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ORIGINAL ARTICLE

Matrix metalloproteinases and risk stratification in patients undergoing surgical revascularisation for critical limb ischaemia

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Key words
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Abstract

Critical limb ischaemia (CLI) is the most advanced form of peripheral artery disease (PAD) and it is often associated with foot gangrene, which may lead to major amputation of lower limbs, and also with a higher risk of death due to fatal cardiovascular events. Matrix metalloproteinases (MMPs) seem to be involved in atherosclerosis, PAD and CLI. Aim of this study was to evaluate variations in MMP serum levels in patients affected by CLI, before and after lower limb surgical revascularisation through prosthetic or venous bypass. A total of 29 patients (7 females and 22 males, mean age 73·4 years, range 65–83 years) suffering from CLI and submitted to lower extremity bypass (LEB) in our Institution were recruited. Seven patients (group I) underwent LEB using synthetic polytetrafluoroethylene (PTFE) graft material and 22 patients (group II) underwent LEB using autogenous veins. Moreover, 30 healthy age-sex-matched subjects were also enrolled as controls (group III). We documented significantly higher serum MMPs levels ($P < 0{\cdot}01$) in patients with CLI (groups I and II) with respect to control group (group III). Finally, five patients with CLI (17·2%) showed poor outcomes (major amputations or death), and enzyme-linked immunosorbent assay (ELISA) test showed very high levels of MMP-1 and MMP-8. MMP serum levels seem to be able to predict the clinical outcomes of patients with CLI.

Introduction

Peripheral artery disease (PAD), also known as peripheral arterial occlusive disease (PAOD), is a common syndrome that affects a large proportion of the adult population and it is often associated with other comorbidities, such as dyslipidaemia, diabetes mellitus and hypertension. PAD may be manifested as intermittent claudication or critical limb ischaemia (CLI), which is the most advanced form and is associated with a higher risk of death and cardiovascular events: 20–25% of patients die at 1 year, and 25–30% undergo major amputation.(1,2)

*The last two authors contributed equally to this work and share the senior authorship

Key Messages

- critical limb ischaemia (CLI) is the most advanced form of peripheral artery disease (PAD) and it is often associated with foot gangrene, which may lead to major amputation of lower limbs, and also with a higher risk of death due to fatal cardiovascular events
- atherosclerosis, PAD and CLI are also characterised by an important inflammatory process occurring in several distinct steps, many of which have been associated with alterations in the activity of matrix metalloproteinases (MMPs)
- this study evaluated variations in serum levels of MMP-1, MMP-2, MMP-8, MMP-9 and MMP-10 in patients affected by CLI, before and after lower limb surgical revascularisation through prosthetic (group I) or venous (group II) bypass and related these values with the 2-year probability of survival and limb salvage
- we documented higher levels of MMP-1 and MMP-8 in patients with poor outcomes in both groups

The major cause for PAD is represented by atherosclerosis (3), and inflammation plays an important role in its development and progression (4). Moreover, elevated levels of C-reactive protein (CRP) and interleukin-23 (IL-23) are associated with lower extremity PAD, as previously reported (5–7).

Atherosclerosis is an inflammatory process occurring in several distinct steps, many of which have been associated with alterations in the activity of matrix metalloproteinases (MMPs) (8). MMPs are a family of zinc-dependent endopeptidases with proteolytic activity against a wide range of extracellular proteins (9) that also contribute extensively to tissue remodelling by degrading extracellular matrix components in diverse vascular pathological processes (10–13). Specifically, MMP dysregulation is associated with leukocyte infiltration, vascular smooth muscle cell (VSMC) migration and intra-plaque matrix remodelling, each of which are key elements in atherosclerotic plaque formation (14,15). Moreover, MMPs seem to be involved in intimal hyperplasia and constrictive remodelling, both responsible for re-stenosis after endoluminal treatment of atherosclerotic lesions (16).

Recent clinical studies have shown an association between PAD and circulating levels of MMP-2, MMP-9, MMP-8 and MMP-10, compared with healthy controls (11–13,17,18). Moreover, an association between MMP-10 serum levels and the severity and poor outcome in patients affected by PAD was reported (17).

Human data on MMP activity in PAD are limited. However, a linear correlation has been demonstrated between plasma MMP-9 levels and the severity of ischaemia in patients with varying degrees of PAD (13).

The aim of this study was to evaluate the variations in serum levels of MMP-1, MMP-2, MMP-8, MMP-9 and MMP-10 in patients affected by CLI, before and after lower limb surgical revascularisation through venous or prosthetic bypass, and to relate these values with the 2-year probability of survival and limb salvage.

Materials and methods

Selection criteria and patients

All patients with CLI, of both sex, aged >20 years, presenting to our institutions and with the indication for surgical revascularisation were recruited and were followed up for at least 24 months. The patients were classified according to the severity of the disease: lower limb rest pain or trophic lesions (Fontaine class III–IV).

Patients with chronic venous insufficiency, or arterial aneurysms, or infected lesions, or with neoplasia, or with generalised or localised inflammatory disease or with severe kidney disease were excluded from the study.

Patients with CLI were randomised to receive lower limb surgical revascularisation through autogenous venous or prosthetic bypass (using synthetic polytetrafluoroethylene – PTFE).

Patients enrolled in this study were followed up through clinical and ultrasonographic examination at 1, 3, 6, 12 and 24 months.

Serial blood samples in order to evaluate MMPs levels were collected three times: T0 (before 24 hours), T1 (after 24 hours) and T2 (after 6 months).

Limb salvage was defined as the absence of major amputation (if performed above the ankle) during the observation period and with the preservation of a functional lower limb.

Laboratory analysis

Serum samples were obtained using serum separator tube and allowing samples to dot for 30 minutes before centrifugation (15 minutes at approximately 1000 *g*); the serum aliquots were stored at −20°C until assay of MMP-1, MMP-2, MMP-8, MMP-9 and MMP-10 serum levels.

Blood count (red blood cells, white blood cells and platelets) was performed on whole blood (ABX Pentra Dx120; Horiba Ltd, Kyoto, Japan). Fibrinogen in the serum was measured using photometric reading (CA 7000; Siemens Healthcare Diagnostics Inc., Deerfield, IL), and CRP was measured using photometric reading (Modular Analytics Systems D 2400; Roche Diagnostics, Indianapolis, IN). Serum creatinine was measured by photometric reading (Roche/HitachiModular Pre-Analytics Plus; Roche Diagnostics).

Approval

This study was approved by the Investigational Review Board, in accordance with the Declaration of Helsinki and the Guideline for Good Clinical Practice. Before the beginning of the study, all participants provided written informed consent. The protocol was properly registered at a public trials registry, www.clinicaltrials.gov (trial identifier NCT02388867).

Results

We evaluated serum MMP-1, MMP-2, MMP-8, MMP-9 and MMP-10 levels and other biochemical values such as red blood cells, white blood cells and platelets, CRP, fibrinogen and creatinine in 29 patients (7 females and 22 males, mean age

Table 1 Demographics of the study population

Demographic and clinical data	*N* (%)
Sex	22 M (75·9%), 7 F (24·1%)
Age (average)	73·4 (range 65–83)
Smoker history	14 (48·3%)
Former	9 (31%)
Current	5 (17·3%)
Diabetes mellitus	25 (86·2%)
Hypertension	19 (65·5%)
Dyslipidaemia	12 (41·4%)
Chronic renal failure on haemodialysis	7 (24·1%)
Myocardial dysfunction	(16, 55·2%)
Medical treatment	
Anti-platelets	19 (65·5%)
Beta blockers	9 (31%)
Statins	12 (41·4%)
ACE inhibitors	8 (27·6%)
Calcium antagonists	12 (41·4%)
Insulin	18 (62%)
Anti-diabetic drugs	7 (24·1%)
Fontaine's classification	
Stage III	1 (3·4%)
Stage IV	28 (96·6%)
Surgical treatment	
LEB using autogenous veins	22 (75·9%)
LEB using prosthetic material	7 (24·1%)

N, number; M, males; F, females; ACE, angiotensin-converting-eEnzyme; LEB, lower extremity bypass.

73·4 years, range 65–83 years) suffering from lower extremity PAD and subjected to surgical bypass in our institution (Table 1). Patients with evidence of systemic sepsis, known neoplastic disease, or any established generalised inflammatory disease were excluded.

The patients were assessed by clinical and ultrasonography examination. At total of 28 patients were in stage IV and only one in stage III, according to the Fontaine's classification. The enrolled patients presented the following risk factors and comorbidities: diabetes mellitus (25, 86·2%), dyslipidaemia (12, 41·4%), smoking (14, 48·3%), hypertension (19, 65·5%), myocardial dysfunction (16, 55·2%) and chronic renal failure on haemodialysis (7, 24·1%). All patients underwent lower extremity bypass (LEB) surgery under locoregional anaesthesia.

Seven patients (group I) underwent LEB using synthetic PTFE graft material: the inflow vessel was the femoral artery in five and the iliac artery in two cases; the outflow vessel was represented by the popliteal artery in three and the anterior tibial artery in four patients.

Group II comprising 22 patients underwent LEB using autogenous veins (great saphenous vein in 20 cases, small saphenous vein in 1 patient, a composite bypass using great and small saphenous vein in 1 case): the inflow vessel was represented by the femoral artery in 18 cases and the popliteal artery in 4 patients; the outflow vessel was the anterior tibial artery in 6, the posterior tibial artery in 11 and the peroneal artery in 5 patients.

Serial blood samples were collected three times: T0 (before 24 hours), T1 (after 24 hours) and T2 (after 6 months).

Moreover, 30 healthy age-sex-matched subjects were also enrolled as control (group III).

We did not document any significant difference between the groups for blood count (red blood cells, white blood cells and platelets), fibrinogen and creatinine; for CRP levels, we found higher serum levels for groups I and II compared with the control group (group III) ($P < 0{\cdot}01$), but there was no significant difference when groups I and II were compared (data not shown).

Significantly higher serum MMPs levels ($P < 0{\cdot}01$) were documented in patients with CLI (groups I and II) with respect to the control group (group III) (Table 2).

Finally, five patients with CLI (17·2%) showed poor outcomes, and enzyme-linked immunosorbent assay (ELISA) test showed very high levels of MMP-1 and MMP-8 (Table 3). Data are expressed as absolute values.

We did not record any significant correlation between serum MMPs levels and characteristic of the enrolled patients (data not shown).

The 2-year overall survival rate was 93·1% (85·7% for group I and 95·4% for group II) and the 2-year overall limb salvage rate was 89·6% (85·7% for group I and 90·9% for group II).

The major complications occurred between the 4th and the 17th month from the inclusion in the study, during the follow-up period (Table 3).

Discussion

PAD is defined as a clinical disorder caused by stenosis or occlusion of the aorta or the arteries of the limbs (19). Atherosclerosis is by far the leading cause of peripheral arterial occlusion in patients aged 40 years or more; however, atheroembolic or thromboembolic disease, in situ thrombosis due to inherited thrombophilia, vasculitis, fibromuscular dysplasia, cystic adventitial disease, entrapment and trauma represent other possible aetiologies. The highest prevalence of PAD occurs in the seventh decade of life, reaching 15% of the persons in this age and, as for other cardiovascular diseases, in certain high-risk populations (e.g. smokers, persons with diabetes mellitus, hypertension, hypercholesterolaemia and hyperhomocysteinaemia) (20). Fewer than 50% of these patients are symptomatic, while many others present slow or impaired gait. The two most important manifestations of the PAOD are: (i) intermittent claudication, defined as a muscle discomfort presenting with pain, ache, cramp, numbness or fatigue, that occurs during exercise and is relieved by rest within 10 minutes (21) and (ii) CLI, defined as limb pain occurring at rest, or impending limb loss caused by a severe blood flow insufficiency of the affected extremity (22). CLI is chronic and complex in its nature and should not be confused with an acute occlusion of the distal arterial tree. With a long-lasting (i.e. months to years) lack of blood supply to the legs, a number of macrovascular and microvascular changes that go further than the simple issue of supply versus demand that characterises PAD occur. Among these, vasomotor paralysis, oedema and endothelial changes of the arterial blood vessels represent the important phenomena that, ultimately, lead to rest pain and/or trophic lesions of the lower limbs (23). In such a scenario, CLI can be considered the end stage of PAD in which the patient complains

Table 2 Serum MMP levels (ng/ml) in enrolled patient, evaluated several times. T0 24 hours before surgery; T1 24 hours after surgery; T2 6 months after surgery. Data are expressed as mean ± standard deviation

	MMP-1	MMP-2	MMP-8	MMP-9	MMP-10
Group I					
T0	4·9 ± 1·2	1325 ± 125	7·1 ± 2·6	135 ± 23	8·5 ± 2·3
T1	4·8 ± 1·3	1310 ± 112	7·1 ± 2·5	138 ± 25	8·4 ± 2·0
T2	4·5 ± 1·2	1250 ± 116	6·9 ± 2·6	137 ± 22	8·5 ± 2·1
Group II					
T0	5·1 ± 2·2	1315 ± 145	7·2 ± 2·1	145 ± 31	8·3 ± 1·9
T1	5·1 ± 2·3	1300 ± 128	7·2 ± 2·3	141 ± 27	8·2 ± 2·1
T2	4·6 ± 2·2	1260 ± 121	6·9 ± 2·5	142 ± 25	8·3 ± 2·0
Group III					
T0	3·2 ± 1·5	722 ± 185	1·7 ± 0·5	22 ± 9	2·9 ± 1·1
T1	3·4 ± 1·4	718 ± 179	1·5 ± 0·6	23 ± 11	2·9 ± 1·0
T2	3·1 ± 1·4	735 ± 198	1·6 ± 0·9	20 ± 8	2·7 ± 0·9

MMP, matrix metalloproteinases.

Table 3 Characteristic of patients with poor outcomes. (MMPs levels are expressed in ng/ml)

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Group of enrollment	I	I	II	II	II
MMP-1 T0	6·3	6·4	5·9	6·1	6·5
MMP-8 T0	8·5	8·7	8·9	8·1	8·3
MMP-1 T1	6·2	7·4	5·8	6·0	7·2
MMP-8 T1	8·6	8·4	8·6	8·2	8·4
Outcomes (complications)	Major amputation	Died for heart stroke	Major amputation	Major amputation	Died for heart stroke
Time point at which the complication occurred	After 4 months	After 5 months	After 8 months	After 15 months	After 17 months

MMP, matrix metalloproteinases.

chronic ischaemic rest pain, ulcers, or gangrene attributable to objectively proven arterial occlusive disease (21) and can be operatively defined as the more severe ends of the Fontaine classification of the PAOD (stage III–IV). However, CLI does not always follow such classification system and can occur even in asymptomatic patients (23).

PAD is a frequent cause of significant morbidity and mortality and can be considered as a marker of subclinical coronary heart disease and stroke, which in turn represent common causes of death in such patients (24,25). The risk of cardiovascular events in these subjects is higher than that in those with symptomatic coronary artery disease (CAD) and is increased by threefold to sixfold when compared with individuals without PAD (25,26). Although they represent approximately only the 1% of those with PAD, patients with CLI have an even greater risk of ischaemic issues than those with PAD alone (22). The overall mortality is 25% at 1 year, 50% at 5 years and 70% at 10 years (1,27,28), while 25–30% undergo major amputation of the affected limbs (2).

In view of the above, an early diagnosis and optimal management are mandatory in CLI. However, whether treating CLI by a surgical or medical way is still a matter of debate: no defined guidelines have been released because of the complex comorbidities (which vary from patient to patient) and the different risks for each procedure (23). In any case, one should be focused on pain relief, improvement of healing rates of ischaemic ulcers, limb salvage, functional outcomes, Quality of Life (QOL) levels and improvement of patient's survival probability. With such aims, predictive indices and risk-stratification of patients with CLI can guide the physician in choosing and tailoring the treatment of PAD. This is even more valiant when newer and ancillary approaches to PAD such as angiosome-targeted revascularisation (29,30), extreme distal bypass (31), gene therapy (32,33), vacuum-assisted closure (VAC) therapy (34,35), spinal cord stimulation (36,37) and prostaglandin E1 infusions (38,39) are considered.

To this date, clinical and biochemical markers have been used as predictors of the functional and prognostic outcomes for patients undergoing intervention for CLI; among these, the most commonly reported are (i) certain intrinsic patient comorbidities at the time of presentation such as diabetes mellitus, CAD, foot gangrene and urgent need of operation(40); (ii) preoperative and postoperative living situation and ambulatory status (41,42); (iii) chronic kidney insufficiency in dialysis regimen, presence of non-healing ulcer or gangrene, individuals aged ≥75 years, a low haematocrit (≤30%), a positive history of advanced CAD (43) and (iv) gender, mode of admission, age on admission, urea, sodium, potassium, haemoglobin, white cell count, creatinine, urea/creatinine (44).

As previously seen, the large majority of aetiological events that result in PAD are somehow linked to inflammation (4). Because of this, inflammatory mediators such as IL-6, tumour necrosis factor-α (TNF-α), neopterin, CRP and IL-23

represent other independent prognostic factors eligible for clinical practice (5–7,45,46).

MMPs are a family of zinc-dependent endopeptidases with proteolytic activity against a wide range of extracellular proteins (9) that contribute extensively to normal physiology (e.g. cell migration, wound healing and tissue resorption). They also seem to play an important role in a number of pathological conditions, both vascular (10–13,47–50) and non-vascular in origin (47,51,49,50). Among the former, alterations in MMP activity have been detected in the course of the atherosclerotic lesion formation (8), this being speculated to be linked to plaque rupture (52), leukocyte infiltration, VSMC migration into the sub-intimal space and intra-plaque matrix remodelling (14,15). In addition, MMPs seem to be involved in intimal hyperplasia and constrictive remodelling, both responsible for re-stenosis after endoluminal treatment of atherosclerotic lesions (16). Finally, even if no selective drug has yet been developed, MMP inhibition using sulodexide (53,54), cilostazol (55,56), minocycline (57) and doxycycline (58) showed to be a useful aid for ulcer prevention and healing in both venous and arterial disease.

With this evidence, an intimate relationship between MMPs and PAD can be presumed; however, human data on MMP activity in CLI is limited. Yet, previous published studies have linked the high levels of both MMP-1 and MMP-8 to a greater risk of endurance of chronic venous and mixed ulcers (59,60). Tayebjee *et al.* demonstrated a linear correlation between plasma MMP-9 levels and the severity of ischaemia in patients with varying degrees of PAD (13). Recent clinical studies showed an association between PAD and circulating levels of MMP-2, MMP-9, MMP-8 and MMP-10, compared with healthy controls (11,12,17,18). A recent study showed the association between MMP-10 serum levels and the severity and poor outcome in patients affected by PAD (18).

In the present study, we have evaluated the variations in the serum levels of MMP-1, MMP-2, MMP-8, MMP-9 and MMP-10 in patients affected by CLI, before and after lower limb surgical revascularisation through venous or prosthetic bypass. Our aim was to firmly relate these values with the 2-year probability of survival and limb salvage of such patients. We documented higher levels of MMP-1 and MMP-8 in patients with poor outcomes in both groups. Interestingly, MMP-1 and MMP-8 are collagenases that are able to initiate cleavage of triple helical collagens I, II and III (61). As type I and III collagens represent the major components of the fibrous cap of atherosclerotic plaques (62,63), we could also speculate a pathogenetic role of these MMPs in such poor outcomes.

In our study, MMP-1 and MMP-8 serum levels were related to all the major complications that occurred in CLI patients during the follow-up period.

The limitation in our study was the relatively small number of patients enrolled. Nevertheless, such sharp results strongly suggest examining in depth the role of MMPs as prognostic factors in the treatment of CLI in terms of probability of survival, limb salvage, QOL and functional outcomes. Furthermore, encouraged by the preliminary results, we also hope that in the future, more selective MMP inhibitors will be introduced in the clinical practice of ulcer healing.

In conclusion, MMP serum levels seem to be effective in predicting poor outcomes in patients with CLI identifying in such way a subcategory of critical patients that need to be monitored more strictly in order to avoid important or fatal complications.

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