs de-regulation. Next, we selected two miRNAs, mir-15a and mir-16 based on their already known role in controlling migratory and apoptotic mechanisms in cancer. To investigate the role of mir-15a and mir-16, we transfected these miRNAs into healthy EPCs. We then analyzed cell functionality in migration and angiogenic assays and we observed that only co-expression of mir-15a/16 blocks EPCs migration and induce apoptosis. Next, we investigated if down-regulation of mir-15a/16 could restore diabetic EPC migratory ability. We observed that inhibition of mir-15a/16 recover FBS and SDF-1 induced migration in diabetic EPCs but no effect was observed in apoptotic assay.

Bionformatic analyses predict BCL2, VEGFA and AKT3 to be target genes of miRNA-15a and -16, which support an anti-angiogenic and pro-apoptotic role of these miRNAs. In diabetic EPCs and mir over-expressing EPCs, we found a down-regulation of AKT3, a protein involved in many EPCs functions like cell cycle progression, migration and survival.

This study demonstrates, for the first time, that the spontaneous migration ability of a well antigenically characterized MNCs subpopulation of cells with EPC phenotype is significantly increased in diabetic patients that manifest at least one major adverse event (restenosis, amputation, death). This is the first evidence generated in a clinical trial that EPC migration could be used as prognostic marker for diabetic vascular complications. At the end of the study, the analysis of the follow up data generated on the entire patient cohort will enable us to generate crucial data on the a new EPC-associated risk for diabetic CLI patients to develop life-threatening vascular complications.

Second, in this work we show for the first time that mir-15a and mir-16 are key regulator of diabetic EPCs pro-angiogenic function. In the future we want to deepen the role of microRNA in diabetic dysfunction because we know that miRNAs have tremendous therapeutic potential for the treatment of vascular diseases associated with aberrant pathological angiogenesis.

In conclusion, EPCs have recently generated considerable attention as potential novel prognostic biomarkers for vascular integrity, and therapeutic clinical approaches using these cells are ongoing. Although the role of EPCs in these processes is well established, the challenge for the next decade is to identify and evaluate methods that increase EPC homing and incorporation, thereby enabling targeted delivery of EPCs to a site of interest. This goal might be achieved through the continued characterization of EPCs in animals and humans, coupled with investigations of the long-term potential of EPCs *in vivo.*

RIASSUNTO

L’Organizzazione Mondiale della Sanità, ha stimato che nel 2030 il numero dei diabetici supererà i 300 milioni, e se consideriamo che nel 1996 la stima era stata di circa 120 milioni, possiamo immaginare quale importanza assuma questo problema nei paesi occidentali. Il diabete mellito (DM) comprende un gruppo di disordini metabolici a diversa eziologia che influenza il metabolismo glucidico, lipidico e proteico ed è caratterizzato da iperglicemia cronica dovuta ad un difetto della secrezione di insulina o dell’azione dell’insulina. Ormai da molti anni, i progressi nella cura della malattia diabetica hanno portato ad un allungamento dell’aspettativa di vita dei pazienti affetti da tale patologia che sostanzialmente non differisce dalla popolazione non diabetica. Oggi i problemi principali per i diabetici sono legati alle complicanze croniche, sia micro-angiopatiche, cioè dei piccoli vasi arteriosi, sia macro-angiopatiche, cioè dei grossi vasi arteriosi, derivanti da tale malattia. La predizione dello sviluppo della patologia si basa su dati clinici quali la presenza o assenza di ischemia, la presenza o assenza di infezione, il tipo di calzatura e il rilascio della pressione, e il controllo glicemico. Anche l’ampiezza e la profondità dell’ulcera sono fattori importanti che influenzano il decorso clinico. Nessuno di questi sistemi, comunque, tiene in considerazione i meccanismi endogeni di riparo implicati nella cicatrizzazione della ferita. La guarigione delle ferite è normalmente assicurata dal concorso di cellule residenti (fibrobasti, cheratinociti, cellule endoteliali) e cellule provenienti della circolazione, tra le quali un ruolo di spicco spetta alle cellule progenitrici endoteliali (EPC). Studi recenti hanno dimostrato che le EPC provenienti da pazienti diabetici mostrano un’alterata proliferazione, adesione e incorporazione all’interno delle strutture vascolari. I meccanismi responsabili di tali alterazioni rimangono per lo più ignoti. Inoltre mancano studi prospettici che consentano di attribuire al deficit delle EPC un significato prognostico in termini di evoluzione della malattia macro e microvascolare.

Questo progetto si propone, come obiettivo primario, di valutare il valore prognostico di cellule EPC in termini di numero, fenotipo antigenico e funzionalità per predire eventi quali mortalità, amputazione maggiore dell’arto e ristenosi post-angioplastica in una coorte di 120 pazienti affetti da diabete di tipo 2 con Ischemia Critica dell’Arto (CLI), reclutati per presenza di ulcera diabetica del piede ed eleggibili per rivascolarizzazione mediante angioplastica. Come obiettivo secondario vogliamo analizzare i meccanismi che causano la disfunzionalità delle cellule EPC diabetiche e in particolare cercare di fornire per la prima volta un profilo di microRNA, potenzialmente coinvolti nei processi pro-angiogenici associati alle EPC diabetiche. Per questo tipo di analisi non sono presenti in letteratura criteri di normalità per cui abbiamo deciso di aggiungere due gruppi di controllo: un gruppo di soggetti non diabetici con ischemia critica dell’arto e un gruppo di donatori sani.

Per poter rispondere al primo obiettivo è necessario attendere la fine dei 12 mesi di follow-up; in questa tesi quindi verranno riportati i dati ottenuti da un'analisi preliminare. Ad oggi, circa il 60% dei pazienti hanno raggiunto un anno di follow-up al termine del quale abbiamo registrato 37 eventi di restenosi, 10 amputazioni e 7 eventi di morte. Non abbiamo osservato nessuna differenza statisticamente significativa analizzando il numero di cellule EPC tra i pazienti che hanno presentato un evento e quelli che non l'hanno avuto. Anche analizzando la capacità migratoria di queste cellule non abbiamo notato nessuna differenza significativa. La nostra analisi preliminare ha dimostrato un aumento significativo nella motilità basale delle EPC nei pazienti con eventi rispetto agli altri. Sulla base di questa analisi la nostra ipotesi è che l'incapacità delle cellule diabetiche di giungere nel sito di ischemia sia dovuta sia ad una aumentata mobilità aspecifica sia ad una alterata risposta migratoria verso gli stimoli e provocando perciò un difetto nel processo di riparo della ferita. Questo dato, molto interessante, dovrà essere confermato alla fine dello studio quando tutti i pazienti avranno raggiunto i 12 mesi di follow-up.
Per quanto riguarda l'obiettivo secondario dello studio abbiamo analizzato tramite analisi citofluorimetrica cellule MNCs subito dopo isolamento sulla base della espressione di marker caratteristici di alcune popolazioni pro-angiogeniche quali CD34 e EPC ($CD45^{dim}$,$CD34^{pos}$,$CXCR4^{pos}$,KDR^{pos}). I risultati ottenuti ad oggi mostrano una diminuzione nel numero delle cellule CD34 e delle cellule EPC nei pazienti ischemici rispetto ai controlli sani. Inaspettatamente, il numero di queste cellule nei pazienti diabetici è più alto se paragonato ai soggetti non diabetici. L'analisi della funzionalità migratoria ha evidenziato una diminuzione delle capacità delle cellule MNC in soggetti ischemici con o senza diabete rispetto ai soggetti sani. Abbiamo inoltre analizzato il fenotipo antigenico delle cellule migrate spontaneamente o in risposta a SDF-1a e delle cellule non migrate. L'analisi citofluorimetrica dimostra una non responsività delle EPC diabetiche allo stimolo migratorio SDF-1α rispetto agli altri due gruppi di pazienti. Questi risultati sono in linea con gli studi già pubblicati e i meccanismi coinvolti in questo difetto non sono ancora completamente noti. Per cercare una possibile spiegazione sono state fatte diverse ipotesi tra le quali citiamo un debole rilascio di cellule del midollo osseo, un'alterata differenziazione o una diminuita sopravvivenza di queste cellule nel circolo sanguigno. In questa tesi abbiamo deciso di focalizzarci sui meccanismi intracellulari di trasmissione del segnale. In particolare, dato il ruolo sempre più importante dei microRNA nel controllo dei meccanismi di regolazione delle cellule endoteliali, quali migrazione, proliferazione e formazione di nuovi vasi abbiamo effettuato una prima analisi per cercare di capire se il diabete provocasse modificazioni nell'espressione di alcuni microRNA nelle EPC. Successivamente ci siamo concentrati su due microRNA, mir-15a e mir-16, il cui ruolo nell'alterata migrazione e sopravvivenza è stato già dimostrato nelle cellule tumorali. A tale scopo abbiamo eseguito esperimenti di over-espressione di questi due miRNA in

cellule EPC e abbiamo osservato come la co-espressione di mir-15a e mir-16 riduca la risposta migratoria e induca anche il processo apoptotico in queste cellule. Successivamente abbiamo voluto verificare se la diminuzione di questi due microRNA nelle cellule EPC di pazienti diabetici potesse permettere il recupero della capacità migratoria di queste cellule. E' stato dimostrato come la down-regolazione di mir-15a e mir-16 sia in grado di ripristinare la funzionalità migratoria di queste cellule. L'analisi bioinformatica dei possibili geni bersaglio di questi microRNAs ha rivelato la presenza di alcuni geni coinvolti nei meccanismi di controllo dell'angiogenesi e del ciclo cellulare quali VEGFa, BCL2 e AKT3. Nelle cellule EPC dei pazienti ischemici con o senza diabete e nelle cellule over-esprimenti mir-15a e mir-16 abbiamo osservato una diminuzione significativa dei livelli di AKT3, proteina coinvolta in numerosi processi di migrazione e proliferazione cellulare. Sulla base dei risultati che abbiamo ottenuto ulteriori studi sono necessari per verificare il coinvolgimento di altri geni bersaglio e per investigare su un possibile ruolo nella disfunzione causata dal diabete. In particolare la nostra attenzione sarà rivolta ai meccanismi di trasduzione del segnale che coinvolgono AKT e la proteina eNOS.

Questo progetto dimostra, per la prima volta, che la migrazione spontanea di una sottopopolazione di MNC definite antigenicamente come EPC è aumentata in pazienti diabetici che hanno avuto un evento quali restenosi, amputazioni e morte. Sulla base di questi risultati è possibile ipotizzare l'utilizzo della migrazione basale delle EPC come fattore prognostico per prevenire le complicanze vascolari di pazienti diabetici. In questa tesi abbiamo anche dimostrato che mir-15a e mir-16 sono regolatori chiave delle funzioni pro-angiogeniche delle cellule EPC. In futuro vogliamo approfondire il ruolo dei microRNA nella disfunzione diabetica anche in quanto è ormai noto che i microRNA hanno un enorme potenziale terapeutico per il trattamento di patologie caratterizzate da alterata angiogenesi.

Negli ultimi anni è emerso come i microRNA svolgano un ruolo fondamentale nel trattamento di patologie vascolari in cui sono presenti alterati meccanismi pro-angiogenici e questo suscita il nostro interesse per meglio comprendere il ruolo dei microRNA nella patologia diabetica soprattutto se complicata da problemi vascolari. L'utilizzo di antagonisti di microRNAs che controllano l'angiogenesi, in grado di essere indirizzati nei siti di ischemia potrebbe essere un valido approccio per cercare di prevenire le complicanze vascolari di alcune malattie.

In conclusione, il nostro studio mostra il ruolo chiave delle EPC nei meccanismi di angiogenesi e suggerisce il loro potenziale utilizzo come biomarcatore dell'integrità vascolare. Approcci di terapia genica che utilizzano queste cellule sono infatti già in corso. Il futuro della ricerca consiste quindi, nell'individuare metodi per migliorare la capacità migratoria e di integrazione di queste EPC nei siti di interesse combinando tecniche sperimentali *in vitro* e *in vivo.*

1. INTRODUCTION

Diabetes is one of the most challenging health problems in the 21st century. The prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 (**Figure 1**).

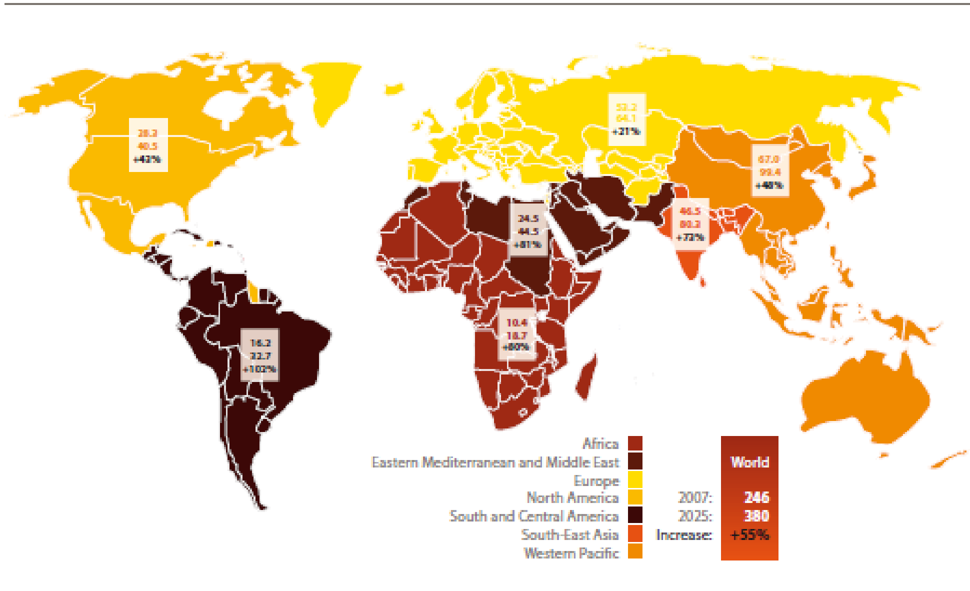


Figure 1. *Global projection for the number of people with diabetes (20-79 age group) 2007-2025 (millions)*

1.1 Etiology of Diabetes

World Health Organization in 1999 described Diabetes Mellitus as a metabolic disorder of multiple etiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. The effects of diabetes mellitus include long–term damage, dysfunction and failure of various organs.

Diabetes mellitus is classified on the basis of etiology and clinical presentation of the disorder as: type 1 diabetes, type 2 diabetes, gestational diabetes mellitus (GDM), and other specific types.

1.1.1 Type 1 diabetes

Type 1 diabetes is sometimes called insulin-dependent, immune-mediated or juvenile-onset diabetes. It is caused by destruction of the insulin-producing cells of the pancreas, typically due to an auto-immune reaction, where they are attacked by the body's defense system. The beta cells of the pancreas therefore produce little or no insulin, the hormone that allows glucose to enter body cells. The disease can affect people of any age, but usually occurs in children or young adults. Type 1 diabetes is one of the most common endocrine and metabolic conditions in childhood. People with type 1 diabetes need injections of insulin every day in order to control the levels of glucose in their blood. Without insulin, people with type 1 diabetes will die. The incidence of type 1 diabetes is increasing, the reasons for which are unclear but are likely to be mainly due to changes in environmental risk factors. Environmental risk factors, increased height and weight, increased maternal age at delivery, and possibly some aspects of diet and exposure to some viral infections may initiate autoimmunity or accelerate an already ongoing beta cell destruction.

1.1.2 Type 2 diabetes

Type 2 diabetes constitutes about 85 to 95% of all diabetes in developed countries. It is characterized by insulin resistance and relative insulin deficiency, either of which may be present at the time that diabetes becomes clinically manifest. The diagnosis of type 2 diabetes usually occurs after the age of 40 years but could occur earlier, especially in populations with high diabetes prevalence. Type 2 diabetes can remain asymptomatic for many years and the diagnosis is often made from associated complications or incidentally through an abnormal blood or urine glucose test. There are several possible factors in the development of type 2 diabetes as obesity, diet, physical inactivity, increasing age and family history of diabetes. In contrast to type 1 diabetes, people with type 2 diabetes are not dependent on exogenous insulin but may require insulin for control of hyperglycaemia if this is not achieved with diet alone or with oral hypoglycaemic agents.

1.1.3 Gestational diabetes

Gestational diabetes mellitus (GDM) is a glucose intolerance of varying degrees of severity which starts or is first recognized during pregnancy. The definition applies regardless of whether insulin is used for treatment or if the condition persists after pregnancy. GDM is also associated with increased risk of obesity and abnormal glucose metabolism during childhood and adult life in the offspring.

1.1.4 Diabetic complications

New progress in the treatment leading to control of hyperglycaemia has resulted in increased life expectation. However, quality of life remains poor due to associated complications, especially affecting the cardiovascular system. These include **macroangiopathy**, large arterial vessels angiopathies (ischemic cardiopathy, arteriopathy of the lower limbs, arteriopathy of the supraortic trunks), **microangiopathy**, angiopathies of the small arterial vessels (retinopathy, nephropathy, neuropathy) (**Figure 2**). The risk for developing complications is influenced by many factors including duration of diabetes and genetic factors. Current treatments have resulted in only a partial reduction in cardiovascular risk, and the management of these conditions remains a major unmet need for those with diabetes.

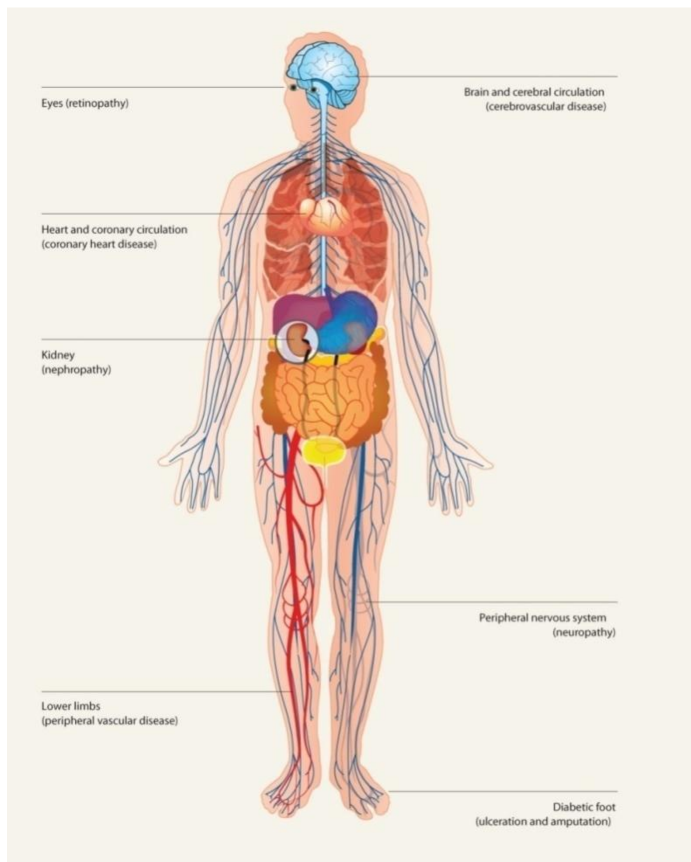


Figure 2. *The major diabetic complications.*

1.1.4.1 Cardiovascular disease

Cardiovascular disease (CVD) is the major cause of death in diabetes, accounting for 50% or more of all diabetes fatalities and disability. CVD include angina, myocardial infarction (heart attack), stroke, peripheral artery disease, and congestive heart failure (CHF). The pathogenesis of CVD associated with diabetes is not yet fully understood. However, because atherosclerotic macrovascular complications occur at an earlier age and with greater severity in people with diabetes, it is likely that its pathogenesis is directly influenced by the diabetic state. Current American Diabetes Association guidelines recommend aggressive treatment for dyslipidemia in diabetic patients, particularly in those with elevated LDL cholesterol levels. Tight glycaemic control achieved with diet, exercise, and some antidiabetic agents may substantially improve the lipid profile and reduce the risk of CVD in some patients. However, most patients will require the use of intensive lipid-lowering therapy to reduce their cardiovascular risk, most commonly with one of the statins or fibric acid derivatives.

1.1.4.2 Nephropathy

Diabetes has become the most common single cause of end-stage renal disease in the U.S.A and Europe; this is due to the facts that *1*) diabetes, particularly type 2, is increasing in prevalence; *2*) diabetic patients now live longer.

The earliest clinical evidence of nephropathy is the appearance of increased urine albumin levels referred to as microalbuminuria, and patients with microalbuminuria are referred to as having incipient nephropathy. Annual screening for microalbuminuria allows the identification of patients with nephropathy at a point very early in its course. Improving glycaemic control, aggressive antihypertensive treatment, and the use of angiotensin converting enzyme (ACE) inhibitors or angiotensin II receptor blockers (ARBs) slow the rate of progression of nephropathy.

1.1.4.3 Neuropathy

Diabetic neuropathy is defined as the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes, after exclusion of other causes. When blood glucose and blood pressure are not controlled, diabetes can harm the nerves. The most commonly affected area is the feet and legs. Nerve damage in these areas is called peripheral neuropathy and could manifest in many ways including loss of feeling in the feet and toes. Peripheral somatic neuropathy, together with peripheral vasculopathy, is the most frequent cause in the formation of diabetic foot ulcers. Autonomic neuropathy contributes to the development of diabetic cardiomyopathy, silent cardiac ischemia, and cardiac arrhythmias. Cardiovascular manifestations of autonomic neuropathy comprise resting tachycardia and orthostatic hypotension. Screening procedures today allow an

early diagnosis of sub-clinical neuropathy, but their use for prevention has not yet entered the common clinical practice. The consequent social costs of an omitted early diagnosis are relevant, considering that several studies and meta-analyses indicate an increase of 3.5 fold in the relative risk of mortality in diabetic patients with cardiac autonomic neuropathy. However, despite the high relevance of this phenomenon, the mechanisms underlying the increase in mortality have not been yet completely understood.

The genesis of the dysautonomy seems to be multifactorial with evidence of the role of oxidative stress, deficit of trophic factors such as nerve growth factor (NGF) and microangiopathy with endonerval ischemia. In the absence of a target for a real mechanistic cure, strict glycaemic control is the only therapeutic action to prevent the evolution of neurological damage.

1.1.4.4 Retinopathy

Diabetic retinopathy is one of the main causes of visual loss in individuals aged 20-64 years old and is present in more than 77% of patients with diabetes mellitus who survive for over 20 years with the disease [1]. Diabetes can harm sight and cause blindness in several ways. The most common cause of blindness in diabetes is macular edema, caused by fluid build-up behind the retina of the eye. A more common complication is background and proliferative retinopathy, which can cause blindness as a result of repeated haemorrhages at the back of the eye. Diabetes also increases the risk of cataracts and glaucoma.

1.1.4.5 Diabetic Foot

Among the complications of diabetes "diabetic foot" plays an increasingly important role; this is definitely the complication which leads to the highest number of hospital admissions and whose cost is enormous. If we consider the forecasts of the World Health Organization for 2030, it is easy to imagine the potential extent of the problem: estimates of this pathology actually state that during their lifetime approximately 15% of diabetic patients will have a foot ulcer which requires medical treatment. The most important problem associated with a foot ulcer in diabetic patients is the risk of major amputation, i.e. carried out above the ankle; although the diabetic population represents around 3% of the general population, over 50% of all major amputations actually involve diabetic patients. Data from several studies have documented that foot ulcers precede approximately 85% of all amputations performed in patients with diabetes. Risk of ulceration and amputation increases 2- to 4-fold with both age and duration of diabetes.

Hence, it is obvious that reducing the number of amputations is a fundamental aim in the treatment of diabetic patients.

Clinical Definition

The term diabetic foot is used when diabetic neuropathy or arteriopathy of the lower limbs compromises the function or structure of the foot.

The first is defined **neuropathic foot** while the second one is **ischemic foot.** These conditions are extremely different from each other: however in most patients, especially those of advanced age, neuropathy and vasculopathy coexist and the term **neuroischemic foot** is therefore used.

Neuropathic foot

Diabetic neuropathy affects the sensory nerves (sensory neuropathy) the motor nerves (motor neuropathy) and the vegetative nerves (autonomic neuropathy); the neuropathic foot is therefore a foot in which the diabetic neuropathy has altered the equilibrium of the muscles, the perception of stimuli, vegetative autoregulation, namely all three nerve components. The ideal treatment would be to identify all those diabetic patients suffering from neuropathy in order to implement a prevention program which could succeed in reducing the risk of an ulcerative wound. Once an ulcer is formed, the requirement is to stimulate the healing as fast as possible.

Fundamentally the treatment of the neuropathic plantar ulcer is based on three points:

- local treatment of the wound (cleaning the ulcer plus dressing)
- the treatment of any infections
- the removal of the ulcerative wound (preventing this worsening on account of the weight of the body while the subject is walking)

It is of fundamental importance to point out the fact that the failure to implement even one of these therapeutic stages drastically reduces the likelihood of the ulcerative wound healing.

Ischemic Foot

The histological characteristics of peripheral obstructive arteriopathy (POA) in diabetic patients do not differ substantially from the arteriopathy of the non-diabetic population. However the clinical characteristics are very different; in diabetic patients, arteriopathy is more frequent, early, rapidly progressive, does not exclude women, even those of fertile age, affects both legs and mainly involves the arteries under the knee. A typical characteristic of the diabetic patient is often the lack of an earlier symptom of peripheral arteriopathy.

International criteria for diagnosing chronic critical ischemia have been re-established several times in the light of new information and new studies. The most recent criteria are those of the TASC (TransAtlantic Inter-Society Consensus) published in January 2000.

TASC criteria are: subject with ulcer or gangrene or pain at rest, pressure on the ankle < 50-70 mmHg or on the big toe < 30-50 mmHg or transcutaneous oximetry < 30-50 mmHg.
Foot ulceration and amputation affect the quality of life and create an economic burden for both the patient and the health care system. Revascularization of the diabetic foot is often complicated by restenosis and thrombosis events, which account for the excess of limb amputation.
At present, two well-established systems are used for wound classification: the 1) Wagner system and 2) the new University of Texas (UT) system, with the latter being a better predictor of outcome.

1) The Meggitt–Wagner classification is the most well-known and validated system for foot ulcers. It is simple to use and has been validated in a number of studies. Higher grades are directly related to increased risk for lower limb amputation. It is considered the gold-standard, against which other systems should be validated (**Table 1**)

Grade	**Description of the ulcer**
Grade 0	Pre- or post-ulcerative lesion completely epithelialized
Grade 1	Superficial, full thickness ulcer limited to the dermis, not extending to the subcutis
Grade 2	Ulcer of the skin extending through the subcutis with exposed tendon or bone and without osteomyelitis or abscess formation
Grade 3	Deep ulcers with osteomyelitis or abscess formation
Grade 4	Localized gangrene of the toes or the forefoot
Grade 5	Foot with extensive gangrene

Table 1. *The Meggitt- Wagner classification*

2) The University of Texas classification system for diabetic foot wounds has recently been proposed and validated by the University of Texas. This system evaluates both depth of the ulcer as in Meggitt–Wagner classification system and presence of infection and ischemia. (**Table 2**). It is simple to use and shown to predict more accurately the outcome of an ulcer (healing or amputation).

Grade				
Stage	**0**	**1**	**2**	**3**
A	Pre- or post-ulcerative lesion Completely epithelialized	Superficial wound not involving tendon, capsule or bone	Wound penetrating to tendon or capsule	Wound penetrating to bone or joint
B	With infection	With infection	With infection	With infection
C	With ischemia	With ischemia	With ischemia	With ischemia
D	With infection and ischemia	With infection and ischemia	With infection and ischemia	With infection and ischemia

Table 2. *The University of Texas classification system for diabetic foot wounds*

Any valid classification system of foot ulcers should facilitate appropriate treatment, simplify monitoring of healing progress and serve as a communication code across specialties in standardized terms.
The "Meggit-Wagner" classification do not take into account the presence of ischemia and infection so the "University of Texas classification system" offers many advantages over the other system and is the most appropriate system devised to date. The two systems do not describe the location of the ulcer and importantly they do not consider parameters related to the patients as poor foot care, emotional upset and denial. To ameliorate these classifications further systems should include other parameters such as location of the ulcer, foot deformities and other factors which may be related to the outcome of an ulcer.

Based on all these considerations the prediction of clinical outcome of diabetic foot relies only on clinical data, including the presence or absence of infection, footwear and pressure relief, and overall glycemic control. However, none of these methods takes into account the endogenous repair mechanisms implicated in wound cicatrisation of the ulcers that are impaired in diabetic patients.

1.2. Endothelial Progenitor Cells (EPCs)

Improvement of neovascularization after critical ischemia is an important novel therapeutic strategy. Growth of new blood vessel in the adult occurs through arteriogenesis, angiogenesis or vasculogenesis [2]. Arteriogenesis is defined as the growth of collateral vessels, whereas angiogenesis refers to the growth of new capillaries by sprouting of preexisting vessels through migration and proliferation of mature endothelial cells (ECs). The concept of vasculogenesis was originally described by Sabin and others, who investigated the embryonic de novo blood vessel formation and assigned the term “angioblast” to the endothelial cell precursor. Now, the term “vasculogenesis” is also used to describe adult blood vessel formation, referring to the mobilization of bone marrow– derived endothelial stem cells, which home to sites of ischemia and contribute to new blood vessel formation [3] (**Figure 3**)

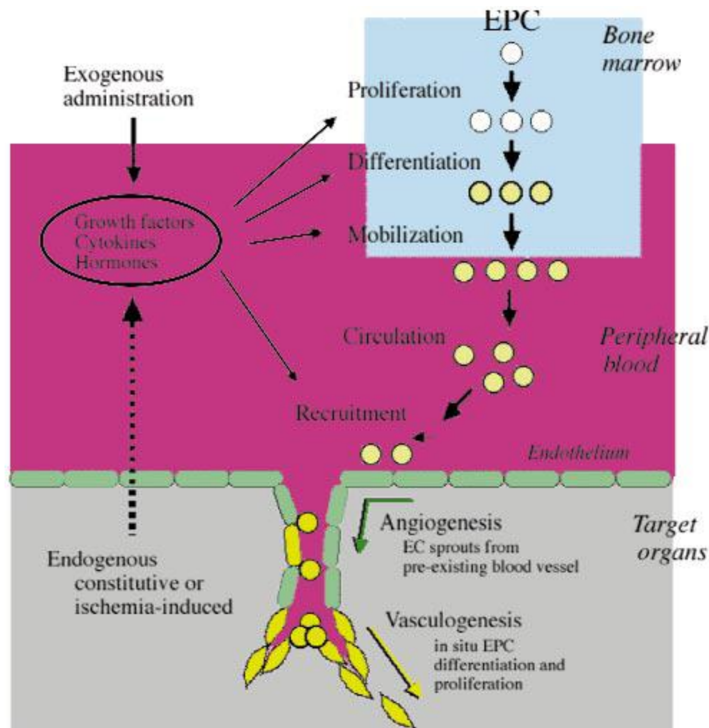


***Figure 3**. Neovascularization encompasses both angiogenesis and vasculogenesis [4].*

The finding that vasculogenesis also contributes to blood vessel formation in the adult offers novel therapeutic strategies for the use of cultivated circulating endothelial progenitor cells (EPCs) or their precursors for cell therapy of tissue ischemia. Endothelial progenitor cells (EPCs) were identified in 1997 by Asahara

and colleagues in human peripheral blood as CD34 positive mononuclear cells (MNC) [5]. In this work they demonstrated that, *in vitro*, these cells have markers of endothelial cells and, in a mouse model of limb ischemia, EPCs incorporated into sites of active angiogenesis. These findings suggest that EPCs may be useful for augmenting collateral vessel growth to ischemic tissues and for delivering anti- or pro-angiogenic agents to sites of pathologic angiogenesis. Based on literature data EPCs are difficult to define precisely because of lack of consensus regarding the best EPCs source, the optimal culture techniques, and the phenotypes and characteristics that are crucial for EPCs identity. Some groups identified EPCs in culture as early outgrowth cells (also called circulating angiogenic cells) or late outgrowth cells (endothelial colony forming cells) [6]. EPCs have also been characterized by the uptake of diacetylated low-density lipoprotein (LDL), by the binding of fluorescently labeled Ulex europaeus agglutinin 1 lectin, or by in-vitro and in-vivo functional assays, such as colony formation, tube formation, and vascular integration. Another conventional method of EPCs identification is based on the coexpression of hematopoietic stem-cell markers (e.g., CD133, CD34) and endothelial-cell markers (e.g., vascular endothelial growth factor [VEGF] receptor-2 or kinase-insert domain receptor [KDR], von Willebrand factor [vWF], endothelial nitric oxide synthase [eNOS]) [7]. Today, scientists accept the definition that EPCs consist of two different subpopulations, termed early and late EPCs [8]. Although both EPCs are derived from MNC and express endothelial cell markers, they have different morphologies and growth patterns [9]. Early EPCs exhibit a spindle-like morphology and the majority of them are derived from CD14(+) subpopulations. Late EPCs, named after their late outgrowth potential, exhibit a cobblestone morphology and are derived from CD14(−) fractions. These two populations have been characterized for production of vascular endothelial growth factor (VEGF), VEGF receptor expression, cytokine secretion, and tube forming activity in vitro and in vivo [10]. Our groups defined EPCs as pro-angiogenic circulating progenitor cells that co-express CD34, KDR and CXCR4, the stromal cell derived-factor (SDF-1) receptor. [11]

Recent studies indicated that bone marrow is the major source of EPCs and that endogenous mobilization of EPCs from the bone marrow into the peripheral blood occurs in response to a physiologic or pathological need for neovascularization and contribute to new blood vessel formation [3, 12]. EPCs recruitment requires a coordinated sequence of events, including chemoattraction, adhesion, and migration. EPCs indirectly support neovascularization by a paracrine mechanism, i.e. they contribute to vascular growth primarily by secreting growth factors, chemokines, cytokines that inhibit cell death, enhance cell proliferation, activate stem/progenitor cells already present in the ischemic tissue and recruit additional progenitor cells to the injury site [13-16].

Detractors of the EPCs isolation protocols argue that most of the available literature should be critically revisited in light of the recent observation that EPCs

have no true progenitor or endothelial properties. Based on all these problems the original antigenic phenotype of EPCs, CD34+KDR+, is still the best compromise in terms of specificity and sensitivity in the clinical setting, even if it might not be completely distinguishable between EPCs, mature endothelial cells and haematopoietic progenitors cells.

1.2.1 Diabetes and EPCs

The inability of EPCs to maintain a normal endothelial homoeostasis and to promote the development of new vessels could lead to accelerated vascular disease and ageing and could block compensatory angiogenesis, thus favoring the development and progression of ischemic syndromes. Interestingly, EPCs alterations have been shown in subjects with diabetes, hypertension, dyslipidemia, cigarette smoking, obesity and family history of cardiovascular disease [17].

Endothelial dysfunction and damage is an early and widespread complication in patients with diabetes and it is also the first stage in the pathogenesis of atherosclerosis. It has been recognized that the endothelial repair process also plays an important role in determining overall vascular health. A damaged endothelium is repaired by being partially covered by resident endothelial cells plus the contribution of circulating EPCs. EPCs reduction and dysfunction has been shown in type 1 and type 2 diabetes: Tepper et al. found that early EPCs from patients with type 2 diabetes exhibit impaired proliferation, adhesion and incorporation into vascular structures [18]; Loomans et al. obtained similar results in patients with type 1 diabetes, by showing a reduced number of early cultured EPCs compared with control subjects [19].

Recently, Egan et al. demonstrated also a reduction in several different subtypes of EPCs in patients with T2D compared to controls.[20]

This reduction is probably involved in the pathogenesis of vascular complications of diabetes, but there are few data on this topic currently available in the literature. In particular Fadini and his collaborators showed a decrease of EPCs levels in diabetic patients which is associated with a high risk of peripheral vascular complications [21].

EPCs have been shown to be altered in all diabetic complications and they appear as a major common underlying pathogenic mechanism [22]. In addition to being a potential pathogenic actor and a marker of the vascular burden, reduced EPCs can be also considered a surrogate marker of increased risk of future adverse events. It has been shown that a low baseline progenitor cell count predicts cardiovascular events in patients with cardiovascular disease [23], chronic renal failure [24] or the metabolic syndrome [25].

1.2.2 Mechanisms of EPCs mobilization and homing

Under steady state conditions, small numbers of EPCs constantly leave the BM, enter tissues, and travel back to the BM or peripheral niches via the blood and lymph [26]. EPCs liberation into the blood increases dramatically after an acute injury or ischemia, thereby providing large numbers of reparative units to the damaged tissue and thus facilitate healing. The travel of EPCs from the BM into the activated tissue is guided by the interplay of several molecular and cellular mechanisms. Initially, the disruption of anchorage mechanisms between EPCs and stromal cells allow the SC to leave the niche, while increased concentrations of chemotactic substances outside the BM niche provide a migratory stimulus [27] [28]. Homing to the target tissue is modulated by a spectrum of mechanisms: loose adhesion via selectins as well as membrane bound growth factors and their ligands/receptors, mediate the rolling movement of the cells along the endothelial layer. Subsequently, integrins mediate invasion of the target tissue, via their interaction with endothelial surface molecules and matrix proteins[29, 30]. Finally, the directional migration of recruited EPCs within the target tissue and their organization into niche-like structures contribute to stable engraftment for tissue repair (**Figure 4**).

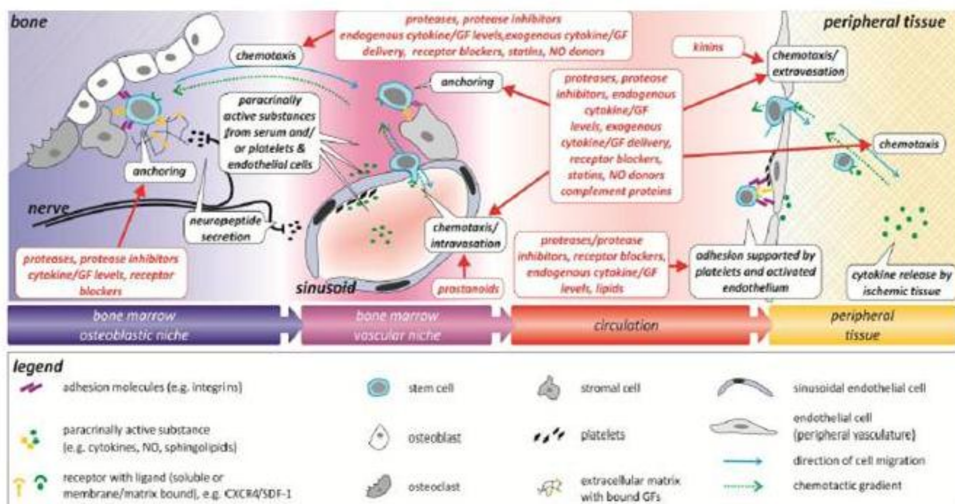


Figure 4. *Overview of endogenous mechanisms modulating EPCs mobilization from the BM and recruitment to the target tissue.*

The most relevant chemokines involved in these processes is Stromal Derived Factor-1*α* (SDF-1a/CXCL12). It has been shown that its expression is up-regulated

during ischemia [27]. Inhibition of CXCR4 partially blocks the homing of progenitor cells to the ischemic myocardium [31]. Other studies demonstrated that inhibition of its receptor CXCR4 significantly reduced SDF-1 induced adhesion of EPCs to mature endothelial monolayer, the migration of EPCs *in vitro* [27], and the *in vivo* EPCs homing in a model of hind limb ischemia [32]. Moreover over-expression of SDF-1 enhanced progenitor cell homing and incorporation into ischemic tissue [33, 34], supporting the idea that SDF-1 play a crucial role for recruitment of circulating progenitor cells. Despite the important role of CXCR4/SDF-1a in EPCs migration, proliferation, survival and angiogenesis, relatively little is known about the signal transduction pathways that mediate these effects in EPCs. Studies in many cell types have implicated both PI3K/Akt and MAPK/ERK signal transduction pathways in the control of directional cell migration and the sensing chemoattractant gradients by the cell. Zheng and colleagues showed that SDF-1a-induced migration of EPCs was CXCR4 dependent using AMD3100, a CXCR4 antagonist. Their results demonstrated that PI3K/AKT/eNOS is crucial for SDF-1 mediated migration of EPCs [35] (**Figure 5**).

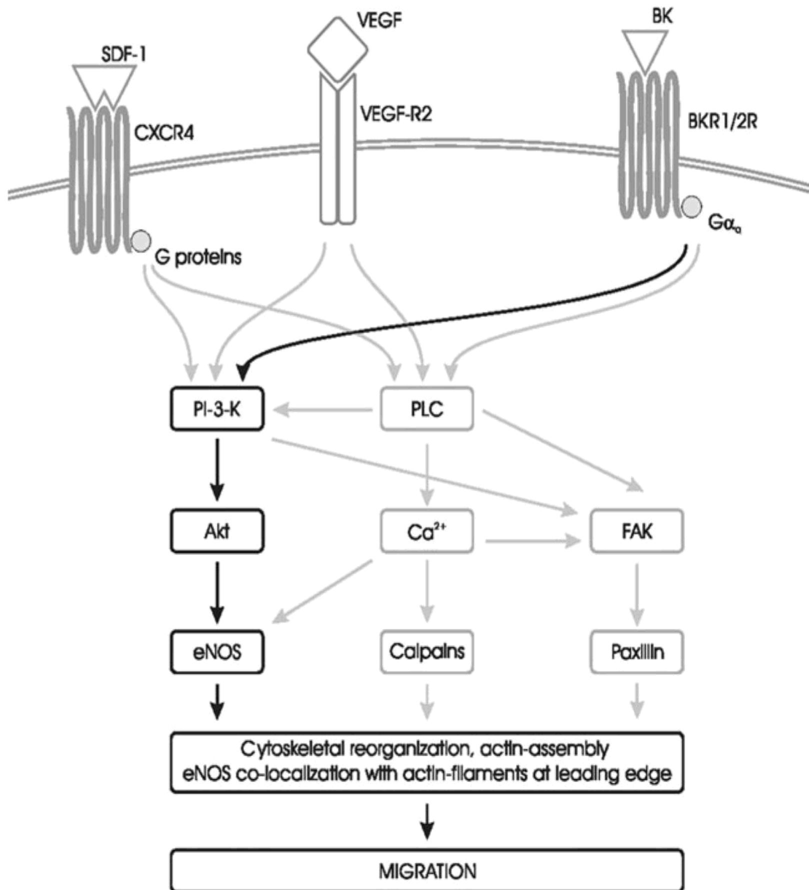


Figure 5*. Intracellular signalling pathways for migration.[36]*

Recent data determined the involvement of additional other chemokines as IL-8/Gro-*α* and its cellular receptor CXCR2, in the homing of CD34 progenitor cells to the ischemic myocardium [37]. Furthermore, ischemia-induced VEGFa acts as a chemoattractant to EPCs [38]. Interestingly, VEGF is sufficient to induce the recruitment of progenitor cells and their perivascular localization via induction of SDF-1 expression by perivascular myofibroblasts suggesting that different cytokines may cooperate during homing of progenitors cells [39].
All these data suggest that chemokines like SDF-1a, IL-8 and probably others are involved in the trafficking of EPCs from the bloodstream to ischemic tissues [40]. So far, mechanistic research has focused mainly on SDF-1/CXCR4 signaling during the recruitment of angio-supportive cells. Nevertheless, kinins play a key role in governing processes of the vessel wall and it was demonstrated their

necessity for recruiting circulating pro-angiogenic cells. In particular, EPCs use proteases, like MMPs and cathepsins, to disrupt the endothelial matrix in the initiation of reparative neovascularization. Madeddu's group has proposed another member of this class, human tissue kallikrein 1 (hKLK1), as a potent factor for promotion of therapeutic neovascularization, as indicated by studies in animal models of ischemia [41, 42]. hKLK1 can exert its effects via kininogen cleavage, thus generating kinins, as bradykinin (BK) or kallidin (kDa). Kinins, such as BK, activate kinin-B_2 receptors (B_2R), which are constitutively expressed on endothelial cells (ECs), and kinin-B_1 receptors (B_1R), whose expression is induced under stress conditions. **(Figure 6)**

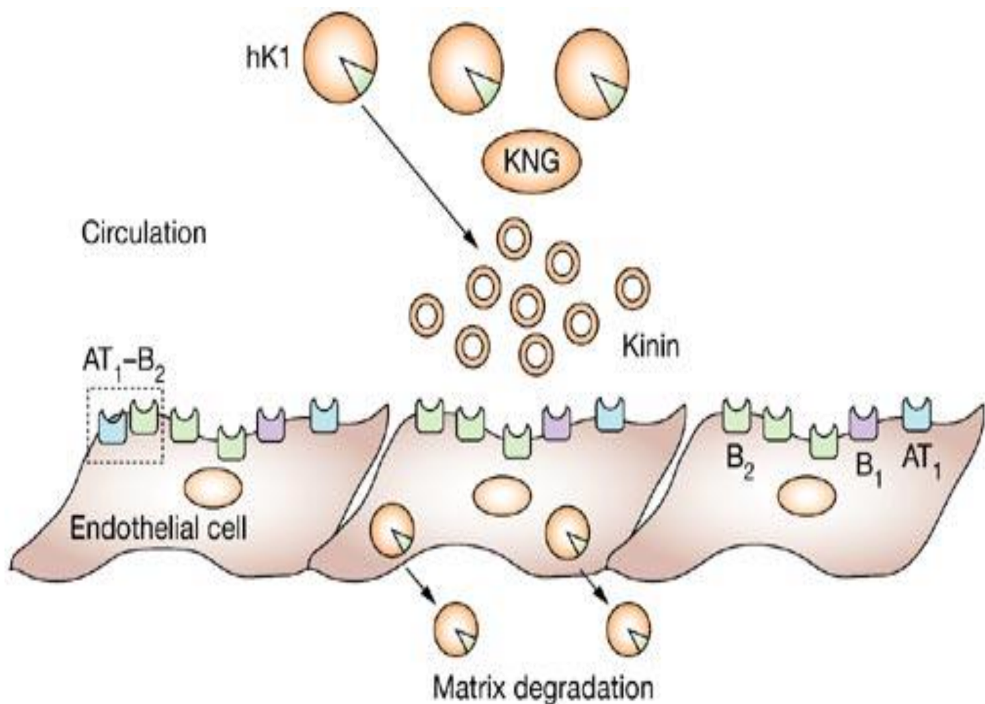


Figure 6. *Components of kallikrein-kinin system [43]*

Overexpression of *hKLK1* promotes a robust and persistent neovascularization through kinin-mediated activation of the Akt-eNOS pathway, and has recently been shown to display tissue specific VEGF dependency. In Stone work, it was demonstrated the fundamental role of endogenous tissue kallikrein in vascular repair and provide novel information on the cellular and molecular mechanisms responsible for the robust arterialization induced by hKLK1 overexpression [44].

Krankel et al. recently demonstrated the importance of the B_2R in the recruitment of circulating pro-angiogenic cell types as well as in the subsequent mounting of revascularization and recovery of blood flow in ischemic tissue [45]. We also revealed that hKLK1 coupling to B_2R signaling is essential for EPCs invasive capacity. Our results showed for the first time that human EPCs are equipped with active endogenous hKLK1 and that hKLK1 is crucial for EPCs invasive and proangiogenic activities by both protease- and kinin receptor-mediated mechanisms. Finally, this study demonstrated that kinin kallikrein system faulty components need to be repaired to restore the pristine EPCs function in diabetes, thus providing the first proof of concept for multiple gene engineering of a pathway crucially implicated in angiogenesis. [11]

1.3. MicroRNA: potential new regulators for EPCs functions in diabetes

MicroRNAs (miRNAs) are a class of endogenous, small, non-coding RNAs, which have been recently discovered to play a major role in gene regulation, including of mammalian cells [46]. MiRNAs characterization has become a major interest in biology and medicine, with implications for the detection and treatment of different pathologies. MicroRNAs are abundant in the vascular system, where they have key roles in development and are likely to be important mediators of vascular system diseases. So far, more than 1400 human miRNA sequences [47] have been identified. A single miRNA can target hundreds of mRNA transcripts for translational repression, mRNA degradation, or inducing mRNA instability. Consequently, accurately determining the full repertoire of miRNA targets is essential for knowing miRNA functional characteristics. MiRNA target gene identification is currently a major bottleneck in miRNA research [48].

MiRNAs are single-stranded RNAs of ~22 nucleotides and are found in both plants and animals. They negatively regulate their targets in one of two ways depending on the degree of complementarities between the miRNA and the target [49]. First, miRNAs that bind with perfect or nearly perfect complementarities to protein coding mRNA sequences induce the RNA-mediated interference (RNAi) pathway. Briefly, mRNA transcripts are cleaved by ribonucleases in the miRNA-associated, multiprotein RNAinduced-silencing complex (miRISC), which results in the degradation of target mRNAs. This mechanism of miRNA-mediated gene silencing is commonly found in plants, but miRNA-directed mRNA cleavage has also been shown to occur in mammals [50]. However, most animal miRNAs are thought to use a second mechanism of gene regulation that does not involve the cleavage of their mRNA targets. These miRNAs exert their regulatory effects by binding to imperfect complementary sites within the 3′ untranslated regions (UTRs) of their mRNA targets, and they repress target-gene expression post-transcriptionally, apparently at the level of translation, through a RISC complex that is similar to, or possibly identical with, the one that is used for the RNAi pathway. Consistent with translational control, miRNAs that use this mechanism reduce the protein levels of their target genes, but the mRNA levels of these genes are barely affected. However, recent findings indicate that miRNAs that share only partial complementarities with their targets can also induce mRNA degradation, but it is unclear if translational inhibition precedes destabilization of the gene targets in these cases. The biogenesis of miRNAs has only recently been elucidated. miRNAs, which generally seem to be transcribed by RNA polymerase II, are initially made as large RNA precursors that are called pri-miRNAs. The pri-miRNAs are processed in the nucleus by the RNase III enzyme, Drosha, and the double-stranded- RNA-binding protein, Pasha (also known as DGCR8), into ~70-

nucleotide pre-miRNAs, which fold into imperfect stem-loop structures. The pre-miRNAs are then exported into the cytoplasm by the RAN GTP dependent transporter exportin 5 and undergo an additional processing step in which a double- stranded RNA of ~22 nucleotides in length, referred to as the miRNA:miRNA* duplex, is excised from the pre-miRNA hairpin by another RNAse III enzyme, Dicer. Subsequently, the miRNA:miRNA* duplex is incorporated into the miRISC complex. The mature miRNA strand is preferentially retained in the functional miRISC complex and negatively regulates its target genes (**Figure 7**).

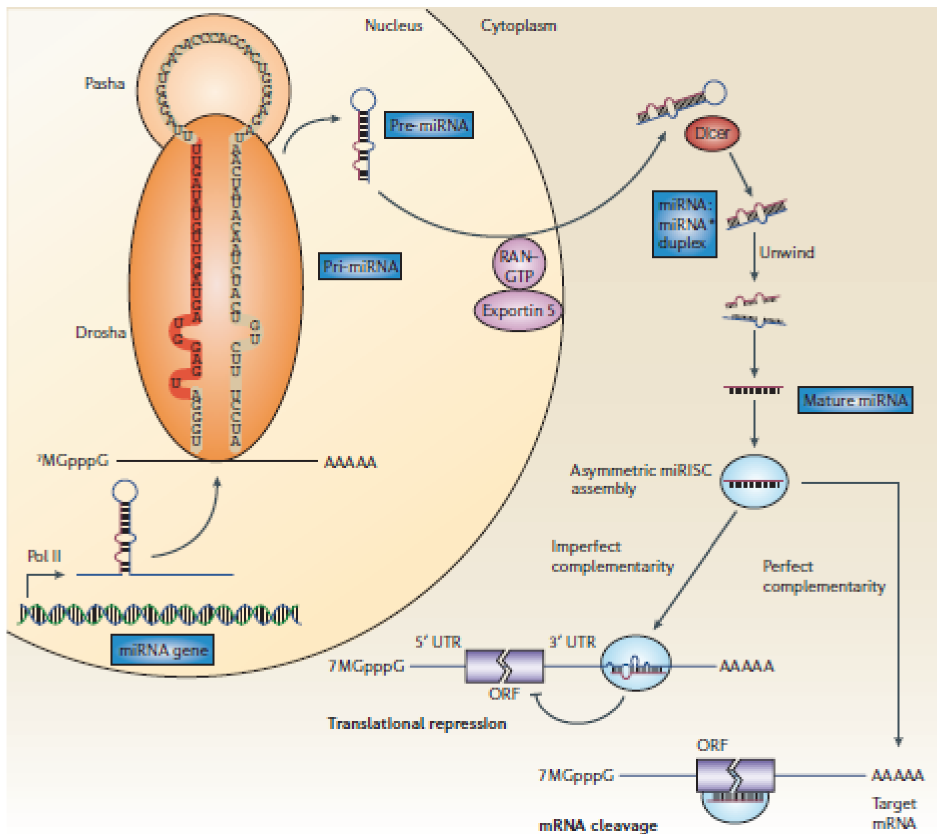


Figura 7. *MicroRNA biogenesis [50]*

The first miRNA gene *lin-4*, was identified in *Caenorhabditis elegans* and controls the timing and progression of the nematode life cycle [51].

Most human miRNAs are found within introns of either protein-coding or noncoding mRNA transcripts. The remaining miRNAs are either located far from other transcripts in the genome, within the exons of noncoding mRNA genes or within the 3′ UTRs of mRNA genes, or they are clustered with other miRNA genes, including

a cluster on chromosome 19 that is comprised of 54 novel miRNAs. MiRNAs can be grouped into families on the basis of sequence homology, which is found primarily at the 5′ end of the mature miRNAs, but whether members of the same miRNA family control similar biological events remains to be seen. Several studies have indicated that the 5′ end of the miRNA is crucial for the stability and proper loading of the miRNA into the miRISC complex, and this end is also important for biological function. Therefore, most bioinformatic algorithms use a 'miRNA seed' that encompasses the first 2–8 bases of the mature miRNA sequence to search for complementarities to sequences in the 3′ UTR of all expressed genes. These studies have revealed that a single miRNA might bind to as many as 200 gene targets and that these targets can be diverse in their function; they include transcription factors, secreted factors, receptors and transporters. [50]

1.3.1 MiRNAs and diabetes

The list of diseases in which dysregulation of miRNAs has been implicated is constantly growing. Compared with cancer, far less is known about the role of miRNAs in diabetes and its complications. The levels of several miRNAs have been reported to be modified in different animal models of diabetes. miR-34a and miR-146a expression is increased in pancreatic islets isolated from diabetic obese mice[52]. Changes in miRNA expression were also observed in different tissues of Goto-Kakizaki (GK) rats, a non-obese model of type 2 diabetes. In the muscle of these rats, two members of the miR-29 family, miR-29a and miR-29b are upregulated compared with healthy rats [53].
MicroRNAs also seem to be involved in diabetes complications. Recent studies demonstrated an involvement in diabetic nephropathy of mir-93, which is regulated by hyperglycemia and controls VEGFa expression [54]. Other groups demonstrated that mir-133 is an important factor for the developing of diabetic cardiomyopathy [55].
Zampetaki et al performed the first plasma microRNAs signature in patients with type-2 diabetes in a large population cohort. They found 5 microRNAs (miR-15a, miR-126, miR-320, miR-223, miR-28-3p) to be deregulated in patients with diabetes[56].

1.3.2 MicroRNAs and angiogenesis

First evidence of miRNAs involvement in the regulation of angiogenesis was found by the inhibition of Dicer and Drosha, two key nucleases involved in miRNA maturation. Knockdown of Dicer or Drosha in vitro inhibits ECs capillary sprouting, migration and tubulogenesis, altering the expression of several key regulators of endothelial biology and angiogenesis [57, 58]. Poliseno et al. identified, for the first time, an endothelial cells miRNAs profile; in particular he found a group of 15 highly expressed miRNAs that could potentially negatively regulate the expression of angiogenic factors [59]. Several microRNAs were described by other groups to be

expressed in EC under normal condition. Moreover, this microRNA expression profile could change if EC are studied in stress conditions or EC are derived from diseased organism [60]. Angiogenesis-regulatory miRNAs can be divided into the pro-angiogenic miRNAs that promote angiogenesis and the anti-angiogenic miRNAs that inhibits angiogenesis:

- *pro-angiogenic microRNAs*:
  mir-126 has been shown to be highly expressed in human endothelial cells. It's important for maintaining vascular integrity during ongoing angiogenesis, targeting SPRED1 and PIK3R2, two negative regulators of VEGF signaling [61];
  mir-130a expression was undetectable in quiescent HUVECs and strongly upregulated after exposure to fetal bovine serum. It down-regulates the anti-angiogenic homeobox protein GAX (growth arrest homeobox) and HoxA5, and functionally antagonizes the inhibitory effects of HoxA5 on tube formation in vitro[62];
  mir-210 is induced by hypoxia in endothelial cells. Mir-210 over-expression enhanced the formation of capillary-like structures and VEGF-induced migration[63];
  Mir-132 expression is induced by angiogenic growth factors, including VEGF-A and bFGF through the phosphorylation of CREB [64]. Moreover, over-expression of miR-132 increases EC proliferation and in vitro networking by targeting p120RasGAP, a GTPase-activating protein.
- *Anti-angiogenic microRNAs:*
  Mir-221/222 inhibits tube formation, migration and wound healing of EC *in vitro* by targeting stem cell factor (SCF) receptor, c-kit. High glucose treatment of HUVECs induced expression of mir-221 and impaired cell migration and reduced expression of c-kit [59];

The role of the **mir-17-92 cluster** in angiogenesis is complex. In fact, mir-18a and mir-19a have been shown to target thrombospondin type 1 repeats (TSR) while mir-17-5p modulate EC migration and proliferation by targeting TIMP1. By contrast, both mir-20a and mir-92a seem to display anti-angiogenesis activity, targeting VEGF-a and integrin-β5 (**Figure 8**).

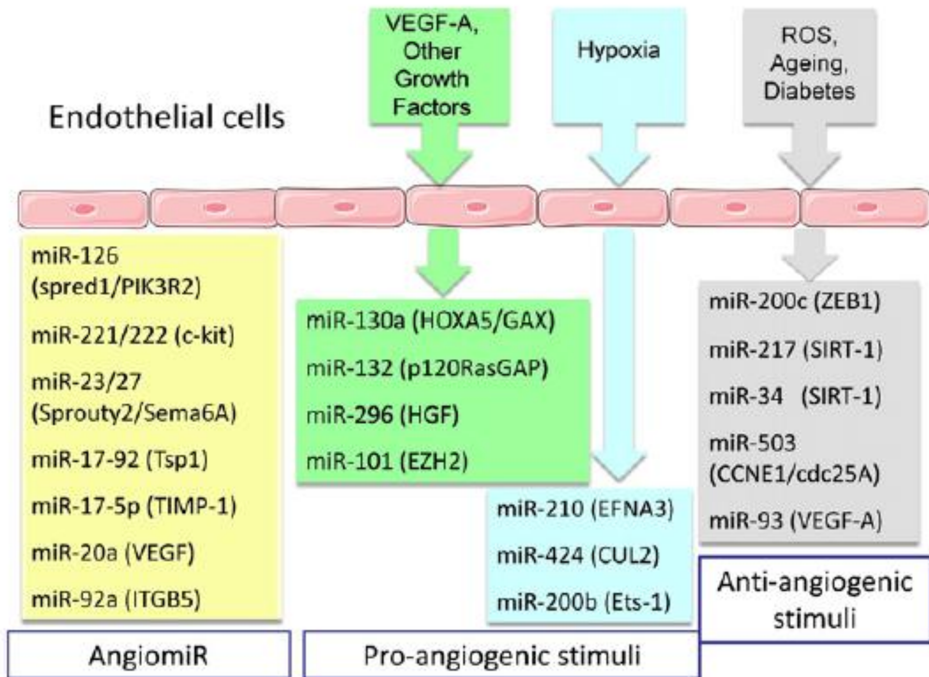


Figure 8. Role of microRNAs in angiogenesis. *The relationship between miRNAs and angiogenesis is bidirectional and it is possible to divide the endothelial miRNAs in three classes: 1) miRNAs that target genes involved in angiogenesis (AngiomiRs), 2) miRNAs whose expression can be modulated by pro-angiogenic stimuli, or 3) miRNAs whose expression can be modulated by anti-angiogenic stimuli.[65]*

2. AIM OF THE WORK

In patients with diabetes, particularly if complicated by ischemic foot lesions, the availability of EPCs is impaired. Different mechanisms may potentially reduce the abundance of circulating EPCs, including impaired mobilization, proliferation, and/or augmented cell loss by necrosis/apoptosis. Moreover, circulating EPCs from diabetic patients show altered function in *in vitro* assays of migration and incorporation into capillary networks. These quantitative and qualitative defects could contribute to the delayed healing of diabetic ulcers as well as to the increased incidence of post-angioplasty restenosis. At the present, however, it remains unknown whether the number of EPCs can be used as a prognostic biomarker.

My project aims to determine the additive value of circulating EPCs in predicting major end-points such as mortality, amputation and post-angioplasty restenosis in diabetic patients with Critical Limb Ischemia (CLI). Furthermore, we aim to obtain mechanistic insights into diabetic progenitor cells impairment and to provide the first characterization of the EPC-associated miRNA with special respect to those potentially involved in the control of angiogenesis.

The project has two major objectives:
A) To validate the prognostic value of endothelial progenitor cells, identified by flow cytometric analysis of antigenic phenotype, in a cohort of 120 patients with type-2 diabetes complicated by CLI. Endpoints are mortality, amputation and post-angioplasty restenosis.
B) To determine the mechanisms responsible for endothelial progenitor cell dysfunction in the perspective of new therapies for the cure of the diabetic foot. In particular we aim to investigate if the microRNA signature could identify high risk patients.

Simultaneous analysis of quantitative and qualitative characteristics of circulating EPCs together with functional signatures of diabetes will allow us to create a cellular profile for prediction of clinical outcome. The biomarkers might be used alone or in combination on a larger scale in patients with initial skin lesions to recognise those that are more prone to develop foot complications.
Compared with previous studies conducted on a limited number of patients, the present one was designed to have sufficient power to draw definitive conclusions on the patho-physiological and clinical relevance of endothelial progenitor cells in diabetic patients as compared with non-diabetic patients affected by foot problems.

3. MATERIAL AND METHODS

Sample size

Since there are no data in the literature concerning the progenitor cells relation with the events considered here (amputations, restenosis, and new atherosclerotic plaques), it is impossible to estimate the number of patients necessary for a study of this kind. From a series of 993 diabetic patients Dr E. Faglia reported a 12% rate of post-angioplasty restenosis. The 5-year incidence for amputation is 84%. Since we don't know the relation between progenitor cells and events, we consider the situation that is described by prevalence of 50% (the most conservative on terms of sample size). On this basis we decided that the prevalence must have an accuracy of 5% with a probability to be showed of 95%. The number of patients calculated is = 91 + 18 (20% drop out) for a total of 109 patients. After one year from the beginning of the enrolment we decided to increase the number of diabetic patients to 120 due to 30% dropout at follow up visit.

Since normal criteria are not already available, to conduct the functional analyses, we enrolled a group of 30 not-diabetic subjects with critical limb ischemia, age- and sex-matched to identify key changes to analyze in the entire group of patients. All patients enrolled were subjected to the same visits of the diabetic group. We also decided to include a third group of 23 healthy volunteers to investigate the role of ischemia in EPCs dysfunction.

Inclusion Criteria

Adult type 2 diabetic patients, both men and women, with chronic critical ischemia as defined by TASC 2007 criteria:

Pain at rest, and/or ulcer or gangrene due to arteriopathy: transcutaneous oximetry < 30 mmHg or pressure on the ankle < 70 mmHg.

Exclusion Criteria

- Cancer with adverse prognosis in months, or chemotherapic treatment
- Ongoing or planned pregnancy
- Lack of consent to participate to the study

Patient enrolment visit:

General comprehensive visit (including: Chest RX, Rest ECG, and glycaemia plus glycosilated haemoglobin measurement)

Ankle arterial pressure

Angiographyc study and angioplasty in the same time

At the enrolment visit and at follow up visit after 12 and 18 months Ecodoppler and Transcutaneous oximetry.

Ulcer severity is measured applying the University of Texas Wound Classification (TUC)

EPCs isolation

Blood samples (35ml) were obtained from a forearm vein and mononuclear white blood cells (MNC) separated on a Ficoll-Paque PLUS (Amersham Biosciences) gradient at 400g. Flow cytometry was used to determine the % of cells expressing respective cell surface antigens (CD45dim, CD34pos, CXCR4pos, KDRpos). Human MNCs were stained for surface antigen expression using combinations of the following antibodies: anti-KDR-PE (R&D Systems), anti-CD45-APC-H7, anti-CD34-PECy7, anti-CXCR4-APC (all BD Pharmingen). For each test, $5x10^5$ to $2x10^6$ total events were analyzed. Fluorescence was analyzed in a FACSCanto flow cytometer using the FACSDiva software (both BD).

For EPCs enrichment, $1x10^7$ MNC/well were plated on fibronectin (Sigma)-coated 6-well plates (BD Falcon) and cultured in EBM-2, supplemented with EGM-2 MV SingleQuots and 10% FBS (EGM-2MV; Cambrex) for 4 days.

Migration Assay

To study directed migration, $5x10^6$ freshly isolated human MNC in 0.5 mL migration medium (EBM-2, 0.1 % BSA) were added in the top chamber of transwell migration inserts (Corning; diameter: 24mm, pore size 3μm) placed inside a 12-well plate. The lower chamber contained 1.2mL migration medium with SDF-1a (100ng/ml). Plain migration medium without SDF-1a was used as controls. The assay was stopped after 16h. After migration, non-migrated cells and migrated cells were analysed by flow cytometry to determine the % of cells expressing respective cell surface antigens (CD45dim, CD34pos, CXCR4pos, KDRpos).

For EPCs migration assay, 5 μm pore-size filters-equipped transwell chambers (Corning) coated with fibronectin were used. Cells ($75X10^3$) were placed in the upper chamber, and allowed to migrate toward SDF-1a (R&D) (100 ng/mL), FBS (10% in EBM medium) or BSA (control).

The assays were stopped after 16 hours at 37°C, and cells that migrated through the filter were fixed on the lower side of the filter and mounted with Vectashield containing DAPI. Five random fields were counted at 20X magnification for each chamber.

RNA extraction and Taq-Man quantitative real time

RNA was extracted using Trizol reagent (Invitrogen) following the manufacturer's instructions. Concentration of the total RNA was determined by the Nanodrop ND1000 Spectrophotometer (Thermo Scientific and the size and integrity of RNAs were assessed using an Agilent 2100 Byoanalyzer.

RNA reverse transcription to measure miRNAs was performed with the TaqMan miRNA reverse transcription kit using the manufacturer's instructions (Applied Biosystems). miR-15 and mir-16 expression was analysed by Applied 7900 Real

Time and normalized to the U6 small nucleolar RNA (snRU6). Each reaction was performed in duplicate. Quantification was performed by the $2^{\Delta\Delta}$ Ct method.
Serum analysis was performed as described in Kroh E et al. 2010[66].

microRNA transfection

EPCs were transfected with 50nM pre-mirna mimics (pre mir-15a and pre-mir-16) or with 50nM mirna inhibitors (anti-mir-15a and anti-mir-16)(Applied Biosystems) using GeneSilencer (Dharmacon). The dose of mimics and inhibitors was selected based on dose response experiments. All experimental control samples were treated with an equal concentration of a non-targeting control mimic sequence, for use as control for nonsequence-specific effects in miRNA experiments. The efficiency of transfection was greater than 95% as assessed by transfection with fluorescently labeled miRNA mimic (mirna mimic-Pe-Cy3)(Applied Biosystems). Verification of the degree of miRNA overexpression and inhibition was determined using quantitative reverse transcription-polymerase chain reaction.

Matrigel Assay

Briefly, $50X10^3$ PHK67-labelled (Sigma) EPCs were added to 8 wells chamber slides pre-coated with 150μl Matrigel (Becton Dickinson), together with $50X10^3$ Texas red-HUVEC in a total volume of 150μl EBM-2 0.1% BSA. After 16 hours incubation at 37°C, floating cells were removed by washing and capillary-like structures were fixed in 2% paraformaldehyde and mounted with DAPI containing fluorescence medium. The assays were performed in duplicate wells. Tube formation was measured by counting the number of intersection points (magnification 20X) in 5 random view fields photographed using a FITC-specific filter setting to concomitantly observe EPCs presence.

Apoptosis assay

Apoptosis was measured quantifying Annexin V positive cells by flow citometry analysis. Briefly, transfected EPCs were stained with 5ul of Annexin V (BD Biosciences) for 15min in the dark. After incubation, Annexin V positive cells were resuspended in 150ul of Annexin Binding Buffer and assed using FACS Canto flow cytometer and FACS Diva Software (BD).

Identification of target genes of mir-15 and mir-16

To determine gene targets of mir-15 and mir-16, five leading miRNA target prediction algorithms (TargetScan 4.1, miRanda, miRDB, MirWalk, RNA22) were used. Western blots for BCL2, AKT3 and β-actin were performed on EPCs lysates from patients or transfected EPCs.

Statistical analysis

Demographic and background information are summarized and displayed using descriptive statistical techniques.

Normality of data distribution was verified with Kolmogorov Smirnov test.

Samples homogeneity was confirmed using Chi Square Pearson test for discrete variables and T- Student’s test for independent sample for continuous variables.

For categorical variables frequency tables are presented, for continuous variables descriptive statistics such as mean, median, standard deviation, IC 95%, minimum, maximum etc. are tabulated.

A p value <0.05 was taken as statistically significant.

Categorical variables were compared with the Chi Square test or Fisher’s Exact test and Cochran Q or Mc Nemar test on the time.

Statistical comparisons between initial and follow-up data for continuous variables were performed using Anova (Two way) test and the Wilcoxon signed rank test.

T-test for independent sample and Mann–Whitney tests were used to compare the subgroups continuous variables.

Analyses were performed with SPSS v.19 software.

4. RESULTS

4.1 Study design

The study has two objectives:

I) To validate the prognostic value of EPCs, identified by flow cytometry analysis of antigenic phenotype, in a cohort of 120 patients with type-2 diabetes complicated by CLI. Events are mortality, amputation and post-angioplasty restenosis. Statistical intermediate analysis of the results obtained is reported in section 4.3.

II) To determine the mechanisms responsible for EPC dysfunction. In particular we aim to investigate the expression of microRNA implicated in the regulation of vascular function.

Two additional control groups have been added in the course of the study to define normal criteria for functional and molecular analyses.

Hence, we have enrolled 3 groups of individuals:

1) **120 out of 120** Diabetics patients with critical limb ischemia (CLI)
2) **20 out of 30** Non diabetic ischemic patients with CLI
3) **23 out of 23** Healthy subjects-controls (no follow up (FU))

In **Table 3,** demographic characteristics are summarized.

	Healthy (n=23)	**Non diabetic CLI (n=20)**	**Diabetic CLI (n=120)**
Age, years	42±8	77±10	71±9
Gender, male (%)	48%	55%	60%
Diabetes year	-	-	18.6±11.9

Table 3. Clinical features of all enrolled groups. *Values are mean±S.D*

4.2 Laboratory evaluations

We received peripheral blood from all the patients enrolled in the study and performed experiments to evaluate the antigenic profile of isolated MNCs and their migratory ability. **Figure 9** illustrates the experimental protocol.

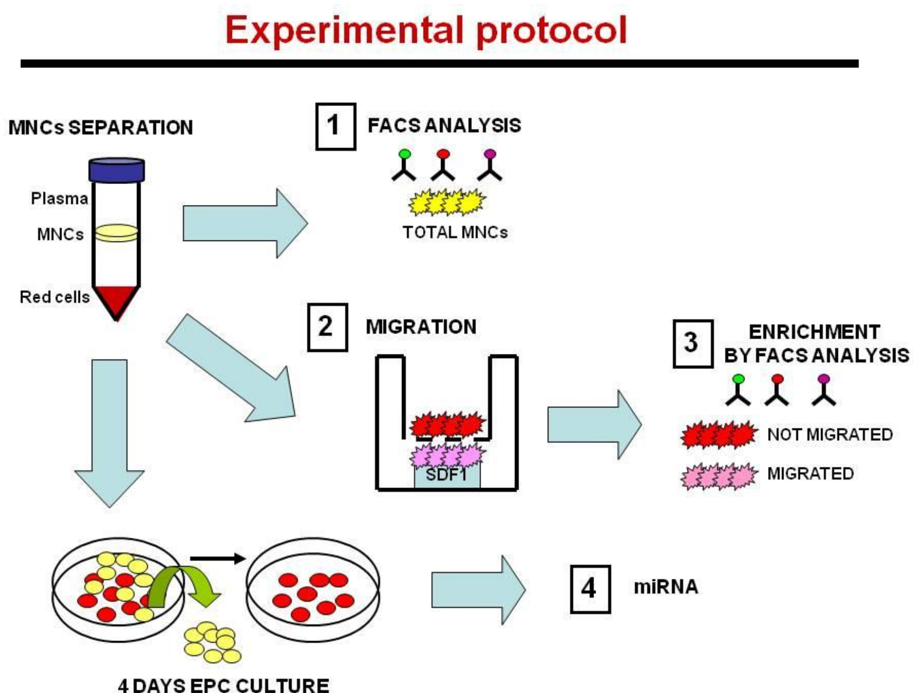


Figure 9. *Scheme of the experimental plan.*

4.2.1 Phenotypic characterization of freshly isolated MNC

Using ISHAGE guidelines, we performed flow cytometry analysis of $CD34^{pos}$ and $CD45^{low}$,$CD34^{pos}$,$CXCR4^{pos}$,KDR^{pos} cells (EPCs) from freshly isolated total MNCs of healthy controls (n=23), non diabetic CLI (n=19), diabetic CLI patients (n=120) (**Figure 10**).

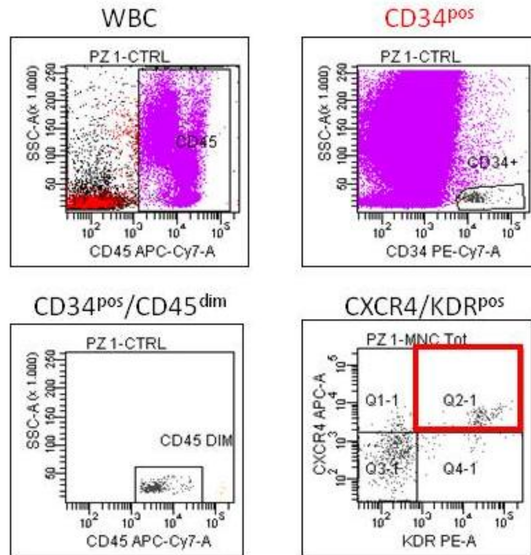


Figure 10. Flow citometry strategy to detect EPCs. *Based on ISHAGE guidelines we performed a flow citometry gating strategy to detect EPCs (red box). EPCs were defined as CD45^{low}/CD34^{pos}/CXCR4^{pos}/KDR^{pos}*

Based on literature data, we decided to analyze these two MNCs subpopulation because it was demonstrated their involvement in reparative mechanism and also the impairment of their functionality caused by diabetes [67].

CD34 positive cells were reduced by ischemia (% CD34 positive cells: Healthy: 0.1190±0.01, non diabetic CLI: 0.07±0.015, diabetic CLI: 0.084±0.006; *p<0.05 vs Healthy) but no differences were observed between patients with or without diabetes.

Ischemia reduced the number of EPCs compared with healthy controls; surprisingly, diabetic CLI patients have higher EPC number compared with non diabetic CLI (Healthy: 0.04±0.012, non diabetic CLI: 0.007±0.001, diabetic CLI: 0.021±0.002, *p<0.05 vs Healthy, #p<0,05 vs non diabetic CLI) **(Figure 11).**

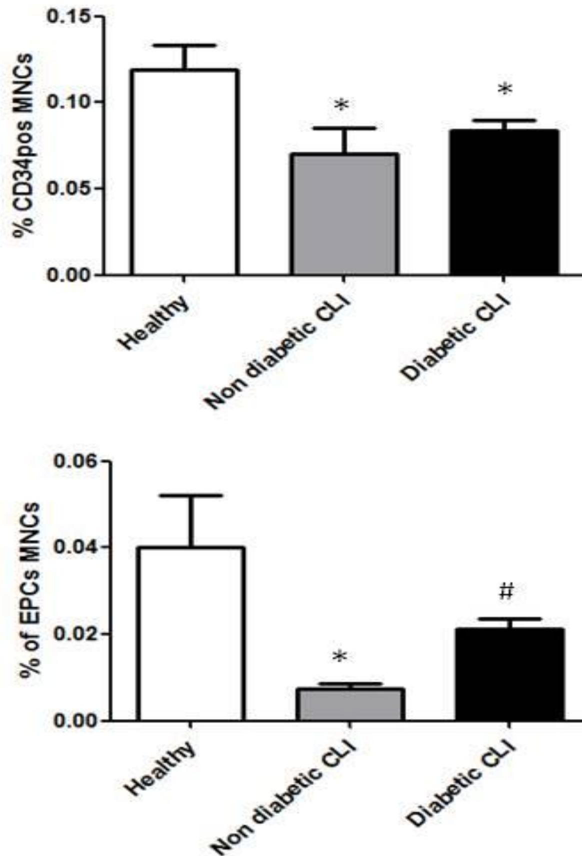


Figure 11. Progenitor cells analysis on total MNC. *Flow cytometry quantification of $CD34^{pos}$ (**upper panel**) and EPCs ($CD45^{low}/CD34^{pos}/CXCR4^{pos}/KDR^{pos}$) (**lower panel**) from healthy, non diabetic CLI and diabetic CLI patients. (*p<0.05 vs Healthy and #p<0,05 vs non diabetic CLI).*

Ischemia and diabetes impairs MNC migratory ability

Transwell migration assay was performed toward SDF-1α using total MNCs from healthy controls, non diabetic CLI or diabetic CLI patients. We found a decrease of MNC migratory activity in ischemic patients with or without diabetes as compared with controls (% of SDF-1α mig cells vs vehicle: healthy: 2.1$\pm$0.11; non diabetic CLI: 1.57$\pm$0.1; diabetic CLI 1.43$\pm$0.05). **(Figure 12)**

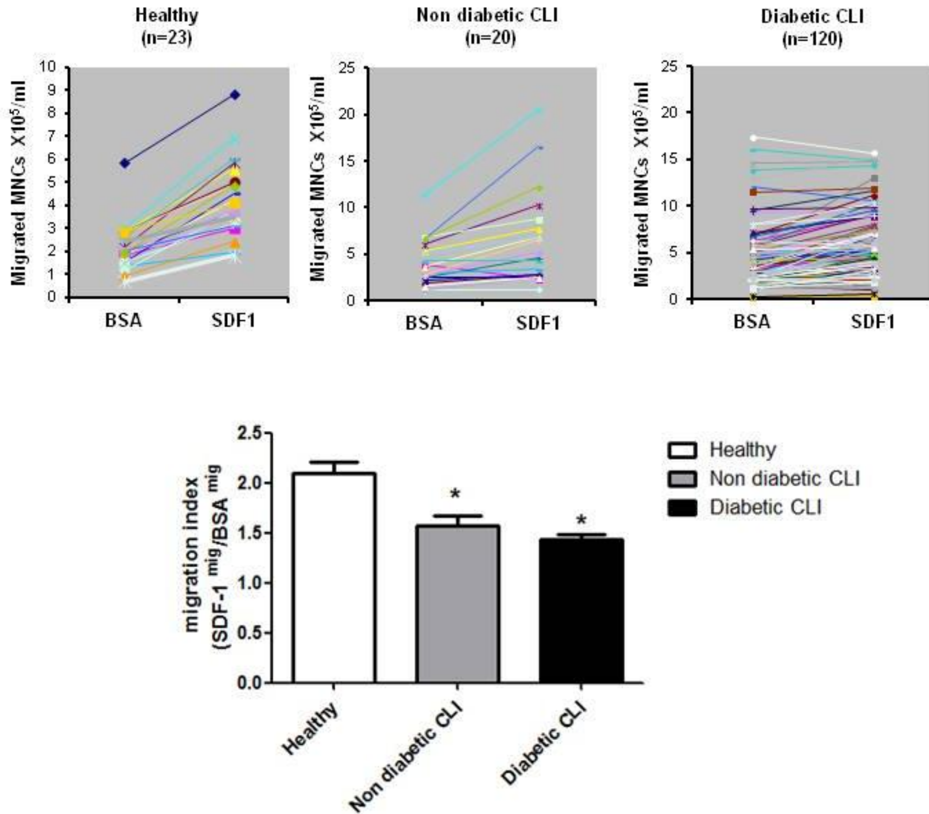


Figure 12. Migratory ability of MNCs. Upper panel *Graphs illustrating MNCs migratory ability in the three groups enrolled.* ***Lower Panel*** *Bar graphs showing MNC migration index. Migration index was calculated as ratio between SDF-1 migrated cells and BSA migrated cells. (*$p<0.05$ *vs Healthy)*

Diabetes impairs SDF-1 driven EPCs enrichment

We then verified whether CD34 positive cells and EPCs can be enriched from total MNCs based on migratory response to SDF1-α. After performing a transwell migration assay toward SDF-1α, we harvested migrating and non-migrating cells from the lower and upper chambers, respectively.

SDF-1α stimulation resulted in a 1.7 and 1.3-fold enrichment of EPCs in the migrated fraction in healthy and non diabetic CLI respectively; whereas, the response of EPCs to SDF-1α was totally abrogated in diabetic patients (**Figure 13**). We did not observe any difference in the percentage of CD34pos after migration in the three groups (data not shown).

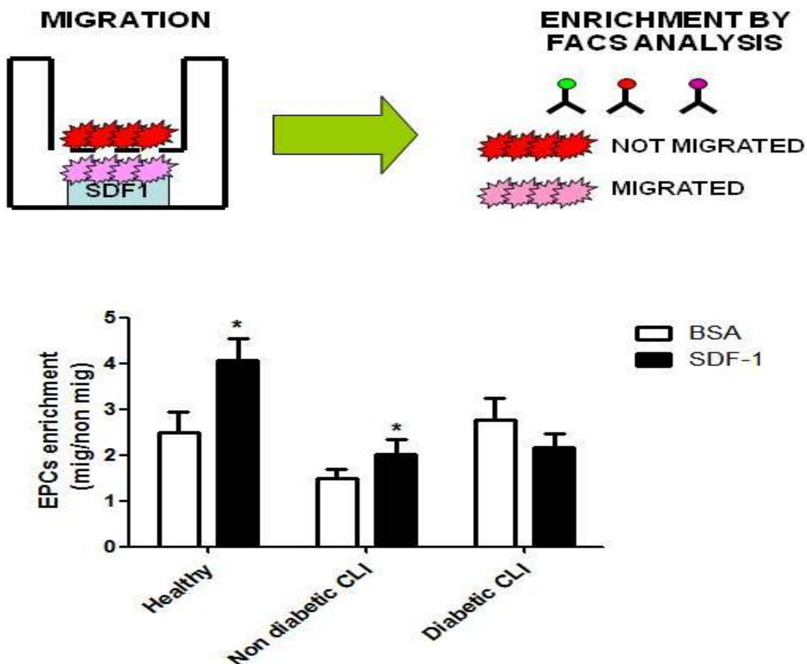


Figure 13. Diabetes impairs SDF-1 induced EPC enrichment. Upper panel *Scheme of flow citometry strategy to detect EPCs enrichment.* ***Lower panel*** *Quantification of MNCs after migration. Data are shown as ratio between migrated cells (mig) and not-migrated (non mig) cells to SDF-1 or vehicle. (*p<0,05 vs BSA).*

Diabetes does not de-regulate CXCR4 expression on MNCs

In order to understand if diabetic EPCs impairment is due to lack of CXCR4, SDF-1 receptor was quantified on the surface of MNCs that express EPC marker. **Figure 14** shows no difference among the two groups using flow cytometry. In line, no difference was also observed in mRNA levels and protein expression of cultured EPCs from the three groups. (data not shown)

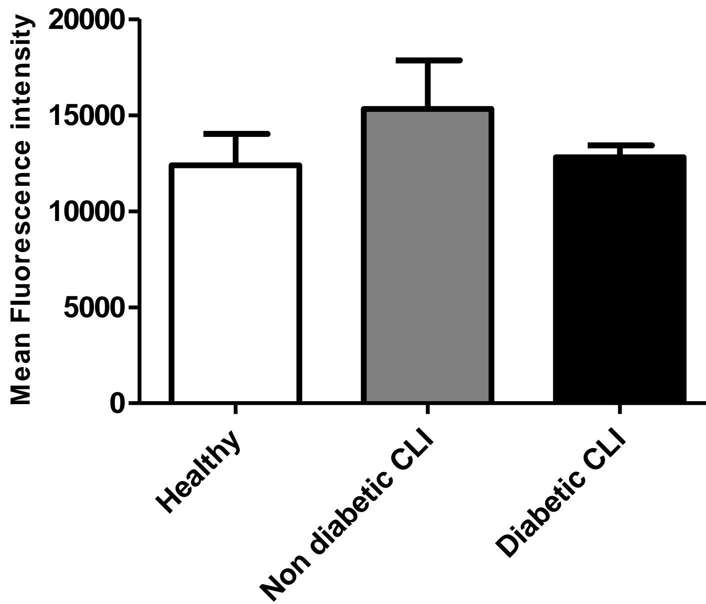
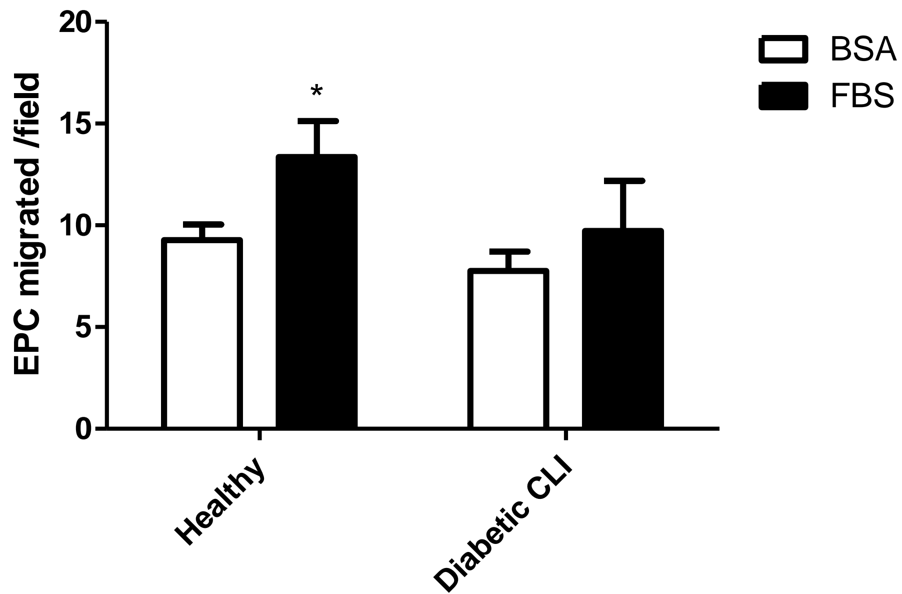


Figure 14. Expression of CXCR4 in MNCs defined as EPCs. *Flow cytometry analysis of CXCR4 on MNCs from all the patients enrolled. Graphs bar are calculated as mean fluorescence intensity ± s.e.m.*

Diabetes impairs the functions of cultured EPCs.

Circulating MNCs give raise to endothelial-like cells in culture on fibronectin. These cells that co-express CD34/CD45/CD14/CXCR4/KDR[11], are named early culture EPCs. We investigated if diabetes induces alterations of early culture -EPC as described in literature. We analyzed EPC migratory ability in healthy individuals (n=5) and diabetic patients (n=5) using FBS (**Figure 15 upper panel**) or SDF-1 as stimulus (**Figure 15 lower panel**).



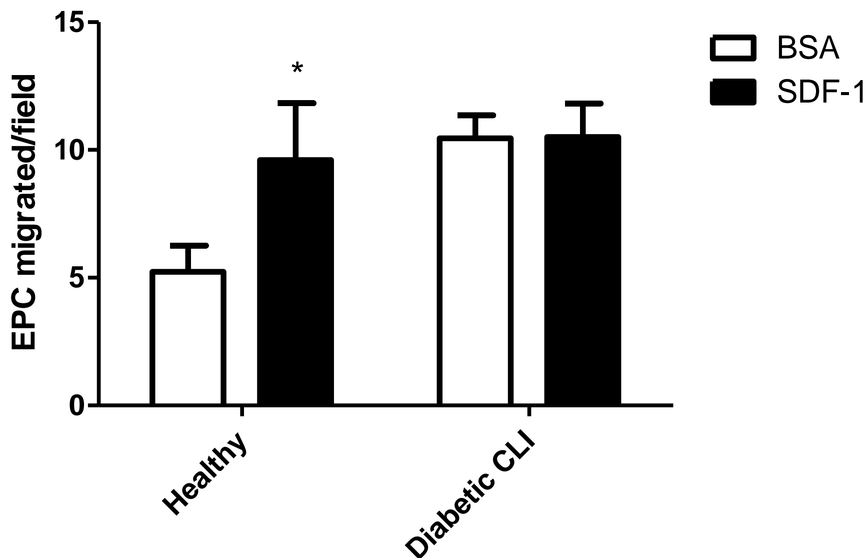


***Figure 15. Diabetes induces early culture EPCs migration impairment**. Graph bar showing number of healthy (n=5) or diabetic (n=5) cultured-EPCs migrated cells vs FBS (**upper panel**) or SDF-1 (**lower panel**) (*p<0.05 vs BSA).*

Then we analyzed the capacity of early culture EPCs to induce capillary-like structures on a matrigel layer and we confirmed that diabetes causes EPC

impairment (**Figure 16 left panel**). We also observed an increase of apoptosis in diabetic cells compared with controls (**Figure 16 right panel**)

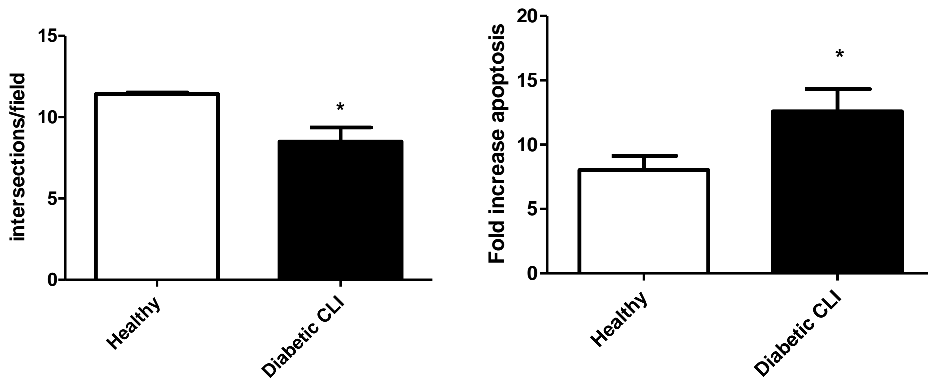


***Figure 16. Diabetes induces early culture EPCs dysfunction. Left Panel**. Graph bars shows a decrease in capillary like structure formation by HUVECs cocultured with diabetic EPCs. (*p<0.05 vs Healthy). **Right Panel**. Diabetes increases apoptosis of EPCs compared to healthy control (*p<0.05 vs Healthy).*

4.3 Intermediate statistical analysis

We here report the intermediate analysis performed on diabetic patients that reached the 12 month follow up visit by September 2011.

Ninety-four out of one-hundred-twenty (94/120) diabetic patients enrolled to the study reached the 12 month follow up, and 58.5% had at least one event registered: restenosis or amputation or death (**Table 4**).

	Events (# of total)
Patients at FU	**94** (58,5%)
Restenosis	**37** (39,36%)
Amputation	**10** (10,64%)
Death	**7** (7.45%)
Restenosis-amputation-death	**1** (1.06)
No events	**39** (41,49%)

Table 4. Events registered at follow-up visit (September 2011).
Percentage of events considering the total number of diabetic patients.

For the purpose of the analysis the diabetic patients group has been subdivided in two subgroups of patient: 1) patients with at least one event (with event), and 2) patients with no events (no event). A detailed description of the patients' characteristics follows.

Diabetic patients with an event were male for the 67.3%. No significant difference was observed considering gender variable (p values=1.00) (**Table 5**).

Gender	With Events		No events		Total	
	n°	%	n°	%	n°	%
Male	37	67.27	27	69.23	64	68.09
Female	18	32.73	12	30.77	30	31.91
Total	55	100	39	100	94	100

Table 5. Analysis of events distribution considering gender as a variable

We did not find a significant association with age between patients with or without events.

Table 6 shows that no difference was observed also considering current anti-diabetic treatments (p values =0.336).

Terapy	With Events		No events		Total	
	n°	%	n°	%	n°	%
Diet	3	5.45	2	5.13	5	5.32
OH (Oral Hypoglycemic)	13	23.64	9	23.08	22	23.4
Insulin	28	50.91	20	51.28	48	51.06
Diet + Insulin	10	18.18	3	7.69	13	13.83
Diet + OH + Insulin	1	1.82	2	5.13	3	3.19
OH + Insulin	0	0	1	2.56	1	1.06
OH + Diet	0	0	2	5.13	2	2.13
Total	55	100	39	100	94	100

Table 6. Analysis of events distribution using diabetic drugs as variable

We also analyzed whether an association exists between other clinical parameters and the presence of events. We found no differences between patients with or without events when considering LDL (p value 0.834), HDL (p=0.272) and triglycerides levels (p=0.796). We found no association when considering smoke (p=0.696)

We also considered if the co-existence of other diseases could be correlated to the occurrence of an event. However, as illustrated in **Table 7** no one of these clinical conditions shows significant correlation with the events.

Clinical outcomes	**With Events**		**No events**		**Total**	
Hypertension	**n°**	**%**	**n°**	**%**	**n°**	**%**
No	26	47.27	10	25.64	36	38.3
Yes	29	52.73	29	74.36	58	61.7
Total	55	100	39	100	94	100
CAD						
No	27	49.09	22	56.41	49	52.13
Yes	28	50.91	17	43.59	45	47.87
Total	55	100	39	100	94	100
CVA						
No	48	87.27	34	87.18	82	87.23
Yes	7	12.73	5	12.82	12	12.77
Total	55	100	39	100	94	100
CKD						
No	37	67.27	23	58.97	60	63.83
Yes	18	32.73	16	41.03	34	36.17
Total	55	100	39	100	94	100
Retinopathy						
No	44	80	34	87.18	78	82.98
Yes	11	20	5	12.82	16	17.02
Total	55	100	39	100	94	100
Neuropathy						
No	47	85.45	35	89.74	82	87.23
Yes	8	14.55	4	10.26	12	12.77
Total	55	100	39	100	94	100

Table 7. Presence of other clinical diseases in diabetic patients group. *List of disease in the table: CAD=Cardiovascular artery disease, CVA=cerebrovascular accident, CKD= Chronic Kidney Disease*

We also compared the values of Transcutaneous PO2 (TC) level, before and after Percutaneous Transluminal Angioplasty (PTA), a parameter that when increased confirms the success of the PTA procedure. We interestingly found significant difference before and after PTA in both of the two groups of patients ($p<0.001$) and also a difference ($p=0.046$) between TC level after PTA between group with or without events as reported in **Table 8**. This data suggests that the improvement in perfusion after PTA is instrumental to a better outcome if associated to amelioration of oxygen diffusion, i.e. less severe microangiopathy.

TC	PTA	NUMBER	MEAN	ST. DEV	CONF. INTERVAL	
With Events	PRE	38	17.53	11.01	13.91	21.15
	POST	38	36.5	19.57	30.07	42.93
No events	PRE	25	20.36	10.21	16.15	24.57
	POST	25	45.12	16	38.51	51.73

Table 8. Values of TC levels before and after PTA in the two groups.

Next, in the attempt to find a correlation between the number and function of EPC and the occurrence of the adverse events (first objective of the study), we analyzed the collected laboratory parameters vs. the events. As illustrated in **Figure 17**, analysis of the number of CD34pos MNCs and EPCs between diabetic patients with or without events did not show any significant difference.

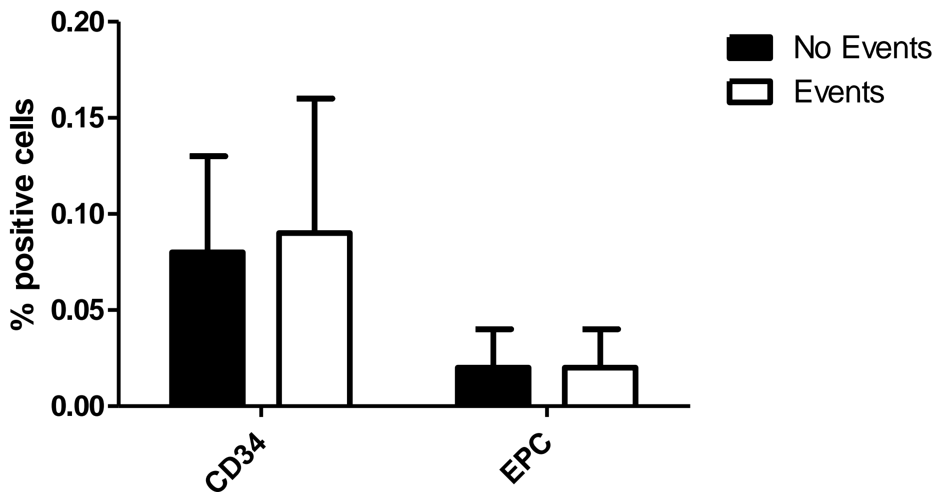


Figure 17. Numbers of progenitors cells in diabetic patients with or without events.

Statistical analysis revealed a significant difference considering the number of SDF-1 migrated cells compared to BSA migrated cells (p<0.001). This result shows that, even if MNC isolated from diabetic patient are less responsive to SDF-1 stimulus compared with healthy or non diabetic controls, they still migrate toward the chemoactractant. When we analyzed migration results, dividing patients with or without events we did not observe any significant difference.
We also analyzed the number of CD34 positive cells of the MNC migrated fraction and we did not observe difference between patients with or without events.

Interestingly, the analysis of EPC number after migration revealed a significant increase in patients with events (p=0.016), but only in the BSA migration fraction (**Figure 18**).

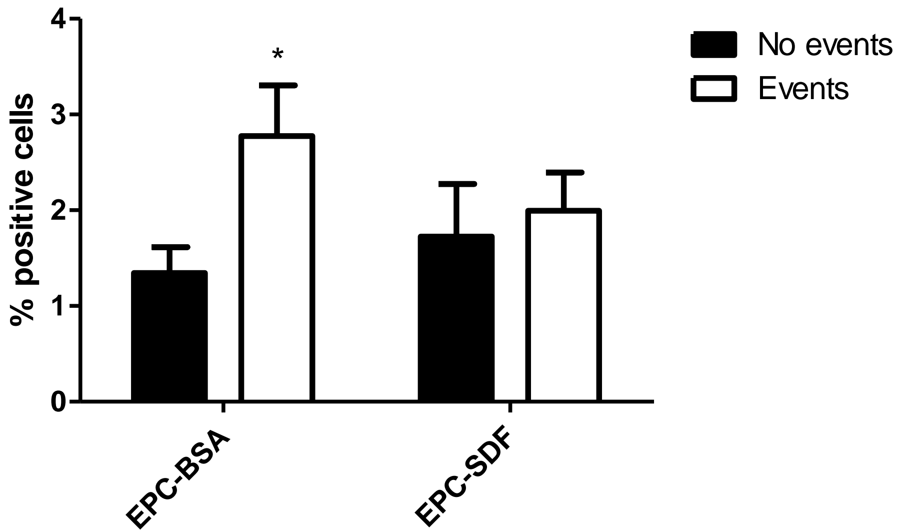


Figure 18. Analysis of EPCs number after migration in the diabetic groups.

Although the above mentioned statistical significance was reached for the analysis of EPC BSA-migrated, we did not obtain significant results when we attempted to identify predictive values using logistic univariate regression. At the end of the study, when all the enrolled patients will reach the follow up and a larger number of events will be recorded, we expect to be able to perform this crucial analysis.

4.4 Mechanism of diabetic EPCs impairment: microRNAs

Ischemia and diabetes de-regulate microRNAs signature

In order to identify other mechanism that potentially explain EPCs impairment in diabetes we analyzed the expression of 29 different microRNAs in 4 days cultured EPCs from healthy and CLI patients with or without diabetes (n=5 for each groups) (**Table 5**).

	Relative expression to snRU6		
	Healthy	**Non diabetic CLI**	**Diabetic CLI**
Mir-1	1,404± 0,58	1,293±1,19	1,408±0,58
Mir-9	1,102±0,24	0,702±0,32	3,264±1,31
Mir-15a	1,019±0,10	0,689±0,35	2,254±0,44
Mir-15b	1,078±0,21	1,405±0,4	1,960±0,21
Mir-16	1,041±0,15	0,73±0,25	1,962±0,27
Mir-17	1,119±0,28	0,426±0,11	1,230±0,25
Mir-18a	1,045±0,15	0,545±0,24	2,068±0,23
Mir-19a	1,137±0,3	0,347±0,21	1,426±0,13
Mir-19b	1,266±0,46	0,406±0,25	1,5±0,11
Mir-20a	1,047±0,16	0,369±0,16	1,394±0,24
Mir-23a	1,160±0,26	1,864±0,37	3,295±0,79
Mir-24	1,076±0,26	0,835±0,18	1,956±0,18
Mir-27b	1,008±0,06	1,052±0,5	2,794±0,67
Mir-92a	1,083±0,19	0,636±0,25	0,724±0,1
Mir-100	1,548±0,77	0,429±0,05	0,528±0,04
Mir-103	1,10±0,19	1,405±0,49	2,882±0,61
Mir-107	1,083±0,2	0,952±0,33	2,731±0,58
Mir-126	1,197±0,28	0,304±0,14	0,980±0,27
Mir-130a	1,073±0,21	0,841±0,41	1,207±0,4
Mir-132	1,037±0,14	0,730±0,15	2,673±0,63
Mir-195	1,044±0,15	0,464±0,21	1,163±0,16
Mir-221	1,035±0,14	0,382±0,17	1,65±0,46
Mir-222	1,133±0,28	0,219±0,05	0,319±0,06
Mir-296	1,305±0,39	2,235±0,29	1,577±0,19
Mir-424	1,027±0,12	0,628±0,28	2,335±1,12
Mir-378	1,073±0,19	1,793±0,53	3,216±1,14
Mir-497	1,129±0,27	0,341±0,13	0,386±0,1
Mir-503	1,096±0,2	0,934±0,26	1,479±0,53

Table 9. MicroRNAs levels in EPCs*. Table shows the relative expression of mirs in EPCs (n=5 patients for each group). MicroRNA expression was normalized to snRU6 expression using the comparative Ct methods. Values are means ±S.E.M.*

Ischemia up-regulates mir-15a, mir-16 and mir-221 expression in EPCs mRNAs.

In different cancer cells, mir-15a and mir-16 cluster have been shown to play an important role in regulating cell proliferation and apoptosis by targeting genes involved in cell cycle progression [68]. Mir-15b and mir-16 have been shown to control the expression of VEGF in carcinoma cell lines [69]. In addition, mir-15b and mir-16 have also been shown to be differentially expressed in endometriosis, in which angiogenesis may be pathogenically involved [70]. Based on these data we decided to investigate the role of mir-15a and mir-16 in diabetic CLI EPCs. We performed mir-15a and mir-16 analysis on healthy (n=17), non diabetic CLI (n=16) and diabetic CLI (n=56). We found an increase of these two microRNAs by ischemia (mir-15a 2-ddCt: healthy:1.4±0.3; non diabetic CLI: 7.7±1.9, diabetic CLI: 5.19±0.8; $p<0.05$ vs Healthy; mir-16 2-ddCt: healthy:1.13±0.12; non diabetic CLI: 4.51±1.13, diabetic CLI: 4.9±0.9; $p<0.05$ vs Healthy). Ischemia also induced the over-expression of mir-221 (mir-221-ddCt: healthy:1.25±0.19; non diabetic CLI: 6.02±1.6, diabetic CLI: 5.15±1.04; $p<0.05$ vs Healthy). This microRNA is potentially involved in anti-angiogenic mechanisms and previous studies also demonstrated that this miRNA is involved in c-kit down-regulation. **(Figure 19**)

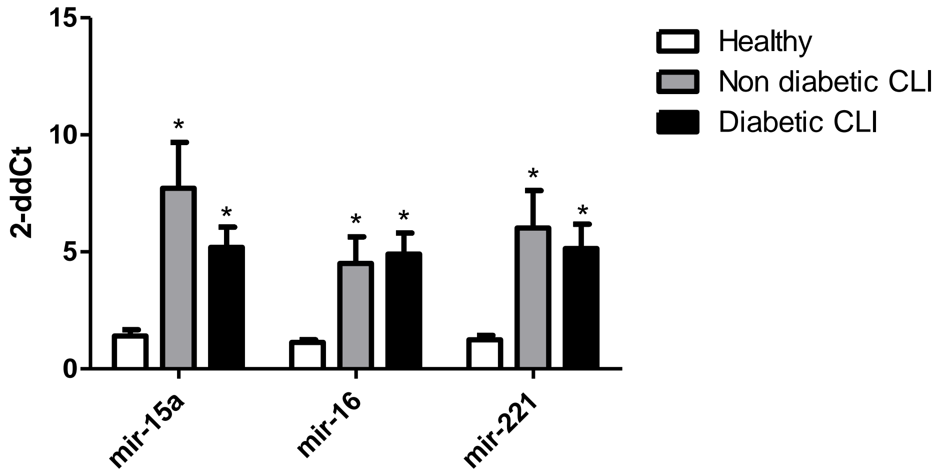


Figure 19. Ischemia significantly increases mir-15a, mir-16 and mir-221 levels in EPCs compared to healthy controls. *Relative expression of mirs in EPCs. MicroRNA expression was normalized to snRU6 expression using the comparative Ct methods. Values are means ±S.E.M (*p<0.05 vs Healthy).*

Mir-15a, mir-16 and mir-221 are all up-regulated in the circulation of ischemic patients with or without diabetes.

Circulating microRNAs levels have been used for diagnostic and prognostic purposes. We analyzed circulating microRNAs levels in serum from all enrolled patients. Ischemia increases serum levels of mir-15a (2-ddCt: healthy:0.99±0.15; non diabetic CLI: 6.89±1.23; diabetic CLI:7.81±1.25; *p<0.05 vs Healthy) mir-16 (2-ddCt: healthy: 1.14±0.19; non diabetic CLI: 2.82±0.63; diabetic CLI:3.87±0.52; *p<0.05 vs Healthy) and mir-221 (2-ddCt: healthy: 1.03±0.18; non diabetic CLI: 3.46±0.56; diabetic CLI:3.24±0.43; *p<0.05 vs Healthy) (**Figure 20**).

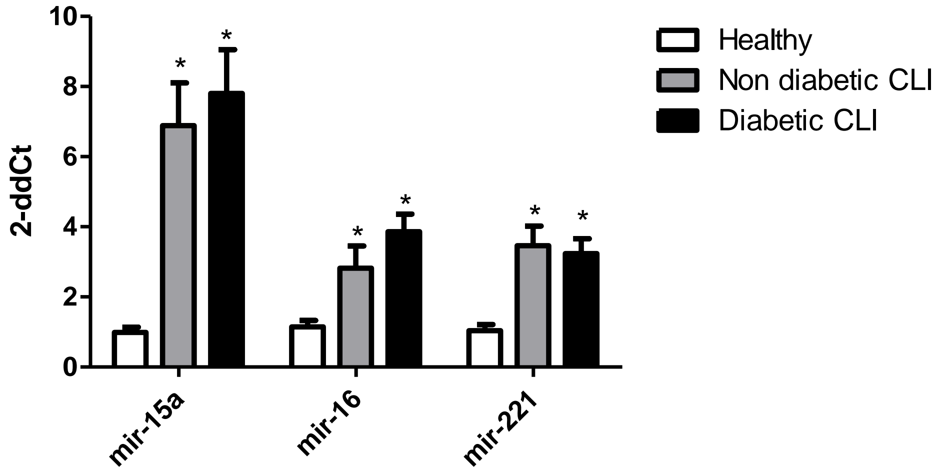


Figure 20. Serum levels of mir-15, mir-16 and mir-221 are up-regulated by ischemia. *Relative expression of serum microRNAs. MiRNA expression was normalized to exogenous cel-mir-39 expression using the comparative Ct methods. Values are means ±S.E.M (*p<0.05 vs Healthy).*

Mir-15/16 over-expression effect on EPCs functionality (migration, apoptosis and pro-angiogenic capacities)

To investigate the functional relevance of mir-15 and mir-16 in EPCs, we transfected pre-microRNA in healthy EPCs.

After confirmation of microRNAs over-expression by qPCR analysis (**Figure 21**), we performed migration assays using unspecific stimulus, FBS, or a specific stimulus, SDF-1. We observed that only combined mir-15 and mir-16 co-expression blocks FBS-induced (n=5) and SDF-1 (n=5) driven EPC migration. (**Figure 22**)

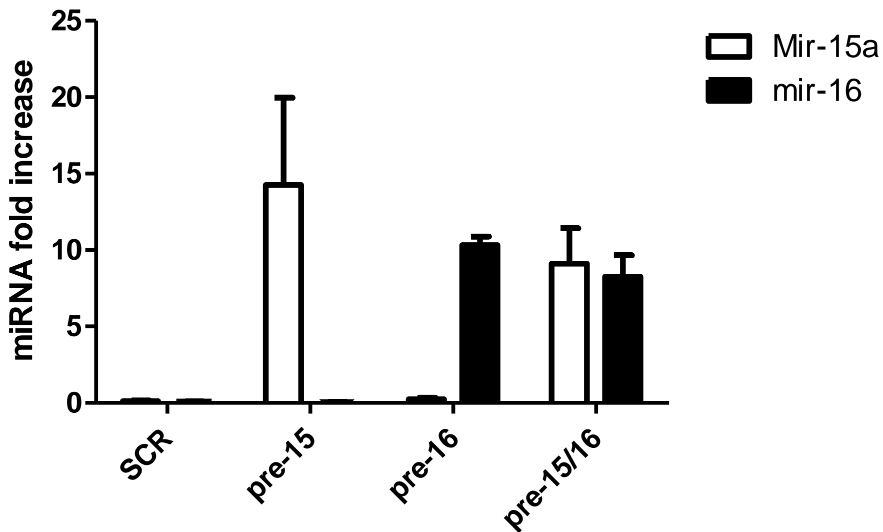


Figure 21. Pre-mir transfection increases mir-15 and mir-16 expression in healthy EPCs. *Relative expression of mir-15a and mir-16 in EPCs (n=3) after transfection. MicroRNA expression was normalized to snRU6 expression using the comparative Ct methods. Values are means ±S.E.M (*p<0.05 vs Healthy)*.

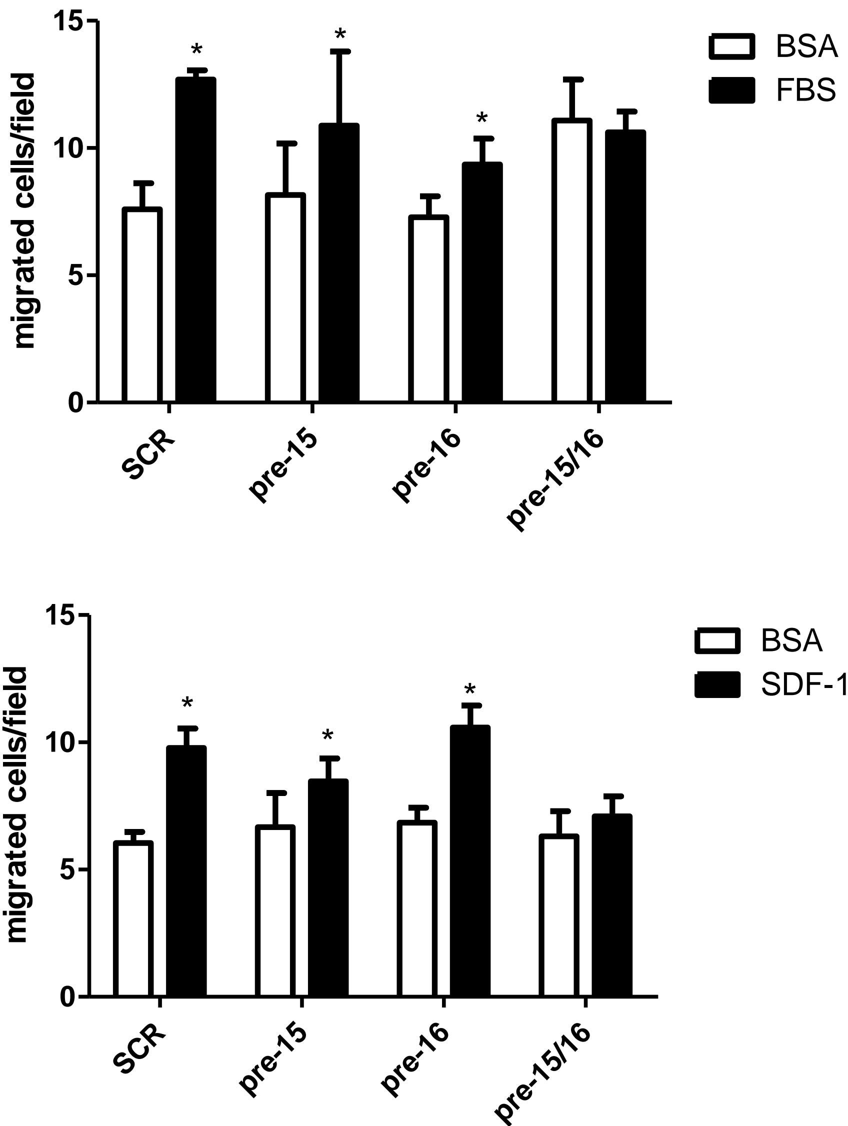


***Figure 22**. **Mir-15 and mir-16 over-expression inhibits EPCs migration**. Graph bars show number of migrated cells vs FBS (**upper panel**) or SDF-1 (**lower panel**) compared to vehicle. We performed experiments on five different donors. For each conditions migrated cells were counted in five random fields (*p<0.05 vs BSA).*

Then, based on literature data showing that mir-15a and mir-16 regulate cell survival in cancer cell lines, we investigated if mir-15 and mir-16 could induce EPCs apoptosis. After transfection of healthy EPCs, we analyzed the percentage of

Annexin V positive cells by flow cytometry. We found that mir-15/16 over-expressing EPCs have higher abundance of Annexin V expression as compared to EPCs transfected with the control vector (SCR). (**Figure 23**)

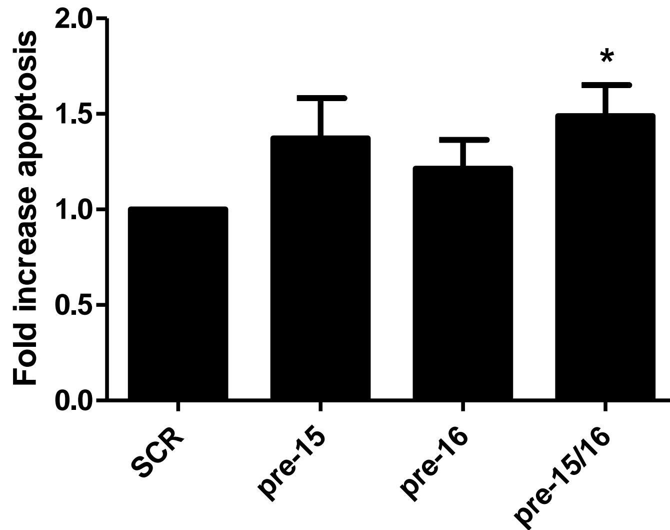


Figure 23. Mir 15/16 increases apoptosis*. Flow cytometry quantification of apoptosis in transfected EPCs. Ten different donors were studied (*p<0.05 vs scrambled).*

Finally, we investigated if mir-15 and mir-16 expression can modulate the pro-angiogenic activity of EPCs. For this purpose, we performed co-culture of microRNA over-expressing EPCs with HUVEC on a matrigel layer. We did not observe any significant difference compared with scramble control. (**Figure 24**)

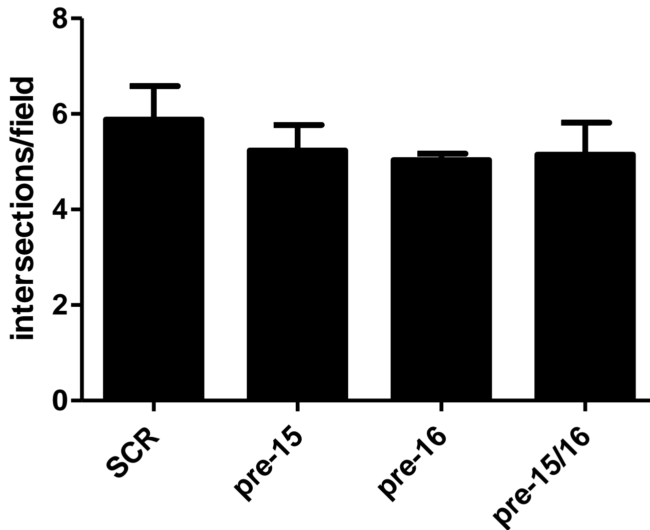


Figure 24. mir-15/16 expression does not alter EPC pro-angiogenic in vitro activity. *Graph bar indicating number of intersections of endothelial network on matrigel layer (n=5).*

AKT3 is a direct target of Mir-15/16 cluster.

Bionformatic analyses predict BCL2, VEGF-a, AKT3 to be target genes of miRNA-15a and -16, which would suggest anti-angiogenic and pro-apoptotic activities of these miRNAs. All these genes have been validated as direct targets of mir-15 and mir-16 [71].

Target genes analysis on EPC lysates from the three groups demonstrates AKT3 down-regulation in ischemic patients both at mRNA (n=5 for each groups) and protein level (n=3 for each groups) as compared to controls (**Figure 25**).

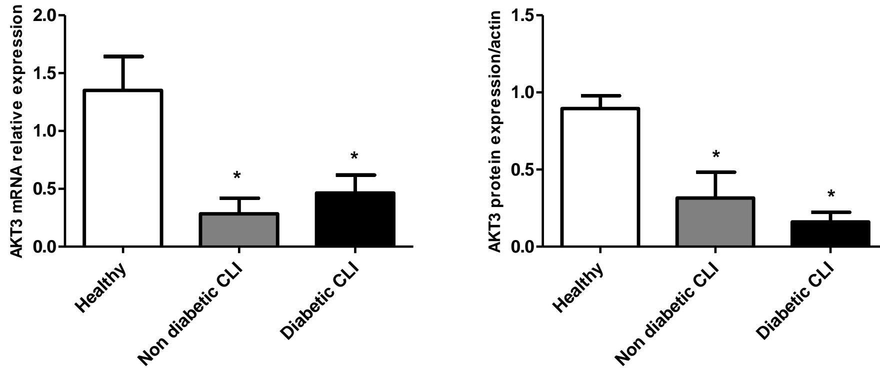


Figure 25. Mir-15 and mir-16 targets AKT3 expression. Left Panel. *We analyzed AKT3 mRNA relative expression by qPCR on RNA extracted from EPCs of the three different groups (n=5 for each group).* ***Right pane****l, we confirmed AKT3 down-regulation by Western blot analysis (n=3 for each group). AKT3 protein levels were normalized by β-actin analysis (*p<0.05 vs Healthy).*

Protein analysis of EPCs lysates revealed no difference in BCL2 expression both in patients and in microRNAs over-expressing EPCs.
Then, we investigated if similar effects are produced by mir-15/16 overexpression. As expected, over-expression of mir-15/16 in healthy EPCs down-regulated AKT3 only at protein level. (**Figure 26**).

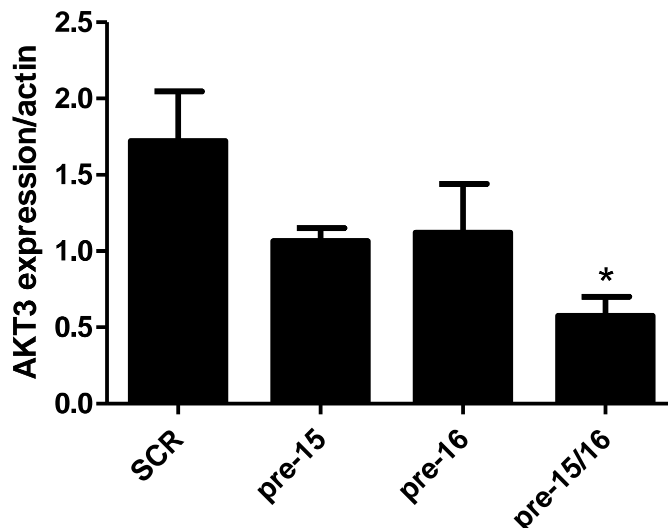


Figure 26. mir-15/16 down-regulates protein levels of AKT3*. Quantification of AKT3 expression in transfected EPCs normalized by β-actin Data is mean of four different experiments. (*p<0.05 vs Healthy*).

No difference was observed in BCL2 expression after up-regulation of mir-15/16 (data not shown).

Inhibition of mir-15/16 rescues diabetic EPC migratory capacity.

To investigate if microRNA down-regulation could restore diabetic EPC migratory ability, we transfected EPCs with oligo anti-mir 15/16. After confirmation of mir-15 and mir-16 inhibition by qPCR (**Figure 27**), we performed *in vitro* migration on diabetic EPCs.

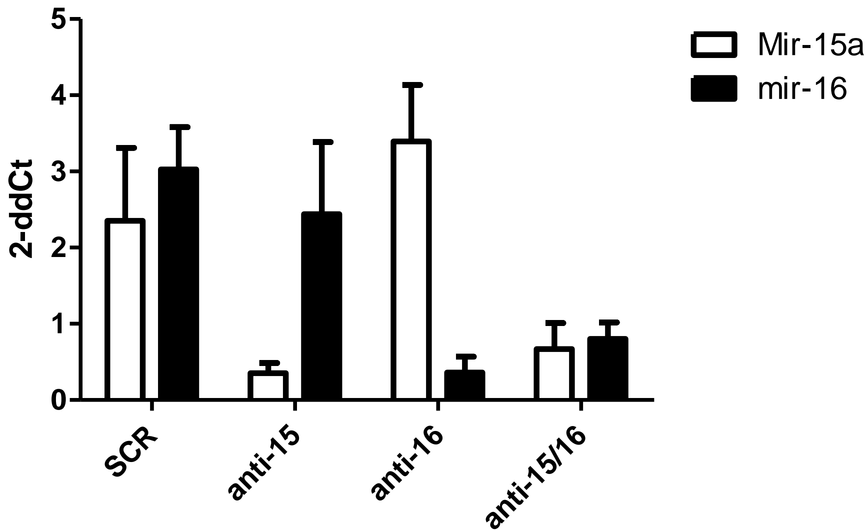


Figure 27. Co-trasfection of diabetic EPCs with antimirs down-regulates mir-15a and mir-16 expression. *Co-trasfection of both anti-microRNAs reduced selected microRNAs expression in diabetic EPCs as compared to scrambled cells. Three different experiments were performed and analyzed by qPCR.*

We observed that only co-transfection of mir-15 and mir-16 rescues FBS or SDF-1-induced migration of diabetic EPCs. (**Figure 28**)

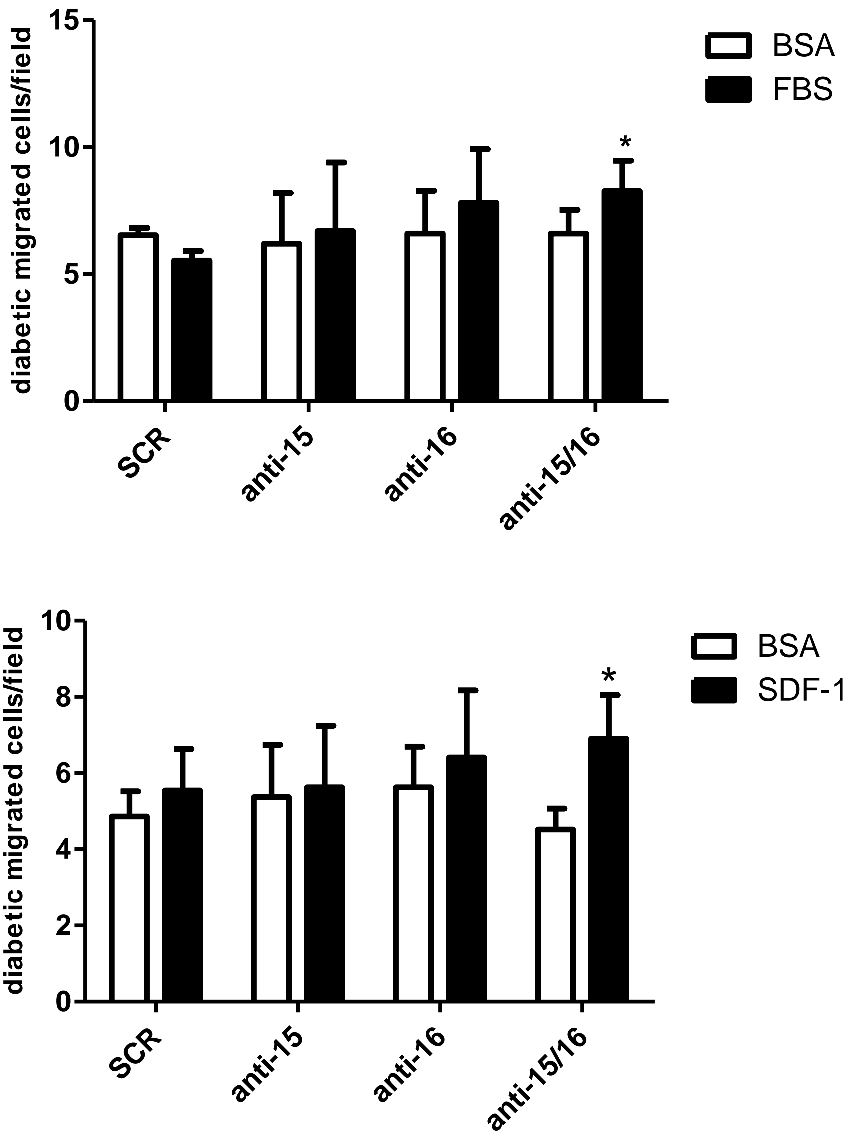


Figure 28. Down-regulation of mir-15/16 rescues diabetic EPCs migration. *Graph bar indicated diabetic EPC migration versus FBS (**upper panel**) or SDF-1 (**lower panel**) compared to scramble. Seven different patients were studied. (*p<0.05 vs BSA)*

To verify if down-regulation has also an effect on diabetes-induced apoptosis, we analyzed the number of Annexin V positive cells. We didn't observe any difference after antimirs transfection (**Figure 29**).

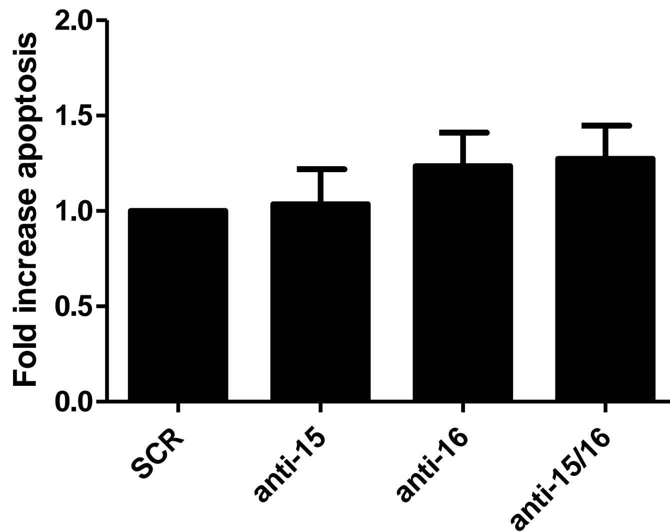


Figure 29. Analysis of apoptosis after microRNAs downregulation. *We performed flow cytometry analysis on diabetic EPCs (n=10) after transfection of anti-mir 15 and mir-16. Apoptosis was evaluated by flow citometry as number of Annexin V positive cells compared to cell transfected with scrambled miRNA*.

Next we investigated if miRNAs inhibition has an effect on diabetic EPC angiogenic capacity. Again, down-regulation of mir-15 and mir-16 in diabetic EPCs (n=5) showed no difference compared to scramble (**Figure 30**).

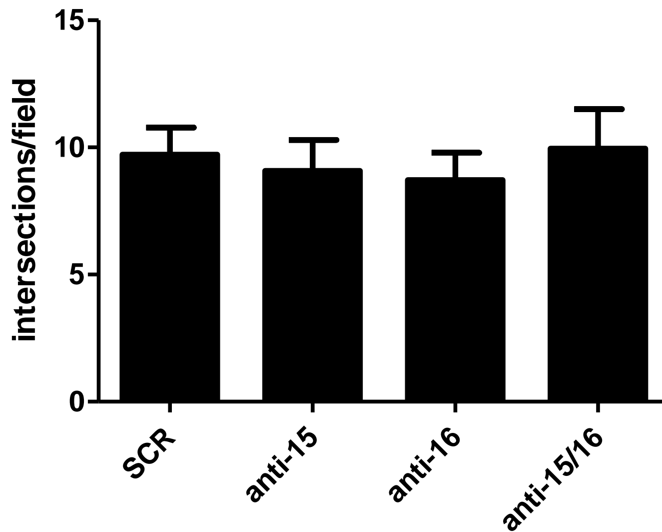


Figure 30. Pro-angiogenic analysis on anti-mir transfected EPC. *Pro-angiogenic capacity was measured as number of intersections in five random fields. We didn't observe any significant difference in matrigel assays*.

5. DISCUSSION

Diabetic foot ulcers represent a serious medical and socio-economic problem throughout the world. Foot problems are associated with significant mortality, morbidity, disability and impairment in the patient's quality of life. Furthermore, despite of the considerable resources devoted to the care of diabetic complications, treatment of the diabetic foot remains an unmet clinical need. Revascularization of the diabetic foot is often complicated by restenosis and thrombosis events, which account for the excess of limb amputation. Considering the impact on the patient wellbeing of the consequences of such peripheral vascular complications, early diagnosis and new therapeutic strategies are of paramount importance.
To date prediction of clinical outcome of the ischemic foot relies on clinical data, including the presence or absence of ischemia, infection, footwear and pressure relief, and overall glycemic control. Although these methods represent to date the best predictors for major disease outcomes, e.g. amputation, none of these data reflects the activity of endogenous repair mechanisms. In particular, EPCs availability and functionality are relevant to vascular repair. It was demonstrated that in diabetic patients the availability of EPCs is impaired [19, 21]. Different mechanisms could potentially reduce the abundance of EPCs, including impaired mobilization, proliferation, and apoptosis [18].

During these years, growing attention is focusing on the possibility of using EPCs as biomarker to predict vascular disease. Fadini et al. demonstrated that the low abundance of CD34 subfraction of MNCs is associated to cardiovascular risk. They hypothesized that progenitor cells count may be used as a surrogate marker of cardiovascular disease [72]. Other groups showed a correlation between progenitor cells and other diseases as cancer [73] or dystrophy [74].

The goal of this study is to determine the additive value of circulating EPCs in predicting major end-points such as mortality, amputation and post-angioplasty restenosis in diabetic patients with Critical Limb Ischemia (CLI). Moreover, we aimed to obtain mechanistic insights into diabetic progenitor cells impairment and to provide the first characterization of the EPC-associated miRNA with special respect to those potentially involved in the control of angiogenesis.
Since normal criteria to conduct functional and molecular analyses were not already available, we enrolled two control groups represented by 1) a group of not-diabetic CLI subjects, and 2) a group of healthy volunteers.

We need to wait the end of the 12 month follow up (FU) to assess the first objective (to determine the prognostic value of circulating EPCs in the development of

diabetic foot complications, namely mortality, amputation and post-angioplasty restenosis). This thesis reports therefore an ad interim analysis of the data.
To date, about 60% of patients have reached one year FU visit and we have registered 37 restenosis, 10 major amputations and 7 death events. About 42% of diabetic patients have no events recorded. Results showed no statistically significant differences in the number of antigenically defined EPCs between complicated (with at least one event) and not complicated (no event) diabetic patients. No difference in the overall migration capacity was observed between the latter two groups. Interestingly, our preliminary data showed a significant increase in the basal migration of EPC in patients with event vs. patients with no event. This result will need to find confirmation when all the FU data will be available at the end of the study. Preliminarily, we propose that the unspecific motility associated with altered directed migration may be indicative of the incapacity of the cells to find their way to injured tissues thereby compromising the healing process. Lack of directional migration associated to increased motility has been referred to alteration in cell polarization and dysfunction of the molecular migratory apparatus[75].

While awaiting for exhaustive and final statistical analysis, we performed an overall analysis of EPC characteristics. To obtain a detailed description of diabetic EPCs number and function, we further analyzed EPC isolated from the three study groups: diabetic CLI, non diabetic CLI, and healthy subjects. Since diabetes mellitus has been shown to adversely affect EPCs' number [76], we first measured the percentage of antigenically-selected MNCs subpopulations with pro-angiogenic potential by flow cytometry and observed a decrease in $CD34^{pos}$ and EPCs in ischemic patients as compared to healthy. These results are in line with data published also by other groups [19, 77]. Unexpectedly however, diabetic patients showed a higher percentage of progenitor cells compared with non diabetic controls. Migration assay showed an impairment of cell motility in chronic limb ischemic patients with or without diabetes compared to healthy subjects. Migration assay results showed an enrichment of EPCs in the controls only; these data are in line with those previously published on an altered migratory function of EPCs from diabetic patients.[7]
Next we confirmed that early culture diabetic EPCs show migratory and pro-angiogenic dysfunctions compared to healthy EPCs [18]. Recruitment and incorporation of EPCs into ischemic tissues requires a coordinated sequence of multistep adhesive and signaling events including chemoattraction, adhesion, and transmigration, and finally the repair of the ischemic injury. Chemoattraction is the fundamental mechanism for the recruitment of progenitor cells to the ischemic or injured tissue. Various studies examined the factors attracting circulating EPCs to the ischemic site. Indeed, SDF-1 has been proven to stimulate recruitment of progenitor cells to the ischemic tissue [34]. These considerations and our present data demonstrate that impairment of diabetic EPCs migratory ability does not

permit the recruitment of these cells into ischemic tissue and potentially causes the failure of wound healing process in diabetic foot lesions.

Mechanisms underlying the reduction of EPCs in diabetes are largely unknown. Weak bone marrow mobilization, impaired peripheral differentiation, and short survival in peripheral blood are all candidates. Several mobilizing factors, such as granulocyte colony-stimulating factor (G-CSF), stromal cell derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF) *via* AKT protein kinase pathway activation and endothelial nitric oxide synthase (eNOS), were demonstrated to mediate EPCs' mobilization, proliferation, and migration.

We therefore attempted to investigate the mechanisms involved in this impairment. Kucia and colleagues also demonstrated that SDF-1 and its receptor CXCR4, play a critical role in regulating hematopoietic cell trafficking [78]. Based also on work of Zheng and collaborators we analyzed the expression of SDF-1 receptor, CXCR4, on EPCs identified by flow cytometry in the total MNCs population. After demonstration that the migration deficit was not dependent on a decrease in the CXCR4 cytokine receptor on the surface of EPCs we focused on mechanisms of intracellular signalling.

Role of microRNAs in diabetic EPC disfunction.

Recently, miRNAs have been shown to regulate EC functions, including proliferation, migration and assembling in branched networks. Caporali et al., published that diabetes up-regulated mir-503 which modulates EC function. Another group demonstrated that mir-34 is involved in EPCs impairment in cardiovascular disease [79]. We therefore considered an involvement of microRNAs in the modulation of EPCs angiogenic capacity in diabetes. To this aim, we performed a preliminary screening on EPCs from the patients enrolled and demonstrated that diabetes and also ischemia induce specific microRNAs de-regulation compared with healthy controls.

Next, we selected two miRNAs, mir-15a and mir-16 based on their already known role in controlling migratory and apoptotic mechanisms in cancer [71, 80, 81]. To investigate the role of mir-15a and mir-16, we transfected these miRNAs into healthy EPCs. We then analyzed cell functionality in migration and angiogenic assays. We observed, for the first time, that only co-expression of mir-15a/16 blocks SDF-1 induced migration in EPCs but single pre-mir-15 or -16 transfection does not modulate EPCs migration underlying a possible cell compensatory mechanism when only one microRNA is over-expressed.

To confirm that this impairment of migration was not SDF-1-receptor-dependent we used an unspecific stimulus, FBS, for migration and we obtained the same results. We also demonstrated that mir-15 and mir-16 together induce EPCs apoptosis but no difference was observed in the pro-angiogenic capacity of these cells in a matrigel assay.

Next, we focused our interest on the involvement of these two microRNAs in diabetic migratory dysfunction. We investigated if down-regulation of mir-15a/16 could restore diabetic EPC migratory ability. Interestingly, we observed that inhibition of both mir-15a/16 rescued FBS and SDF-1 induced migration; but no effect was observed in apoptotic and angiogenic assay.
Our data demonstrate that inhibition of microRNAs expression could potentially increase the recruitment of diabetic EPCs in the ischemic tissues to repair the injury.
Bionformatic analyses predict BCL2, VEGFA and AKT3 to be target genes of miRNA-15a and -16, which support an anti-angiogenic and pro-apoptotic role of these miRNAs. In diabetic EPCs and mir over-expressing EPCs, we found a down-regulation of AKT3, a protein involved in many EPCs functions like cell cycle progression, migration and survival. Our data suggest that the regulation of this protein is at a post-transcriptional level because AKT3 mRNA levels were not affected after microRNA over-expression.
Recently, Chamorro et al demonstrated that mir-16 and 424, which share targets genes with mir-15, may participate in the control of angiogenesis in multiple ways [82]. They demonstrated that those miRNas also modulate the expression of KDR, FGFR1 and VEGF. Based on this evidence, we propose further functional studies to validate predicted target genes and correlate the expression changes with EPCs dysfunction in diabetes. In particular, we propose to investigate pathways like AKT/eNOS that could explain EPCs impairment due to diabetes.

In conclusion, EPCs have recently generated considerable attention as potential novel prognostic biomarkers for vascular integrity, and therapeutic clinical approaches using these cells are ongoing. In this scenario, this study newly demonstrates that the spontaneous migration ability of a well antigenically characterized MNCs subpopulation of cells with EPC phenotype is significantly increased in diabetic patients that manifest at least one major adverse event (restenosis, amputation, and/or death). This is the first evidence generated in a clinical trial that EPC migration could be used as prognostic marker for diabetic vascular complications, thus opening the way to future clinical use of EPC as new biomarker for disease progression. We believe that at the end of the study, the analysis of the follow up data generated on the entire patient cohort will enable us to generate crucial data on the a new EPC-associated risk for diabetic CLI patients to develop life-threatening vascular complications.

Second, we showed for the first time that miRNA 15 and 16 are crucial regulators of diabetic EPC pro-angiogenic functions. miRNAs have tremendous therapeutic potential for the treatment of vascular diseases associated with aberrant pathological angiogenesis.

FUTURE PERSPECTIVES

We hypothesize, based on literature data and our own results, that hyperglycaemia and diabetes modulate the expression of microRNAs, like mir-15a and mir-16 in EPCs. These expressional changes may be relevant for induction of alteration in the paracrine capacity. Therefore, measurement of growth factors and cytokines in conditioned medium is a matter of future research.

In addition, we plan to investigate the anti-angiogenic action of mir-221 in EPCs. Preliminary results showed that in diabetic EPCs mir-221 is over-expressed and its validated target gene, the stem cell factor (SCF) receptor, c-kit, is down-modulated. Hence, migration towards SCF and pro-angiogenic assay are considered in the future to investigate the importance of this microRNA in EPCs functionality. Next we will perform mir-221 over-expression or down-regulation on healthy or diabetic EPCs respectively.

In conclusion, the identification of miRNAs regulating angiogenesis might be a meaningful approach for therapy. Thus, we hypothesize in the future that the use of miRNA mimics may be an attractive provasculogenic strategy to implement recovery of ischemic tissues. Interestingly, there is now direct evidence that synthetic miRNA mimics can be systemically delivered [83] and support the promise of miRNAs as a future targeted therapy for diabetic vascular complications.

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