Heliyon 8 (2022) e08756

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Assessment of androgen receptor, IGF-IR and insulin receptor expression in male patients with severe peripheral artery disease

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ARTICLE INFO

Keywords:
Peripheral artery disease
IGF-I receptor
Insulin receptor
Androgen receptor
PAD
AR
IGF-IR
IR

ABSTRACT

Background: Peripheral artery disease (PAD) of the lower limbs is a common condition that can affect quality of life. Androgen receptor (AR) can exert sex-specific effects on metabolic system, endothelial function and vascular tone. IGF-I receptor (IGF-IR) and insulin receptor (IR) may also be involved in the aforementioned functions. The aim of this study was to evaluate AR, IGF-IR and IR expression in the arterial vessel walls of PAD patients.
Results: This is a cross-sectional study examining 30 males with PAD undergoing open surgery procedures. Mean age was 75.9 ± 8.8y. All patients belonged to Rutherford stage 4–6. Median expression levels of IR, IGF-IR and AR significantly decreased from stage 4–6 ($p < 0.05$).
Significance: The study evidenced a progressive decrease of IR, IGF-IR and AR expression as the severity of disease increased. Altered levels of IR, IGF-IR and AR following PAD may be useful for the clinical evaluation of these patients.

1. Introduction

Peripheral artery disease (PAD) of the lower limbs is one of the most common clinical manifestations of atherosclerosis and can be considered a major cardiovascular disease (CVD) with important morbidity and mortality, also affecting the quality of life (QoL), thus representing an important public health concern. From a clinical point of view, PAD may be initially symptomatic with intermittent claudication (IC). In critical limb ischemia (CLI), an advanced stage of PAD, there may also be rest pain, together with leg skin ulceration, a complication that may even lead to limb loss by amputation [1, 2]. From a pathophysiological point of view, in the presence of atherosclerosis, endothelial activation and dysfunction lead to hemodynamic alterations such as turbulent flow, abnormal shear stress, loss of potential energy, anomalies of vascular tone [3]. This determines functional impairment during leg exercise in early stages, with IC onset, and in advanced stages, during the CLI stage, also with rest pain because of an important demand/perfusion mismatch [3]. Both endothelial dysfunction and vascular tone are also regulated by sex hormones [4, 5] and both incidence and prevalence of PAD have usually been found to be higher in men than in women [6]; moreover, males generally have a higher risk of developing cardiovascular disease during the reproductive period compared with females of the same age.

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https://doi.org/10.1016/j.heliyon.2022.e08756
Received 14 June 2021; Received in revised form 10 October 2021; Accepted 10 January 2022

This sex related difference in developing the disease was hypothesized to be related to the protective role of estrogens in female subjects, and harmful effects of androgens in male individuals [7]. Furthermore, the androgen receptor (AR) seems to have a role in neointima formation, in influencing the size and the composition of atherosclerotic plaques, and vascular smooth muscle cell (VSMC) activity [8]. Moreover, sex-specific effects on the metabolic system are well-known and associated with different risk profiles for cardiovascular disease and they have also been related to differences in sex hormone activity [9]. In fact, insulin and IGF-I signaling is mediated by hormone interaction with IGF-I receptor (IGF-IR) and insulin receptor (IR) that are members of subclass II of the tyrosine kinase receptor super-family. These pathways, on one hand, participate in metabolic homeostasis, and on the other hand, they have a direct role on VSMCs, and in some stages of the atherosclerotic process [10, 11, 12].

In this study we aimed to evaluate IR, IGF-IR and AR expression levels in the arterial wall of male patients with PAD undergoing open revascularization surgery.

2. Results

Descriptive baseline characteristics of patients are represented in Table 1. The whole population was characterized by old age (75.9 ± 8.8 years) and very-high cardiovascular risk. In fact, prevalence of type II diabetes, dysmetabolism and smoking habit were 53.3%, 76.7% and 60%, respectively. Previous CVD was documented in 56.7% of the whole population. According to the Rutherford classification, patients belonged to stage 4–6. Specifically, we observed 56.7% of patients with ischemic rest pain (Stage 4), 16.7% of patients with minor tissue loss (Stage 5) and 26.7% with the more severe condition of major ulceration or gangrene (Stage 6). Moving from the mildest to the most severe Rutherford category, frequencies of dysmetabolism ($p = 0.020$), previous CVD ($p = 0.015$), prevalence of type II diabetes ($p = 0.007$) were significantly increased. Prevalence of amputation ($p < 0.001$) and CKD ($p = 0.033$) were also more frequent in the major ulceration or gangrene category compared with the other risk categories. HR have been evaluated using median values given the skewed distribution. Median levels of IR, IGF-IR and AR were decreased going from ischemic rest pain to major ulceration or gangrene categories, with a very high statistical significance ($p < 0.05$ for the three variables, Figure 1A–C). Distribution of antidiabetic agents varied between Rutherford categories. Oral glucose lowering drugs ($p = 0.009$), metformin ($p = 0.003$) and glargine insulin ($p = 0.044$) were used more by the patients with ulceration or gangrene. This latter category was also characterized by more frequent use of statins. Treatments followed by patients at basal visit (hospital admission) are reported in Table 2. Multivariable adjusted analyses are depicted in Table 3. Stages 5 and 6 of the Rutherford classification were associated with significantly lower levels of IR, IGF-IR and AR. The use of metformin was significantly associated with lower levels of AR ($p = 0.042$), this effect not being significant for IGF-IR and IR. Computed VIF values were all below 10, so that we maintained these models as adjusted ones. At interaction analysis, we did not find any significant interaction effect between covariates included in Table 3, thus meaning that all the significant effects were independent of each other.

3. Discussion

The association of male gender with increased atherosclerosis, and, more specifically, with PAD, has promoted interest in the role of androgen signaling in this disease [6, 8]. The influence of sex hormone receptors on vascular system are generally known, and, in a previous study, we also evaluated the effects of estrogen receptors in venous disease [31]. The presence of AR in vascular cells has been identified some

Table 1. Basal characteristics of male patients, overall and by peripheral artery disease categories.

Variables	Overall (n = 30)	Rutherford Stage			
		Stage 4 Ischemic rest pain (n = 17)	Stage 5 Minor tissue loss (n = 5)	Stage 6 Major tissue loss or gangrene (n = 8)	*p*
Age ± SD, *years*	75.9 ± 8.8	76.5 ± 7.4	76.6 ± 6.1	74.1 ± 13.1	0.812
Dysmetabolism, %	**76.7**	**64.7**	**100**	**87.5**	**0.020**
Smoking habit, %	60.0	70.6	40.0	50.0	0.375
Previous CVD, %	**56.7**	**47.0**	**60.0**	**75.0**	**0.015**
Hypertension, %	80.0	76.47	100	75.00	0.471
Diabetes, %	**53.3**	**29.4**	**100**	**75.0**	**0.007**
Amputation, %	**26.7**	**0**	**0**	**100**	**<0.001**

Values in bold are of particular statistical significance. SD, standard deviation; CVD, cardiovascular disease; AR, Androgen Receptor; IGF-IR, IGF-I receptor; IR, insulin receptor.

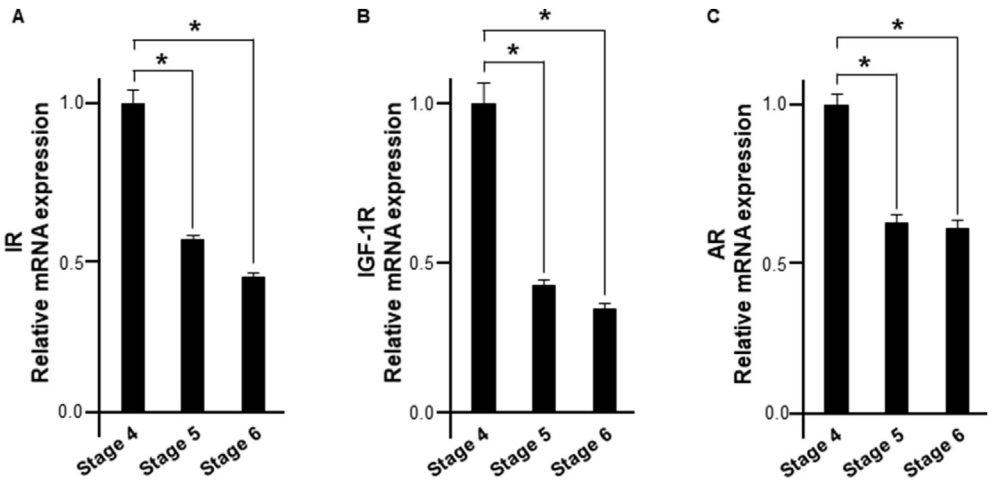

Figure 1. PAD progression significantly correlates with decreased IR, IGF-1R and AR expression levels. (A) Relative changes in IR expression levels during PAD progression from stage 4 to stage 6 of Rutherford classification. (B) Relative changes in IGF-1R expression levels during PAD progression from stage 4 to stage 6 of Rutherford classification. (C) Relative changes in AR expression levels during PAD progression from stage 4 to stage 6 of Rutherford classification. Results shown are representative of three independent experiments and are given as means ± SD. * indicates $p < 0.05$.

Table 2. Treatments followed by patients at basal visit (hospital admission).

Therapies	Rutherford Stage			
	Stage 4 Ischemic rest pain (n = 17)	Stage 5 Minor tissue loss (n = 5)	Stage 6 Major tissue loss or gangrene (n = 8)	*P*
Oral hypoglycemic agents, %	**23.5**	**100**	**50.0**	**0.009**
Metformin, %	**17.7**	**100**	**50.0**	**0.003**
Glargine Insulin, %	**5.9**	**40.0**	**12.5**	**0.044**
Antihypertensive agents, %	76.5	100	75.0	0.471
Statins, %	**35.3**	**40.0**	**62.5**	**0.015**
Antiplatelet agents, %	88.2	80.0	62.5	0.324
Anticoagulants, %	5.9	20.0	12.5	0.628
Calcium channel blockers, %	29.4	40.0	12.5	0.511

Values in bold are of particular statistical significance.

decades ago [13], and later studies have shown that these receptors exert a multitude of actions in endothelial cells (ECs) and VSMCs [7, 8]. Specifically, AR may function both through binding of androgens and through androgen-independent pathways [14], and it also has a role in regulating glucose and lipid metabolism in males [5]. The role of androgens and AR mediated effects are pivotal in the regulation of vascular tone as androgens cause arterial vasodilation by inducing relaxation of VSMCs and contribute to vasorelaxation through the induction of Nitric Oxide (NO) production by ECs. These mechanisms also support EC growth and proliferation that are crucial for endothelial repair in case of vascular damage and endothelial dysfunction that can lead to cardiovascular disease onset [7]. Therefore, androgens seem to have an atheroprotective effect both via AR-dependent and -independent signaling. Taken as a whole, the androgen–AR system protects vascular remodeling through multiple and important signaling pathways [15,16]. Furthermore, the androgen–AR system modulates angiogenic events in males, but not in females both *in vitro*, and in animal models suggesting this effect operates in a sex-specific modality [17].

Moreover, Hu et al showed that androgen deprivation therapy (ADT) led to increased risk of PAD [18] and other studies showed that ADT may increase cardiovascular risk in terms of morbidity and mortality [19, 20, 21, 22, 23].

Growth factors, including insulin-like growth factors (IGFs), also have a role in the regulation of VSMCs and ECs. From a metabolic point of view, in experimental animal studies, AR exerts protection against diet-induced atherosclerosis and modulates body composition and lipid metabolism [24]. The insulin and IGFs (IGF-I and IGF-II) signaling is mediated by hormone interaction with IR and IGF-IR [11, 12]. Beneit et al. showed reduced IGF-IR expression in complicated versus non-complicated regions of human atherosclerotic plaques postulating an increased apoptosis of VSMCs, thus promoting plaque instability [25]. Moreover, defective insulin signaling also plays an important role in cells that is related to the atherosclerotic process [26].

Current evidence shows that IGFI deficiency is closely related to metabolic syndrome, and its clinical manifestations such as impaired lipid profile, insulin resistance, increased glucose levels, obesity, and CVD [27].

To our knowledge, this is the first study examining the expression of three types of receptors in a high-risk population, namely in patients with PAD who underwent surgical revascularization. The main finding of the present analysis is that the expression levels of IR, IGF-1R and AR show a significant descending trend as the severity of PAD increases, moving from the ischemic rest pain risk category to minor tissue loss and to major tissue loss or gangrene. These data are in accordance with previous findings describing an increased atherosclerotic risk and lower AR expression [7, 16–18]. We also found a similar trend when testing the adjusted levels of IGF-IR and IR. Intriguingly, these estimates remain significant even after adjusting for major confounders such as antidiabetic pharmacological agents that directly interfere with receptor expression. Once again, these findings seem to support those from previous studies which reported an inverse relationship between insulin signaling and plaque instability or atherosclerotic risk [25, 26].

High morbidity, mortality, and major amputations are common events in CLI patients although these outcomes seem to be improved over the course of time, both for improved medical care, including care for the associated comorbidities, and advances made in revascularization options [28]. Revascularization, via endovascular means (e.g., percutaneous transluminal angioplasty or stenting procedures) or open surgery (e.g. bypass), is performed for patients with CLI. Nevertheless, up to 30% of CLI patients are not considered ideal for such interventions (being unfit for vascular interventions or for high peri-operative risk) [29]. Therefore, improving our understanding of the main pathophysiological events in PAD could effectively help researchers to identify precise steps

Table 3. Multi-adjusted linear regression analysis on the correlates of AR (A), IGF-IR (B) and IR(C).

Variables	β (Standard Error)	*P*
A)		
Dysmetabolism, *yes vs. no*	1.78 (0.7)	0.029
Glargine insulin, *yes vs. no*	-2.77 (2.1)	0.198
Metformin, *yes vs. no*	**1.08 (0.41)**	**0.042**
Rutherford	-	-
Stage 4	Ref.	Ref.
Stage 5	**-1.34 (0.50)**	**0.023**
Stage 6	**-2.42 (1.05)**	**0.005**
B)		
Dysmetabolism, *yes vs. no*	3.11 (1.4)	0.019
Glargine insulin, *yes vs. no*	-1.19 (1.9)	0.555
Metformin, *yes vs. no*	-2.18 (1.72)	0.217
Rutherford	-	-
Stage 4	Ref.	Ref.
Stage 5	**-1.73 (0.42)**	**0.015**
Stage 6	**-2.21 (1.04)**	**0.001**
C)		
Dysmetabolism, *yes vs. no*	-2.32 (0.84)	0.011
Glargine insulin, *yes vs. no*	1.91 (1.07)	0.100
Metformin, *yes vs. no*	0.72 (0.93)	0.444
Rutherford	-	-
Stage 4	Ref.	Ref.
Stage 5	**-1.79 (0.75)**	**0.004**
Stage 6	**-1.22 (0.45)**	**0.010**

Values in bold are of particular statistical significance.

for development, progression and treatment outcome that could be extremely useful in the clinical setting.

This study describes the relationship of AR, IGF-IR, and IR in arterial wall of CLI patients. It analyzes their level of expression according to the clinical stage. We noted a progressive decrease of the expression of IR, IGF-1R and AR receptors as the severity of the disease increased. Taken together, it seems that IR, IGF-IR and AR might exert a protective role against PAD, but the most critical point, to be elucidated is how to apply these concepts into clinical practice.

3.1. Limitations of the study

The limit of this study is the small number of patients and the lack of a control group. Therefore, further investigations are needed to expand our findings and to provide a clinical and practical perspective that can be applied in patients with PAD.

Declarations

Author contribution statement

Michele Andreucci, Damiano Cosimo Rigiracciolo, Marcello Maggiolini and Raffaele Serra: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Michele Provenzano: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Umberto Marcello Bracale., Nicola Ielapi, Diletta D'Iuorno, Ashour Michael, Pasquale Mastroroberto, Giuseppe Filiberto Serraino: Analyzed and interpreted the data; Wrote the paper.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

The clinical trial described in this paper was registered at ClinicalTrials.gov under the registration number NCT04710186.

Acknowledgements

Damiano Cosimo Rigiracciolo was supported by Italian Minister of University and Research [MIUR, D.D. n. 3407/2018]-PON R&I 2014–2020 'AIM Attrazione e Mobilità Internazionale'. Damiano Cosimo Rigiracciolo and Marcello Maggiolini acknowledge (i) the special award namely "Department of Excellence 2018–2022" (Italian Law 232/2016) to the Department of Pharmacy, Health and Nutritional Sciences of the University of Calabria (Italy), (ii) Sistema Integrato di Laboratori per L'Ambiente—(SILA) for providing lab tools.

STAR methods

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Data and code availability

This was a cross-sectional clinical study examining 30 consecutive male patients who underwent surgical intervention for peripheral arterial revascularization between August 1st2018 and December 1st, 2020. The cohort was originally built to collect information about the expression of AR, IGF-IR and IR in patients with peripheral artery disease referred to a Vascular Surgeon Specialist. The study was approved by the Institutional Review Board of Interuniversity Center of Phlebolymphology (CIFL) International Research and Educational Program in Clinical and Experimental Biotechnology (Approval number: ER.ALL.2018.39.A.), and all patients gave written informed consent. The protocol was properly registered at a public trials' registry, www.clinicaltrial.gov (trial identifier NCT04710186). Moreover, all methods were performed in accordance with the relevant guidelines and regulations. Inclusion criteria were patients with age >18 years, with severe peripheral artery disease that needed surgical repair. Patients with life expectancy <6 months, advanced liver or heart disease and active malignancies were excluded.

Materials availability

At the moment of hospital admission, clinicians collected the medical history including previous CVD (CVD: stroke, coronary heart disease, heart failure, peripheral vascular disease), presence of dysmetabolism, history of arterial hypertension and diabetes, previous amputation, and history of chronic kidney disease (CKD). Therapies practiced at home, encompassing hypoglycemic oral agents, insulin, blood pressure lowering drugs, statins, antiplatelet and anticoagulants were also collected. For the purpose of this specific study, patients were stratified according to the following Rutherford clinical stages of PAD: ischemic rest pain, minor tissue loss and major tissue loss or gangrene [30].

Experimental model and subject details

Samples obtained from the arterial wall of male patients affected by PAD undergoing surgery were collected and immediately preserved at -80 °C and were processed as follows according to a previous experience [31]. Briefly, arterial tissues were excised, homogenized using a motor driven homogenizer and total RNA was isolated using Trizol reagent (Invitrogen, Milan, Italy), in accordance with the manufacturer's instructions. To prevent confounding findings from gDNA contamination, on column DNase digestion was performed during RNA purification steps. Then, total RNA was quantified spectrophotometrically, and quality was checked by electrophoresis by agarose gels stained with ethidium bromide. Only samples that were not degraded and showed clear 18 S and 28 S bands under ultraviolet light were used for reverse transcription polymerase chain reaction (PCR). Total cDNA was synthesized from the RNA by reverse transcription using the murine leukemia virus reverse transcriptase kit (Life Technologies, Milan, Italy). The reverse transcription reaction mix was made as follows: total RNA, 5X First-Strand Buffer, 0.1 M DTT, dNTP mix consisting of 10 mM each dATP, dGTP, dCTP and dTTP, recombinant ribonuclease inhibitor (RNase OUT™), murine leukemia virus reverse transcriptase (MLV-RT) and sterile distilled water. Random hexamer and oligo-dT primer sets were used to ensure representative cDNA synthesis from total RNA. All the steps were performed following the protocol provided by the manufacturer. Specific primers for β-actin, AR, IGF-IR and IR were designed using Primer Express version 2.0 software (Applied Biosystems). β-actin was used as a reference control [32,33]. To minimize the risk of gDNA amplification for β-actin reference primers, a regular PCR reaction was performed by using total RNA template instead of cDNA and no amplification for β-actin was detected. In addition, evaluation of AR, IGF-IR and IR expression levels was performed by using oligonucleotide sequences complementary to the ends of adjacent exons to avoid the possible risk of contamination with the amplified genomic DNA. Therefore, the sequences were as follows: β-actin

forward 5′- AAGCCACCCCACTTCTCTCTAA-3′ and reverse 5′-CACCTCCCCTGTGTGGACTT-3’; AR forward 5′-TGCCCAT TGACTATTACTTTCC-3′ and reverse 5′-TGTCCAGCACACACTA-CACC-3’; IGF-IR forward 5′-TGGTGGAGAACGACCATATCC- 3′ and reverse 5′-CGATTAACTGAGAAGAGGAGTTCGA-3’; IR forward 5′-CGTGGAGGATAATTACATCGTGTT-3′ and reverse 5′- TGGT CGGGCAAACTTTCTG-3’. The PCR efficiency for all primer pairs used in the study was between 90-100 %. RT-PCR reaction (total volume = 10 μL) was made as follows: 5μL of Fast SYBR™ Green Master Mix, 3 μL of nuclease free water, 0.5 μL of each forward and reverse primers and 1 μL of cDNA template. Ct values for all the genes examined were as follows: Ct < 20 for β-actin; Ct of 27–33 for AR, IGF-IR and IR, respectively. Assays were performed in triplicate; RNA expression values were normalized using β-actin as internal reference and then calculated as relative fold induction. No amplification was observed in negative template control (NTC) conditions.

Quantification and statistical analysis

Continuous variables were reported as either mean ± standard deviation (SD) or median and interquartile range [IQR] based on their distribution. Comparison among Rutherford risk categories was assessed by Student t-test, one-way ANOVA or Kruskal-Wallis test. Categorical variables were depicted as percentage and analyzed using the Chi-square test. From the univariate associations between hormone receptor (HR) levels and the other clinical or laboratory variables we built multivariable linear regressions using the continuous HR levels as dependent variables. Selection of variables to be included in the models was performed by a “knowledge-driven” selection based on the biological relationships between dependent and independent variables [34]. Multicollinearity was assessed with variance inflation factors (VIF), which is a measure of the degree to which a single predictor variable can be expressed as a linear combination of the remaining predictor variables; values greater than 10 were a cause for concern [35]. First order interactions in the multivariable models have been also tested. Data were analyzed using STATA version 14 (Stata Corp. College Station, TX, USA) and ggplot2 package of R software 3.3.1 (R Foundation for Statistical Computing, Vienna, Austria).

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