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Alexandre Nuzzo

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Université de Paris

École doctorale 563 MTCI

*UMR1124, Toxicité Environnementale, Cibles Thérapeutiques,
Signalisation Cellulaire et Biomarqueurs
Department of Surgery and Cancer, Imperial College London*

**Identification of metabolomic-based biomarkers of acute
mesenteric ischemia: a cross-sectional diagnostic study**

*Identification de nouveaux biomarqueurs d'ischémie
mésentérique aiguë : une étude diagnostique transversale*

Par Alexandre Nuzzo

Thèse de doctorat en Biochimie

Dirigée par le Professeur Dominique Gauguier
et co-encadrée par le Professeur Marc-Emmanuel Dumas

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Titre : Identification de nouveaux biomarqueurs diagnostiques d'ischémie mésentérique aiguë par une approche métabolomique

Résumé : La présentation clinique de l'ischémie mésentérique aiguë (IMA) n'étant pas spécifique, son identification au sein des autres urgences abdominales reste un défi majeur en gastroentérologie et médecine d'urgence. Dans une étude transversale menée entre janvier 2016 et mars 2018 ont été inclus prospectivement 1) les patients admis dans notre unité spécialisée pour une IMA confirmée par angioscanner et 2) des témoins admis aux urgences du même hôpital pour douleur abdominale aiguë non ischémique. Chaque patient a eu un prélèvement de plasma à l'inclusion. Chaque plasma a été analysé par 2 méthodes complémentaires, la spectroscopie de masse haute-résolution (MS) couplée à une chromatographie en phase gazeuse (GC-MS) et la spectroscopie par résonance magnétique nucléaire (1H-RMN). Nous avons inclus 126 patients dont 47 cas d'IMA et 79 témoins. La comparaison des échantillons sanguins des patients avec une IMA à patients témoins ayant une douleur abdominale aiguë d'origine non ischémique a permis l'identification d'un panel de métabolites plasmatiques (n=13, correspondant à des acides aminés, sucres et fragments lipidiques). Les concentrations plasmatiques de ces métabolites étaient significativement différentes entre les 2 groupes de patients en analyses univariée et multivariée ajustée sur l'âge et le sexe. Le développement de cette combinaison de biomarqueurs par un algorithme de machine learning sur 85% de la cohorte (n=108) a permis l'identification des patients avec IMA avec une aire sous la courbe ROC (AUROC) = 0.89. Après validation interne effectuée sur les 15% restant de la cohorte (n=18), tous les cas d'IMA étaient identifiés (AUROC = 1.00). Ces résultats prometteurs nécessitent une validation dans un cohorte indépendante.

Mots clefs : ischémie intestinale, colite ischémique, infarctus mésentérique, infarctus intestinal

Title: Identification of metabolomic-based biomarkers of acute mesenteric ischemia: a cross-sectional diagnostic study

Abstract: As the clinical presentation of acute mesenteric ischemia (AMI) is not specific, its identification among other acute abdomen remains a major challenge in gastroenterology and emergency medicine. In a cross-sectional study conducted between January 2016 and March 2018, we prospectively included 1) patients admitted to our specialized unit for an AMI confirmed by computed tomography angiography and 2) controls admitted to the emergency department of the same hospital for acute non-ischemic abdominal pain. Each patient had an inclusion plasma sample. Each plasma was analyzed by 2 complementary methods, high-resolution mass spectroscopy (MS) coupled with gas chromatography (GC-MS) and nuclear magnetic resonance spectroscopy (1H-NMR). We included 126 patients including 47 cases of AMI and 79 controls. Comparison of blood samples from patients with AMI to control patients with acute abdominal pain of non-ischemic origin allowed the identification of a panel of plasma metabolites (n = 13, corresponding to amino acids, sugars and fragments lipid). The plasma concentrations of these metabolites were significantly different between the 2 groups of patients in univariate and multivariate analyzes adjusted for age and sex. The development of this combination of biomarkers by a machine learning algorithm on the 85% of the cohort (n = 108) allowed the identification of patients with AMI with an area under the receiver operating curve (AUROC) = 0.89. After internal validation carried out on the remaining 15% of the cohort (n = 18), all AMI cases were identified (AUROC = 1.00). These promising results require validation in an independent cohort.

Keywords: intestinal ischemia, ischemic colitis, mesenteric infarction, intestinal necrosis

A mes grands-parents,
Papi et Mamie Michel, Mamie Sandra, Papi Pascal

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Abbreviations

¹H-RMN: hydrogen-1 nuclear magnetic resonance spectroscopy

AAA: aortic aneurysms patients

AMI: acute mesenteric ischemia

CA: celiac artery

CMV: cytomegalovirus

CPMG: Carr-Purcell-Meiboom- Gill sequence

CT: contrast-enhanced computed tomography

ELISA: enzyme-linked immunosorbent assay

ER: emergency room

FDR: false discovery rate

GCMS: gas chromatography-mass spectrometry

IFABP: intestinal fatty-acid binding protein

IMA: inferior mesenteric artery

ISC: intestinal stroke center

JRES: J-resolved

LAMBDA: Learning Algorithm for Multivariate Data Analysis

MEMBAS: Membership Margin Based Attribute Selection

MS: mass spectrometry

NOESY: Nuclear Overhauser Effect Spectroscopy

OPLS-DA: Orthogonal Partial Least Squares- Discriminant Analysis

PCA: principal component analysis

PCT: Procalcitonin

ROMS: Retrograde open SMA stenting

SMA: superior mesenteric artery

SURVI: Structure d'URgences Vasculaires Intestinales

US : United States of America

UV: ultraviolet

General introduction

Acute mesenteric ischemia (AMI) is one of the most severe of vascular and digestive emergencies, leading to an early and high mortality due to the rapid and unpredictable constitution of intestinal infarction. Late-stage necrotic AMI is the form most commonly recognized by physicians, but usually of poor prognosis. Although the natural course of AMI consistently leads to multiple organ failure and death within a few hours or days, the early stage of the disease still paradoxically poses a major diagnostic challenge to physicians. Recently, the development of specialized intestinal stroke units offering 24/24 dedicated care to AMI patients has shown dramatically improved survival and decrease in intestinal resection rates in patients diagnosed and treated at an early stage. This represents not only a therapeutic progress, but also a major diagnostic hope. Indeed, the recruitment of these patients into dedicated medical and research teams is an essential prerequisite for scientific progresses and nurtures hope for the future discovery of new diagnostic tools for an early diagnosis, the clinical application of which could bring major changes in the recognition, epidemiology, treatment and prognosis of the disease.

In this thesis, we will first review the current medical and scientific knowledge on AMI and try to explain why such a life-threatening emergency still is so difficult to recognize at an early stage. Then, in a cross-sectional human study of patients with AMI and controls with abdominal pain of another origin from the intestinal stroke center of Beaujon Hospital, Paris, France (SURVIBIO study, ClinicalTrials.gov Identifier: NCT03518099) we will first study common laboratory tests readily available to clinical practice, and then assess the diagnostic value of promising candidate biomarkers highlighted from the scientific literature. Finally, we will explore new diagnostic avenues through untargeted plasma metabolomics in collaboration with the expert leading team of Imperial College London, UK.

Chapter 1

Background

1. ACUTE MESENTERIC ISCHEMIA

Acute mesenteric ischemia (AMI, or intestinal ischemia) represents the most severe and the most challenging of digestive and vascular diseases. The progression to mesenteric infarction (or intestinal necrosis) is an irreversible complication potentially leading to large intestinal resection and short bowel syndrome or death. (1) Thus, any intestinal ischemia should be recognized and treated at a reversible, early or chronic stage. (2) In the absence of validated and available biomarkers, the diagnosis requires a high degree of clinical suspicion and an urgent specific therapy with two linked objectives: saving the gut, to save the life of the patient. (3) Developed on the model of “stroke units”, a new intensive care unit dedicated to the acute care of AMI offering expert multimodal and multidisciplinary 24/7 management has been implemented in Paris, France. The first results of this intestinal stroke center have shown high rates of survival without intestinal resection in most patients. In this paragraph, we will develop the latest scientific and clinical knowledge on AMI as well as the treatment strategy developed in our intestinal stroke center.

1.1. Definition of mesenteric ischemia and anatomy

Mesenteric ischemia is defined by 1) an intestinal injury, secondary to 2) a vascular insufficiency in the territory of the splanchnic vessels, in 3) the absence of an alternative diagnosis. The perfusion disorder can be acute or chronic, non-occlusive (low-flow states, vasospasm) or occlusive (venous or arterial thrombosis or embolism), segmental or extensive.(1, 4)

The digestive tract vascularization is ensured by the three main arteries originating from the anterior face of the abdominal aorta (Figure 1 and Table 1):

- the celiac artery (CA) which supplies the stomach, the duodenum, the first centimeters of the jejunum, the bile ducts, the pancreas and the spleen;
- the superior mesenteric artery (SMA) which supplies the small bowel, the ileocecal valve and the right colon;
- the inferior mesenteric artery (IMA) which supplies the colon from the right angle to the upper rectum. The middle and lower rectums are supplied by the middle and lower rectal arteries, branches of the internal iliac arteries.

The topography of digestive ischemic lesions generally depends on the territories of vessel involved. Consequently, the distal occlusion of a branch of the SMA (embolism or vasculitis of small vessels, for instance) may lead to a segmental and focal ischemia, while a proximal thrombus is more likely to be complicated by extensive intestinal and right-side colon necrosis. Conversely, non-occlusive ischemia is more likely to present as multifocal ischemia, involving watershed areas of the GI tract with large superficial mucosal injuries rather than wall deep.

The GI tract arteries are terminal (straight vessels), perforating the digestive wall on the mesenteric edge, dividing into intramural arterioles penetrating through each villus up to its summit and connecting on a wide submucosal capillary network without constituting an arterial-venous shunt. These capillaries form intramural venules which run parallel to the arterioles, at the base of the villus. This micro-vascular network facilitates the diffusion of oxygen at the base of the villus and makes of the top of the villus the most sensitive area to ischemia. The villous venules merge into straight veins and empty into the mesenteric veins and the portal system. The right and left parts of the colon are vascularized by the superior and inferior mesenteric arteries, respectively. Left-side colon ischemia, which represents the most common type of digestive vascular injury, is generally mild, self-limited and the consequence

of a transient decrease in microvascular blood flow from a non-occlusive (vasospasm) and/or drug-induced origin.(5) Instead, ischemia affecting the right colon is associated with a vascular lesion of the SMA in 25% of patients and then should be considered as a segmental form of AMI. (6)

Figure 1. Gastrointestinal tract vascularization

Source: Anatomy of the human body. 20th edition. Gray Henry, 1918.

The superior mesenteric artery supplies the entire small bowel and the right side of the colon (A), whereas the left side of the colon is supplied by the inferior mesenteric artery (B). The portal circulation delivers the majority of the blood flow to the liver (C).

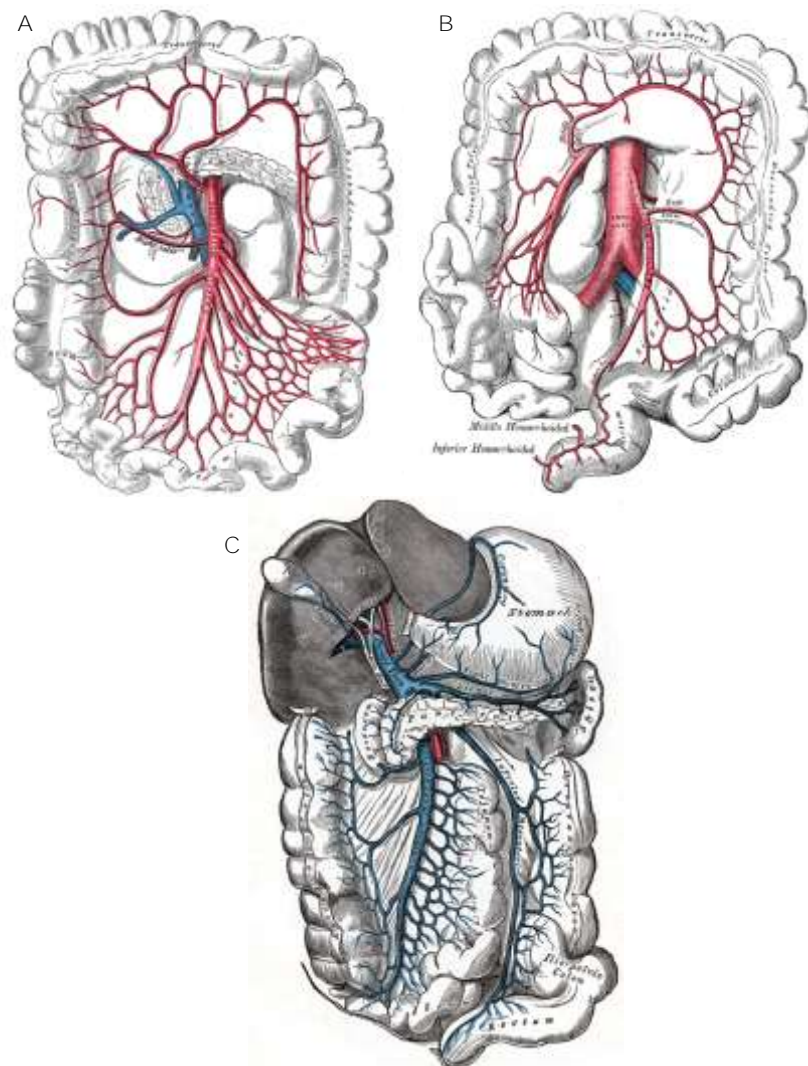


Table 1. Intestinal injury location depending on the mechanism and the type of vascular insufficiency

Splanchnic vessel	Organ supplied	Intestinal injury location	
		Proximal occlusive ischemia <i>(ex : atherosclerotic thrombosis)</i>	Non-occlusive, or distal occlusive, ischemia
Celiac artery	Stomach, Duodenum, proximal jejunum, Gallbladder, Pancreas, Liver, Spleen	Gastric & duodenal or duodeno- jejunal ulcers, cholecystitis, pancreatitis, spleen infarcts	Gastro-intestinal segmental and multiterritory ischemia
Superior mesenteric artery and vein	Jejunum, ileum Right and traverse colon	Extensive small bowel and/or right-side colon ischemia	
Inferior mesenteric artery and vein	Left-side colon, upper rectum	Left-side colon ischemia with frequent rectum sparing	

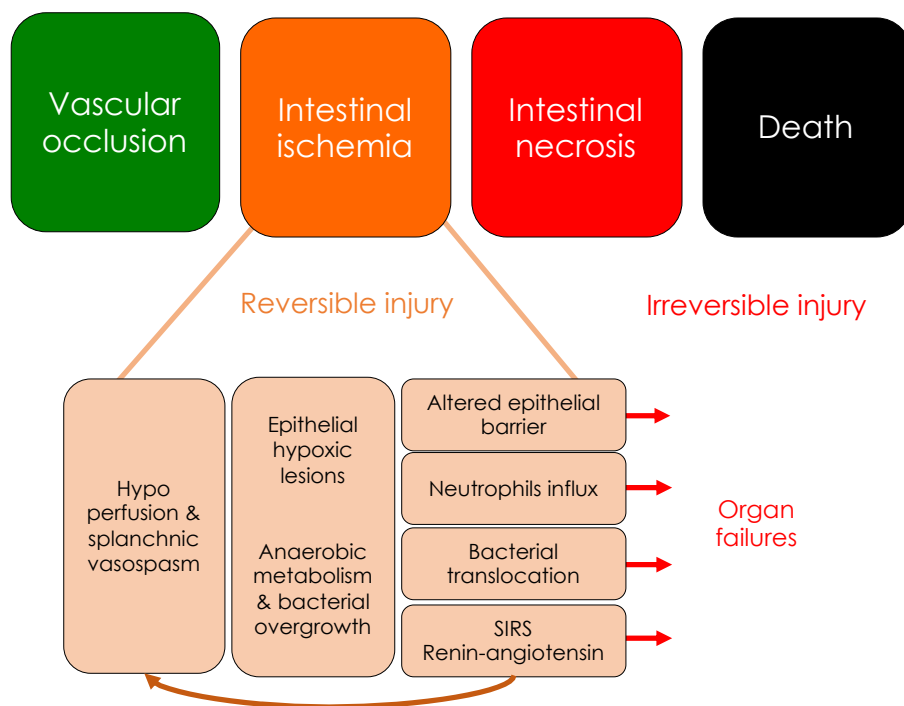
1.2. Pathophysiology

Acute mesenteric ischemia (AMI) should be considered as one stage of a multistep process (Figure 2) leading, from a digestive vascular insufficiency, to intestinal necrosis, organ failure and death. Ischemia begins early and superficially and then spreads in deep and surface of the intestinal wall. (Figure 3) Vascular insufficiency is initially the trigger for an inadequacy between inputs and requirements for energy substrates by overcoming the adaptive processes of a digestive territory. This loss of homeostasis results from a sudden decrease or interruption

of the splanchnic-mesenteric blood flow. The decrease in splanchnic blood flow in the proximal circulation induces a deep extension of the ischemia which then becomes transmural and gangrenous.

Figure 2. Multistep pathophysiology of acute mesenteric ischemia

Intestinal ischemia should be considered as the reversible stage of a pathophysiological multistep process leading to necrosis and death

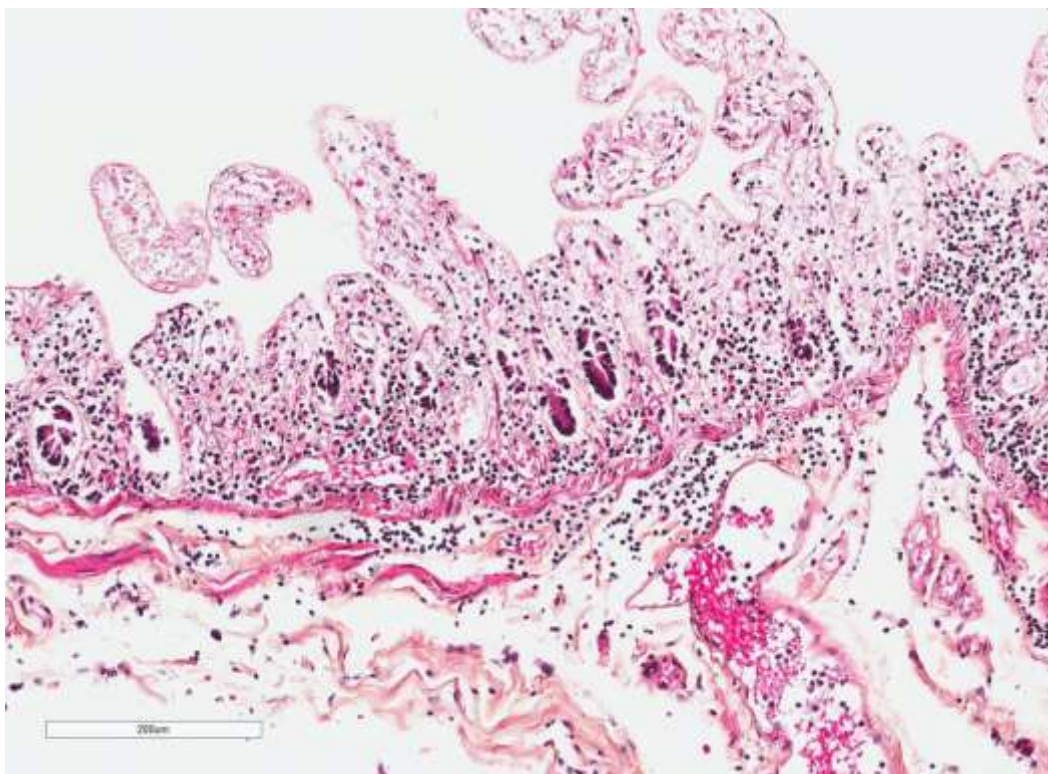


Conversely, when perfusion abnormalities relate to intra-parietal arterioles, lesions of ischemia remain superficial. Intestinal vascular insufficiency leads to hypoxia, first with mucosal and submucosal consequences. The hypoperfusion of the intestinal mucosa is responsible for an early hypoxic cellular desquamation of the intestinal villi. Polymorphonuclear neutrophils are early major actors that adhere and migrate to the ischemic site to ensure the removal of tissue debris during necrosis. Mucosal and submucosal cells switch to anaerobic glycolysis with local

production of lactate initially fully metabolized by the liver. The increase of intracellular acidosis achieves to block the anaerobic metabolism and the membrane pumps of ionic and acid-base regulation. This leads to a profound alteration of cellular homeostasis and, ultimately, to cell death by apoptosis. (7-9)

Figure 3. Pathological analysis of acute ileal ischemia

Acute ileal ischemia characterized grossly by congestive small bowel loops and superficial mucosal ischemia starting at the tip of the villi.



Initially, there is a dissociation between high porto-mesenteric blood lactate levels and normal peripheral blood lactate levels due to the active liver metabolism.(4) Systemic lactic acidosis is, therefore, a late phenomenon, which often indicates intestinal necrosis and the onset of organ failures. (10) Associated endothelial lesions can lead to platelet, pro- and anti-thrombotic agents (protein C, S, and antithrombin) consumption that cause the haemorrhagic syndrome.

Furthermore, the intestinal neuro-hormonal regulation of vasomotricity is associated with the activation of the renin-angiotensin-aldosterone system, to try to maintain a mucosal oxygen extraction rate. This induces a reflex splanchnic arterial vasospasm, irrespective of the initial vascular mechanism, that may prolong and worsen ischemia despite the revascularization treatment. This vasoconstriction accompanies, for example, situations of hypovolemia, during which digestive ischemia develops before clinical hemodynamic instability. (11, 12)

The disruption of the epithelial barrier resulting from mucosal alterations leads to interactions between microorganisms, bacterial antigens, endotoxins of the intestinal lumen and the mucosal and submucosal immune system. The stimulation of innate immunity will result in local then systemic inflammatory pathways activation such as TLR, NF-KB or TNF. (13, 14)

Through the bloodstream, bacteria, endotoxins, cells degradation products and activated immune cells translocate and promote systemic inflammation response syndrome (SIRS).

Cytokines, chemokines, cellular and bacterial debris can also reach the pulmonary circulation from the lymphatic circulation and thus cause acute respiratory distress syndromes.(15, 16)

The translocation of bacteria and/or bacterial products into the mesenteric lymph nodes and/or bloodstream is reported in intestinal ischemia in up to 25-100% of experimental animal models.

It further increases the SIRS making of the ischemic small bowel an authentic infection site.(17) The absence of a rapid recovery of a sufficient digestive perfusion leads to irreversible transmural necrosis with peritonitis. Without intestinal resection, the SIRS leads to multiple organ failures and death. (16)

In the model of the “gut origin of sepsis”, the gut was considered to be the ‘motor’ of multi-organ failure.(11, 15, 16, 18) Aside from its barrier function, the gut contains growth factors, adenosine, and hormones, which are potential mediators for the modulation of intestinal inflammation and repair, due to their roles in cellular proliferation, differentiation, migration, apoptosis, and autophagy.(19-23) Physiologically, the gut could initiate and propagate sepsis

due to the ability of bacteria, endotoxins, and other antigens to translocate, along with the production of pro-inflammatory cytokines and toxins. (11) In The 'Three Hits Model' Deitch et al. added the phenomenon of reperfusion injury. (24) In the 'Gut-Lymph' theory, bacteria, cellular components, immune cells, cytokines and chemokines generated by the injured gut travel via the lymphatics to reach the pulmonary circulation, activating alveolar macrophages, and contributing to acute lung injury, acute respiratory distress syndrome and multi-organ failure related to AMI. (15, 16, 25) The systemic consequences of bowel ischemia and necrosis are lethal in most patients in the absence of curative treatment including revascularization. (26, 27) However, reoxygenation of the digestive mucosa can also paradoxically worsen epithelial and vascular lesions, due to an oxidative burst mechanism causing the influx and death of neutrophils with the formation of neutrophil extracellular traps and the secretion of their granular content. (28)

Another mechanism of epithelial self-digestion would contribute to aggravate this alteration of the gut barrier function via proteolysis of tight enterocytic junctions by bilio-pancreatic secretory enzymes.(22) This quite recent concept describes the effect of pancreatic enzymes on the intestinal barrier altered by ischemia. Self-digestion contributes to the worsening of intestinal ischemic lesions and the development of the related systemic inflammatory response. Degradation products of pancreatic enzymes, residues of bacterial products pass through the lymphatic, hematogenous or peritoneal barrier and are likely to induce a loco-regional but also systemic reaction. (20, 21) In animal models, inhibition of these enzymes results in a decrease in intra-parietal micro-bleeding, in the systemic inflammatory response, and even in mortality in some studies.(22) The action of these enzymes would involve degradation of inter-enterocytic tight junction's proteins such as E-cadherin. Moreover, these enzymes would also induce a cleavage of the pro-metalloproteinases into active metalloproteinases.(23)

Mesenteric vein thrombosis can be anterograde (primary occlusion of the straight veins, generally secondary to an enteritis or an intra-abdominal inflammatory process) or retrograde (primary occlusion of the portal vein, generally secondary to portal hypertension). In this context, the occurrence of intestinal ischemia may be more likely in case of the occlusion of second order radicles of the superior mesenteric vein and in the context of arterial insufficiency / associated arteriosclerosis.(19) Downstream the portal/mesenteric vein thrombosis, the hepatic consequences are weakly marked due to rapid compensatory mechanisms such as hepatic arterial vasodilation and the development of a cavernoma, visible as early as a few days after thrombosis.

1.3. Epidemiology

1.3.1 Incidence

Given that the clinical diagnosis of mesenteric ischemia is still a challenge, the incidence of digestive vascular diseases is still difficult to assess and then potentially largely underestimated. However, it is likely increasing due to an increase in the population at cardiovascular risk and improvements of its recognition by the CT scan.(27)

Based on autopsy studies in Sweden, Acosta et al. estimated an incidence of 13/100,000 person-years between 1970 and 1982, when autopsy rate was 87%.(29, 30) In a US comprehensive database and population analysis in Maryland, Crawford et al. reported a statewide admission rate of 10/100,000 inhabitants per years during 2009-2013.(31) These figures are consistent with epidemiological studies on chronic mesenteric ischemia with an estimated incidence of 9.2/100,000 inhabitants per years from a Dutch prospective database between 2014 and 2019.(32) The incidence of arterial AMI increases exponentially with age, with an incidence of 25/100,000 person-years after 70 years old, and a peak incidence of 217/100,000 person-years after 85 years-old .(29, 30, 32)

1.3.2 Causes

Main causes of mesenteric ischemia can be classified according to the two main mechanisms of vascular insufficiency, often associated: 1) occlusive mesenteric ischemia (85%), due to atherosclerotic lesions, embolism or thrombosis, involving splanchnic artery (70%) and/or vein (15%), and 2) non-occlusive mesenteric ischemia (15%) caused by mesenteric vasospasm secondary to a systemic low flow or a vasoconstrictive drug. Each of these forms can occur on pathological vessels (atheroma, dissection, dysplasia, vasculitis) or on healthy vessels. In the latter case, the origin of the occlusion is often due to a systemic cause of thromboembolism (cardiogenic embolism, thrombophilia, myeloproliferative syndrome) and/or a regional risk factor (inflammatory or infectious intra-abdominal process or neoplasm, portal hypertension). The main cause of arterial mesenteric ischemia is atherosclerosis. Although the prevalence of significant splanchnic arterial atheromatous stenosis in the general population is high (17 to 50%), most are asymptomatic.(33) In this setting, the incidence and predictive factors for the onset of mesenteric ischemia are unknown. Other causes of mesenteric arterial occlusion include embolism, vasculitides, infectious arteritis, fibromuscular dysplasia, median arcuate ligament syndrome, dissection and hypercoagulability.(1, 4) Their respective prevalence and incidence have not been precisely evaluated.

Causes of venous mesenteric ischemia include portal hypertension, intra-abdominal inflammatory processes (pancreatitis, inflammatory bowel disease, sepsis, trauma), malignancies, hematologic disorders, and thrombophilia.

Risk factors for non-occlusive mesenteric ischemia include vasoconstrictors (noradrenaline, cocaine) and hypovolemia (sepsis, hypotension, dialytic depletion).(18, 34)(Table 2) The knowledge on the risk factors of AMI might be incomplete given that half of patients, no prior cardiovascular or prothrombotic history are found.(35)As a result, we believe that AMI should be considered regardless of the patient's age and cardiovascular history.

1.3.3. Prognosis

In a systematic review of 45 studies including 3692 patients, acute mesenteric ischemia (AMI) was consistently fatal without adequate treatment.(26) Despite treatment, mortality varied from 40% (venous ischemia) to more than 80% (arterial ischemia), the main prognostic factors being the precocity of the diagnosis and treatment, the occurrence of transmural necrosis, the mechanism of ischemia (venous vs arterial) and the age of the patient.(36) In the 2010s, our most recent results have shown that multimodal and multidisciplinary SURVI management made it possible to obtain a survival of 91%, without intestinal resection in 58% of cases,(4, 37) making AMI a potentially disease fully reversible at its early stage.(2) However, the late recognition of the diagnosis and intestinal necrosis, at the stages of peritonitis and multi-visceral failure, explain the mortality still reported today by other centers and the prevalence of short bowel syndrome in survivors.(38)

Table 2. Main causes of mesenteric ischemia

Mesenteric ischemia		Causes and risk factors
Occlusive	Vascular disease	<ul style="list-style-type: none"> - Atherosclerosis - thrombosis - Embolism (cardiogenic and arteriogenic) - Aneurysm, dissection, - Fibromuscular dysplasia - Vasculitides, neuro-endocrin tumors, radiation enteritis - Vascular injury (endovascular or open vascular surgery, trauma, compression by a mesenteric malignancy)
	Hypercoagulability	<ul style="list-style-type: none"> - Intra-abdominal inflammatory process, cancer or surgery - Inherited thrombophilia (prothrombin gene mutation G20210A, factor V Leiden, antithrombin, protein S and C deficiencies, increased factor VIII) - Hematological disorders (polycythemia, myelofibrosis, thrombocythemia, JAK2 V617F mutation, antiphospholipid antibodies, paroxysmal nocturnal haemoglobinuria) - Malignancies - Oral contraceptive, pregnancy, obesity - Portal hypertension, congestive heart failure - Other : CMV, nephrotic syndrome, SARS-COV-2 infection
Non-occlusive		<ul style="list-style-type: none"> - Low flow states (hypovolemia, shock, dialysis) - Extra-corporal life support, clamping in vascular surgery - Toxic / iatrogenic (cocaine, amphetamine, catecholamines) - Sickle cell disease, leukostasis - Heavy exercise (marathon runners)

1.4. Diagnosis of mesenteric ischemia

1.4.1 Clinical suspicion

In the absence of a biomarker allowing a rapid non-invasive diagnosis, the clinician's only weapon remains his diagnostic suspicion and confirmation by the contrast-enhanced abdominal CT scan. At this stage, the time of onset of acute abdominal pain should be considered as the

starting point for a countdown leading, without prompt and adequate treatment, to intestinal necrosis and death. Contrary to popular belief, most patients with AMI present to the emergency department at a potentially reversible, but still insufficiently recognized early stage. Indeed, 50% of patients present initially with no known cardiovascular history, without surgical abdomen, without organ failure and without elevation of plasma lactate.(35, 39, 40)

The acute abdominal pain is constant, apart from the particular case of the intensive care patient receiving a sedation.(1, 4) Pain can be inaugural or succeed to symptoms of chronic mesenteric ischemia in 30% of patients, the diagnosis of which is most often overlooked.(29, 35) Pain is typically sudden ("vascular") or rapidly progressive, intense and requiring opioids, continuous and relentless, peri-umbilical or diffuse, and contrasts with an abdominal palpation initially falsely reassuring. It can be associated with vomiting (48%), diarrhoea (31%), digestive haemorrhage (18%) and an inflammatory biological syndrome which, inconstant and / or too late have no diagnostic value.(37, 40)

1.4.2. Diagnostic imaging

The cornerstone of the diagnosis is contrast-enhanced computed tomography (CT). Its excellent reported sensitivity and specificity (41) suggest that it may be used as the first-line imaging technique. However, lower sensitivities were reported when the CT scan was performed during both arterial and venous phases (83%) or venous phase only (72%)(35), or when in the real-life setting, the clinical suspicion was not always mentioned to the radiologist,(42) resulting in either an inappropriate IV contrast protocol and/or an analysis that did not focus on the mesenteric vessels.(43) Finally, the unenhanced CT scan should be avoided because it does not detect signs of AMI, causing a significant delay in diagnosis.(35)A recent study focused on the impact of contrast-enhanced multidetector CT on the survival in patients with acute superior mesenteric artery occlusion; in-hospital mortality rate was 42% for

patients who underwent contrast-enhanced multidetector CT, *versus* 71% for patients not examined with CT (43). As a result, CT should be performed as quickly as possible after the onset of symptoms in all patients, and include contrast-enhanced images to visualize both mesenteric vessels and digestive structures, including in the presence of renal insufficiency, the risk of overlooking an AMI greatly exceeding that of the injection of the contrast agent.(1, 4, 44, 45)

CT plays a double role in patients with AMI. First, diagnosis is reached based on the combination of two sets of features that parallel the pathophysiology of the disease: 1/ vascular insufficiency, and 2/ ischemic intestinal injury. Second, CT should help identify negative prognostic factors, suggestive of extensive necrosis, or complications which will guide the treatment of revascularization,(46) and the indication for digestive surgery.(37)

Vascular imaging findings

Intra-luminal defects or occlusions of the mesenteric vessels are highly specific for the diagnosis (94-100%), but reported sensitivity is rather low (12-15%) (47). Yet, in our experience, vascular anomalies are encountered in more than 75% of patients. In occlusive forms of AMI, CT allows visualization of the site of the vascular obstruction and helps distinguish emboli from thrombosis. It also depicts other vascular anomalies such as calcified or non-calcified plaques, or rare dissections. In non-occlusive mesenteric ischemia, CT may show narrowed veins, flattened inferior vena cava (48), diffuse irregularities or stenoses of the SMA and SMA branches, and poor visualization of intestinal arcades and intramural vessels (49), as described by Siegelman et al with angiography (50).

Bowel imaging findings

Numerous findings associated with intestinal ischemic injury, including bowel wall thickening or thinning, spontaneous wall hyperattenuation on unenhanced CT acquisition, decreased or absent bowel wall enhancement, bowel dilatation, pneumatosis intestinalis and portal venous

gas, peritoneal fat stranding and ascites. These features are often associated, and bear different prognostic value.(44) (Figure 4)

Prognostic value of CT

CT helps distinguish early from late forms of AMI by depicting imaging features of necrosis or complications. (Figure 4) From this perspective, radiologists should not only recognize bowel ischemia on CT, but also differentiate it from bowel necrosis that requires surgical resection. Free intraperitoneal gas is the only pathognomonic finding of bowel perforation, and therefore of wall necrosis in patients with AMI.(51) Yet, it is a delayed feature, and patients need to be diagnosed and treated before. Another important finding is pneumatosis intestinalis. Importantly, pneumatosis intestinalis may occur in ischemic bowel segments that have not yet undergone transmural infarction. Duron et al. found that 47% of patients with AMI showing pneumatosis still had viable bowel, with only partial mural ischemia without transmural infarction on surgical or pathological analysis.(52) Patients with associated portomesenteric venous gas are more likely to have transmural infarction than those with pneumatosis intestinalis alone. In AMI of arterial origin, bowel dilatation and decreased wall enhancement have been shown to be more frequent in cases of bowel necrosis.(10) A recent prospective study from our group identified both features in univariate analysis, with necrosis in 68% of cases when bowel wall enhancement was decreased, and in 64% when bowel loops were dilated. Only bowel dilatation was retained in multivariate analysis.(37)

Figure 4. The contrast-enhanced abdominal CT-scan: the cornerstone of the diagnosis



A-B : Axial view, (A) showing a spontaneous hyperdensity of the intestinal wall at the non-contrast time, not to mistake with wall enhancement (B) unchanged after contrast injection.

C-D : Sagittal view, arterial phase, 90% stenosis by thrombi of the superior mesenteric artery (black arrows) before (C) and after intra-arterial thrombolysis (D)

E-F : CT-signs of transmural intestinal necrosis. (E) Axial view, venous phase, porto-mesenteric extensive thrombosis (arrows) with small bowel dilatation and decreased wall enhancement of the proximal jejunum (white arrowheads) when compared to normal ileum (black arrowheads). (F) Sagittal view, venous phase, small bowel dilatation and feces sign (arrows)

1.5. Treatment of acute mesenteric ischemia

1.5.1 Management in dedicated stroke units

In the 2010s, we showed that a multimodal and multidisciplinary management of AMI, focused on preserving intestinal viability in a specialized intestinal stroke center, could decrease the rate of intestinal resection, as well as improve short- and long-term survival.(53) These results demonstrated that intestinal ischemic injury during AMI is potentially reversible, and that intestinal necrosis could be considered an unwanted outcome and a late complication.(2, 37) Following these results, we created in 2016 a 8-bed dedicated intestinal stroke unit at Beaujon Hospital, Paris University, France, that provides 24/7 standardized multimodal and multidisciplinary care to AMI patients referred from all hospitals in the Paris area.

Preventing the progression from reversible to irreversible intestinal ischemic injury should be a primary goal in the management of AMI. Indeed, in our experience mortality goes from 2% to 35 % when intestinal ischemia is treated at the stage of irreversible necrosis.(54) The treatment strategy developed in our ISU simultaneously follows three main objectives: (Figure 5)

- 1) Prevent worsening, SIRS and organ failure by a specific medical protocol for intestinal ischemia (Table 3);
- 2) Preserve the non-necrotic intestine by systematic revascularization;
- 3) Resect intestinal necrosis according to a non-invasive risk score (Figure 5), before its complications (perforation, peritonitis)

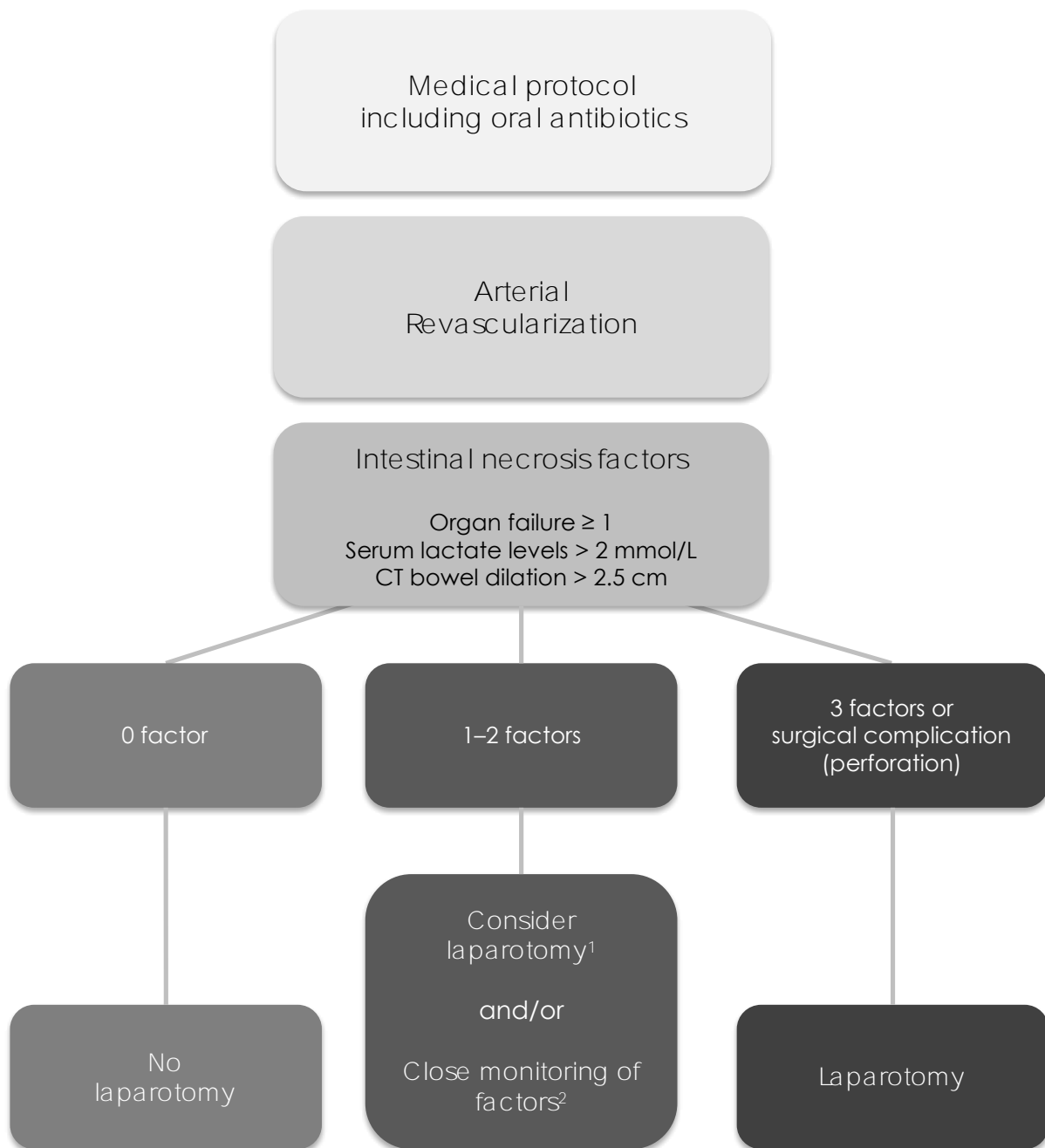
This multidisciplinary emergency strategy is coordinated by a gastroenterologist and requires a structure with available 24-hour revascularization and intestinal resection, in an intensive care environment and in collaboration with an intestinal failure unit. A specific medical protocol targeting each step of the pathophysiological process of intestinal ischemia is administered upon diagnostic confirmation, regardless of the mechanism and form of the AMI.

Systematic administration of oral antibiotics yielded a protective effect against transmural intestinal necrosis (HR = 0.16, 95% CI: 0.03-0.62, p = 0.01).(54) Oral antibiotics may prevent intestinal necrosis by (1) decreasing the luminal bacterial load which interacts with the intestinal innate immune system through the disrupted epithelial barrier, (2) limiting local and systemic inflammation which results from bacterial interaction/translocation, and (3) improving their luminal bioavailability in the context of vascular occlusion or low flow. Given the life-threatening risk of translocation in AMI, most experts recommend early and widespread use of antibiotics consistent with our experience.(1, 36, 55, 56)

The effectiveness of the treatment is judged on the disappearance of the pain. Any persistent digestive clinical symptom (pain, food intolerance, haemorrhage, persistence of a failure) should suspect for residual ischemia. Exclusive parenteral nutrition is offered until the ischemia is not lifted, especially when there is a pre-existing undernutrition or a short bowel syndrome. Although a Chinese study suggests that enteral nutrition should be systematically considered, many biases inherent in the retrospective and univariate nature of the analysis limit its interpretation.(57) In practice, oral and / or enteral feeding should be resumed gradually only after joint disappearance of pain and biological inflammatory syndrome.

In the particular case of non-occlusive ischemia, the therapeutic priority is to restore and maintain sufficient splanchnic hemodynamic by treating the cause of hypoperfusion (hypovolemia, sepsis, heart failure, haemorrhage), by preferring as much as possible fluids to the use of catecholamines (Figure 6). Despite the absence of vascular occlusion, the similar ischemic injury of the digestive mucosa justifies the maintenance of the common medical protocol. In the absence of rapid improvement, an arteriography with intra-arterial infusion of vasodilators (papaverine) is recommended. Surgical treatment is therefore offered as a last step in case of persistent patient worsening or immediately in the event of peritonitis / perforation.(57)

Figure 5. Treatment algorithm used in the intestinal stroke unit



¹ Consider laparotomy, especially when the factors value is high and when a revascularization is not feasible

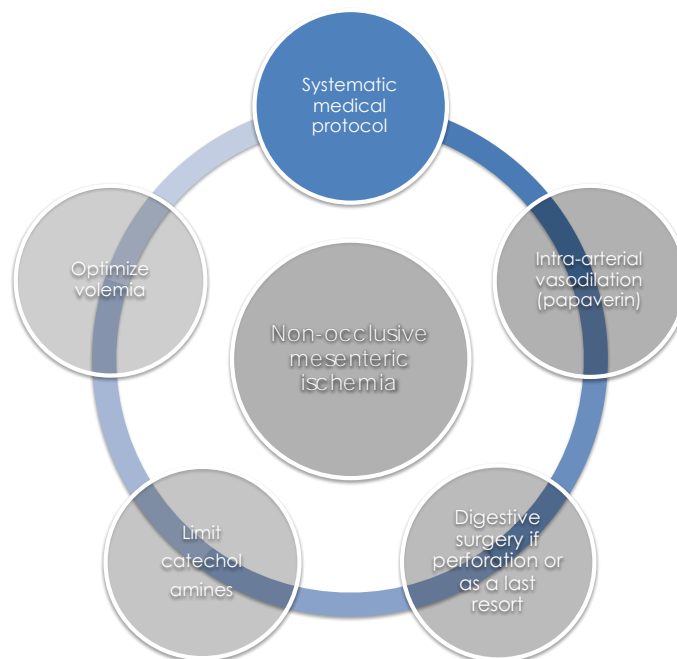
² Consider close monitoring, especially when the factor value is close to the upper normal cut-off and when an improvement is expected with successful revascularization

Table 3. Systematic medical protocol provided in the intestinal stroke unit (Corcos et al. 2013)

Common medical protocol	<p>Blood volume resuscitation Mean arterial pressure >65 mm Hg Urine output >0.5 mL · kg⁻¹ · h⁻¹ Curative unfractionated heparin therapy Anti-Xa target: 0.4–0.8 Oral digestive decontamination PO gentamicin 80 mg/d PO metronidazole 1.5 g/d IV proton pump inhibitors IV pantoprazole, 80 mg/d Oxygen therapy Food resting PN if prolonged >5 d</p>
Additional medical protocol	<p>IV aspirin 100 mg/d If arterial thrombosis or revascularization IV piperacillin-tazobactam 12 g/d If SIRS or organ failure Upper gastrointestinal aspiration If ileus Blood transfusion If hemoglobin level <9 g/dL</p>

IV, intravenous; PO, orally; SIRS, systemic inflammatory response syndrome.

Figure 6. Treatment principles of non-occlusive mesenteric ischemia



1.5.2 Revascularization

As with any vascular emergency, early revascularization is the only therapy that can prevent / limit irreversible necrosis and its life-threatening complications.(53) Depending on the accessibility of vascular lesions and local expertise, arterial revascularization is ideally carried out by percutaneous first and before any digestive surgery in order to preserve a maximum of viable small intestine.(4, 46)

Endovascular strategies

If a vascular impairment is reachable, percutaneous revascularization (intra-arterial thrombolysis, angioplasty-stenting, thrombectomy) should be considered. Recent literature reports a success rate of endovascular revascularization of 87%.(58) The choice of techniques depends on the etiology, and the localization of the vascular occlusion.

To date, no randomized controlled studies have compared an up-front endovascular approach with open surgery for the treatment of AMI. Because AMI is a relatively rare and urgent condition, such trials are unlikely to be performed. Most published studies are therefore retrospective monocentric series, with all methodological biases associated with such study design. A group from the University Hospital of Kuopio, Finland, recently reported a consecutive series of patients with AMI treated over a five-year period. Endovascular treatment was applied as first-line in 88% of the patients.(59) Mortality was acceptable (32%) yet the endovascular strategy failed in 50% of the patients, and a surgical bypass was finally achieved. The 30-day mortality rate was lower than that reported by Endean et al. after surgical strategy alone (62%).(60) Recently, a review conducted by Zhao et al. reported that radiological revascularization should be considered as a first-line therapy in patients with a low suspicion of intestinal necrosis.(61)

The analysis of population registers makes it possible to obtain data of greater value. Swedvasc, the Swedish vascular register, founded in 1987 comprises more than 90% of all vascular

surgical procedures in a country of 9.5 million inhabitants. Two publications from Swedvasc reported the results of revascularization of the superior mesenteric artery for AMI for the periods 1987-98 and 1999-2006.(62, 63) Overall, total surgical activity quadrupled from 1999 to 2006, while the number of endovascular revascularizations increased sixfold. If overall mortality decreased, this decline was observed only in patients treated with endovascular strategy. Long-term survival was also better after endovascular strategy. The difference between the two periods could be explained by a difference in patient severity, but the length of the resected intestine was similar in both groups and the endovascular strategy was identified as an independent factor of survival in multivariate analysis (odds ratio 3.7). However, as in the Kuopio Hospital experience, one of the main reasons for successful endovascular strategy was the possibility of surgical revascularization if the former fails.

Similar observations emerge from the analysis of the National Inpatient Sample (NIS) based in the United States.(64) The NIS is a database of 20% of the hospitalization episodes of nearly 1,000 hospitals. Of the 679 patients with AMI treated between 2005 and 2009, 514 (76%) were treated by surgical revascularization and 165 (24%) by endovascular procedure. The proportion of patients who had endovascular repair increased from 12% in 2005 to 30% in 2009. Mortality was 39% after surgery and 25% after endovascular procedure. Among survivors, the proportion of patients requiring total parenteral nutrition was also significantly higher after surgery than after endovascular strategy (24% versus 14%).(64) Thus, although Level I evidence is lacking, this data seems convincing.

This approach of surgery *versus* endovascular revascularization, seems to us to be limited and erroneous. If endovascular interventions are sometimes performed alone, optimal patient management must combine the two approaches in a complementary perspective, the benefits of each being able to support those of the other. Indeed, the majority of patients treated with the endovascular route have laparotomy and intestinal resection in a second phase. However,

the mean length of the resected small intestine is significantly shorter than with surgery alone.(46)

Open vascular surgery

Vascular surgery is often required to restore the mesenteric revascularization in order to improve the prognostic of the AMI (increase survival and prevent intestinal failure). The most common situations when revascularization is done surgically are:

- surgical abdominal exploration is needed and urgent
- surgical exploration has already been done by the visceral surgeon
- endovascular procedures failed to restore the mesenteric revascularization
- vascular surgeon and/or radiologist consider that the case not appropriate for endovascular revascularization
- contraindication to endovascular technique (local thrombolysis for example)
- visceral surgeon is not familiar with endovascular procedures but is able to perform basic

vascular surgery (embolectomy for example)

In our experience, the three most common surgical vascular procedures to restore the blood flow in the superior mesenteric artery (SMA) are: (1) the SMA embolectomy, (2) the retrograde SMA bypass, (3) the retrograde open SMA stenting. The vascular surgeon may need to harvest a segment of saphenous or femoral vein. Hence, we recommend having one of the thighs prepared in the surgical field in anticipation of possible access. A radiologic system to perform arteriography during the procedure is mandatory (C-arm fluoroscopy or hybrid operating room). All the vascular surgical procedures need systemic heparinization.

SMA embolectomy

Anterior exposure to the SMA for straightforward embolectomy is achieved by elevating the omentum and transverse colon, the small intestine is wrapped and retracted to the right. A segment of the proximal SMA between the middle and the right colic branches is isolated.

Circumferential dissection is required to isolate and to control any of the jejunal branches in this segment. The artery is open transversely if the SMA has a sufficient diameter and is not too atheromatous. For diminutive or diseased vessels, we firmly recommend performing a longitudinal arteriotomy using a patch closure (prosthetic or a saphenous patch). Great care must be taken to avoid damage or rupture to fragile collaterals of the SMA. When necessary, small Fogarty embolectomy catheters are used. After all macroscopic clot is cleared, we can consider administering a single dose of thrombolytic agent into the distal mesenteric vessels. An arteriography can be performed at the end of the procedure to control the quality of the revascularization.

Retrograde SMA bypass

Antegrade bypass using the distal thoracic aorta or suprarenal aorta as the inflow source is not recommended in case of AMI. A retrograde bypass based on the infrarenal aorta, a previous aortic graft, or the iliac artery is strongly preferred with the advantage of avoiding cross-clamp the aorta. Most retrograde reconstruction deal with the only SMA, but reconstruction of the common hepatic artery can also be achieved by tunnelling the graft retroperitoneally or via the transverse mesocolon. As the graft assumes a C-shaped configuration, it is important to avoid graft elongation, angulation or kinking. Synthetic bypass grafts (Dacron or externally supported polytetrafluoroethylene) are preferred because of the better size match, ease of handling, availability and kink resistance. The choice of conduit is heavily influenced by the degree of abdominal contamination and the perceived risk of subsequent infection. Therefore, if good-quality vein is available (saphenous vein or femoral thigh vein), it is preferred in the presence of significant peritoneal soilage. In the presence of a diseased SMA, an endarterectomy may be necessary before doing the distal anastomosis. Reports suggest that retrograde grafts perform as well as antegrade grafts.(65)

Retrograde open SMA stenting (ROMS)

The ROMS technique uses a hybrid approach via midline laparotomy to expose the SMA combined with endovascular retrograde stenting. Because several of these patients already have an indication for laparotomy to address advanced bowel gangrene or ischemia, direct surgical exposure of the SMA allows expeditious access for direct puncture. The proximity of the sheath to the lesion affords excellent support to cross a difficult occlusion with less risk of distal embolization by occlusion of side branches. Furthermore, primary stenting is an excellent method of revascularization that avoids the need to reconstruct the vessel by surgical bypass, minimizing surgical dissection and potentially eliminating the need to harvest a vein or to use a prosthetic graft for conduit.

We recommend the use of covered balloon expandable stent, 7mm diameter stent are most often used.(66, 67) In our experience, a majority of the SMA needs an endarterectomy and we recommend performing a longitudinal arteriotomy using a patch closure (prosthetic or a saphenous patch). ROMS during emergent laparotomy for AMI is a very promising technique and an attractive alternative to emergent surgical bypass.

1.5.3. Intestinal resection

Non-invasive predictors of transmural necrosis

The cornerstone of acute mesenteric ischemia surgical management in the acute phase is the diagnosis of intestinal necrosis, that will not be reversible despite revascularizations procedures.(3, 46, 68) However, the identification of patients with intestinal necrosis has been shown as difficult. Indeed, no clinical sign or laboratory study is specifically associated to intestinal necrosis,(55, 56) as even serum lactate might be normal in necrotic patients or elevated in patients with reversible ischemia, due to dehydration and decreased oral intake.(55) In this setting, we have performed a prospective cohort study of 67 patients with acute mesenteric ischemia, of which 34% presented with intestinal necrosis.(37) From this overall population, we identified which signs were associated to intestinal necrosis in multivariate

analysis. Our results suggested that organ failure, elevated serum lactate ≥ 2 , and bowel loop dilation (defined as a diameter >2.5 cm) on CT scan were independent factors predictive of intestinal necrosis. On the basis of these findings, the rate of intestinal necrosis in our study population rose from 3% in patients with no predictive factor to 38%, 89%, and 100% in patients with 1, 2, and 3 factors, respectively. We therefore consider in routine practice that all patients presenting with 2 or more factors should undergo immediate explorative surgery to assess for intestinal viability.(37) (Figure 5)

If a surgical exploration is decided, this procedure should be, whenever possible, carefully planned according to the feasibility of a revascularization procedure. In highly instable patients and/or in patients with overt peritonitis, we advocate a primary blood flow restoration procedure prior to surgical resection, as this might reduce the extent of intestinal resection and reduce the risk of secondary necrosis of the remnant bowel.(46) In all other patients, we routinely perform a radiological or surgical revascularization procedure prior to the explorative laparotomy, for the same reasons.

Surgical approach

Very few studies reported the use of laparoscopic approach for the management of acute mesenteric ischemia.(69-73) Despite this small series, the European Society for Trauma and Emergency Surgery guidelines does not recommend the use of minimally invasive approach for acute mesenteric ischemia, due to the paucity of reported evidence.(55) In our experience, laparoscopic approach is not the gold standard approach for the management of acute mesenteric ischemia as: (1) the complete visualization and viability assessment of the small bowel and colon might be difficult, (2) patients' tolerance of the pneumoperitoneum might be compromise, especially in instable patients, (3) surgical revascularization procedure requires an open approach. We therefore usually perform an open approach in our patients, reserving

laparoscopic approach in cases of difficult diagnosis and especially in non-occlusive patients.(71)

Bowel viability assessment

Most of the time, necrotic bowel will be clearly identifiable during the laparotomy, and will be based on the bowel colour, motility and bleeding of cut ends.(55, 74, 75) (Figure 7) As described before, this viability should be assessed after revascularization procedure, whenever possible. However, viability assessment might sometimes be difficult, especially in cases of hypotension, vascular impairment, and the concurrent use of vasopressors. In such patients, the use of doppler ultrasound of the vascular arcade,(76) fluorescein angiography,(77) and indocyanine green angiography(78) have been reported but failed to gain widespread of use. In our practice, we usually perform a frozen section examination in order to avoid unnecessary extensive resection.

Figure 7. Acute arterial mesenteric infarction

Resection specimen showing transmural intestinal necrosis from arterial intestinal ischemia



Damage control surgery

The strategy of Damage Control Surgery includes an abbreviated laparotomy with resection of necrotic bowel and the absence of anastomosis or stoma(74, 79, 80), in an attempt to reduce the operative time and to prioritize the resuscitation in intensive care unit. For many surgeons, it has become the standard of care for acute mesenteric ischemia management. However, this strategy imposes the realization of a second-look surgery, which is usually performed 48 hours after the primary procedure.

In our routine practice, we do not advocate such strategy for all patients. As stated in the European Society for Trauma and Emergency Surgery guidelines published in 2016, Damage Control Surgery might only be performed in patients with severe septic shock,(55) which in fact represent a minority of patients. We favorize a strategy that includes the realization of stomas of all resected bowel segments, as this might have several advantages: (1) To avoid unnecessary second look laparotomy – indeed, Park *et al.* reported the outcomes of patients operated on for acute mesenteric ischemia and reported that an additional bowel resection was performed during the second look in the minority of patients (11/23), thus exposing the majority of patients to the risk of an unnecessary laparotomy;(81) (2) The presence of a stoma allows the direct evaluation of the remnant bowel viability, as the mucosa can be easily evaluated and, if necessary, biopsied; and (3) this strategy does not expose the patient of the complications of intestinal anastomosis, as anastomotic leakage is frequent in those patients that will receive intensive resuscitation and vasopressor injections.(55) We therefore never perform any anastomosis in the acute phase on acute mesenteric ischemia management. In this setting, during bowel resection, care should be taken to facilitate the intestinal continuity restoration. Double end stomas should be preferred over single stomas in separate incisions, as this will allow to perform intestinal continuity restoration through an elective approach, rather than a midline laparotomy.

Extensive bowel resection

In severe patients, with extensive intestinal necrosis requiring large bowel resection, the surgeon might face a philosophical decision whether to expose the patient to a short bowel syndrome, requiring total parenteral nutrition, or not to do anything. Such decision is highly difficult and should, whenever possible, always be discussed intra-operatively with the intensivists, the gastroenterologist, and the nutritionist in charge of the patient. As even some patients with very short bowel syndrome might be completely weaned of parenteral nutrition after intestinal continuity restoration and small bowel reversal,(82) in parallel with the advent of Teduglutide for such patients,(83) we propose an aggressive strategy regarding small resection, limiting at its maximum the indications of no resection. Our decision is routinely based on the associated comorbidities, and the long-term probability of patient discharge from medical assistance.

1.6. Follow-up and rehabilitation

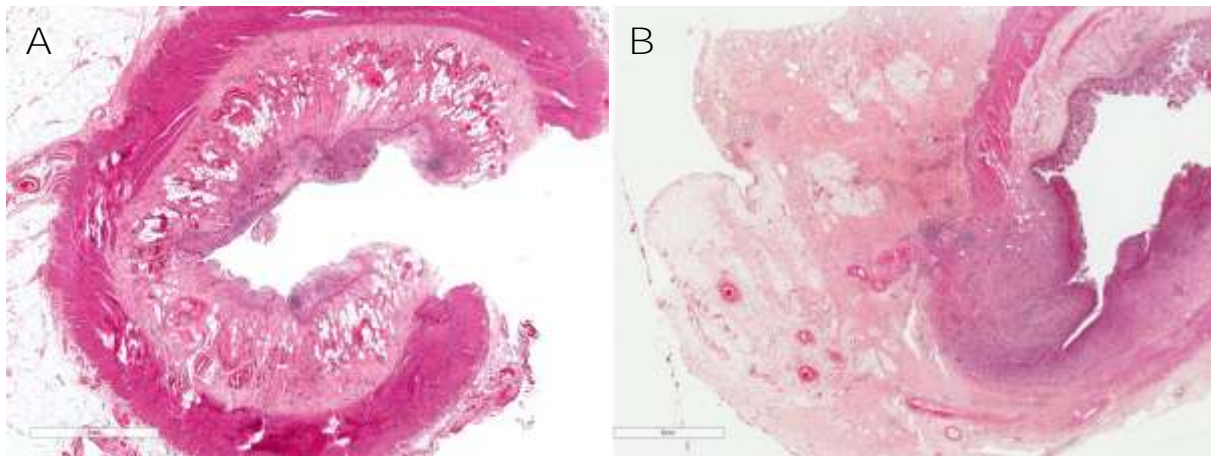
1.6.1 Post-ischemic stenoses and disorders

When it does not progress to transmural necrosis, any mesenteric ischemia can cause functional intestinal "sequelae", especially dysmotility disorders. In most cases, these lesions may appear when the intestinal injury has exceeded the healing capacity mechanisms and has progressed to stenosing parietal fibrosis and / or chronic mucosal ulcers. Occurring within a variable delay after refeeding, post-ischemic disorders present as pseudo-occlusive syndrome, chronic diarrhoea and / or septic episodes of digestive origin (microbial translocations). The treatment is generally surgical. In some cases, the motility disorder is transient and could be linked to the intestinal smooth muscle response to ischemia-reperfusion. Indeed, recent physiomorphological studies of gastrointestinal peristalsis by magnetic enteromyography, have shown that peristalsis was early and significantly decreased in the case of chronic mesenteric ischemia and normalized after revascularization.(84) This could explain some functional GI

disorders, such as gastroparesis, which refeeding after revascularization could unveil.(85)(Figure 8)

Figure 8. Histopathological picture of post-ischemic enteritis

- (a) *Resection specimen showing a non-penetrating chronic post-ischemic enteritis: the mucosa is destroyed, ulcerated with submucosal fibrosis, increased angiogenesis, and normal underlying muscularis propria.*
- (b) *Resection specimen showing a chronic post-ischemic enteritis with fistula : the mucosa is ulcerated with a deep fistula infiltrating a fibrosed muscularis propria.*



1.6.2 Nutritional, vascular and intestinal rehabilitation

In the event of multiple vascular involvement, primary revascularization of the superior mesenteric artery should always be offered. If this is impossible, and due to the collateral network between the digestive arteries, revascularization of the celiac trunk and / or the inferior mesenteric artery can lead to resolution of AMI.(86) In the extreme, in the patient not eligible for revascularization, we observed clinical improvement in 4 of the 6 patients in a series after infusion of iloprost (ilomedine®), a prostacyclin analog having a peripheral vasodilator, antiaggregating and immunomodulatory effect and used in the Raynaud syndrome, critical ischemia of the lower limbs and high blood pressure.(87)

Proximal and/or extensive resection of intestinal necrosis can be complicated by a transient or definite short bowel syndrome. The care of these patients must be provided in collaboration with an expert intestinal failure unit.(39, 88) Rehabilitation after mesenteric infarction is threefold:

- Nutritional: screening and correction of hydro-electrolytic, caloric and / or vitamin / trace element deficits, and parenteral support;
- Vascular: detection of persistent ischemia related to a residual vascular stenosis, and secondary cardiovascular prevention with long-term anti-thrombotic treatment;(89)
- Digestive with the restoration of continuity with possible interposition of a reverse loop.(90)

2. BIOMARKERS OF ACUTE MESENTERIC ISCHEMIA

This section on acute mesenteric ischemia biomarkers was published as a review in the journal Clinical Chemistry and Laboratory Medicine in 2018 (91) Peoc'h K, Nuzzo A, Guedj K, Paugam C, Corcos O. Diagnosis biomarkers in acute intestinal ischemic injury: so close, yet so far. Clin Chem Lab Med. 2018 Feb 23;56(3):373-385. doi: 10.1515/cclm-2017-0291. PMID: 28841570 Review.

2.1.Clinical unmet needs in the diagnosis workup of ischemic intestinal injury: urgent need for a biomarker

Early diagnosis of AMI requires a high degree of suspicion faced with any abdominal pain, especially when the pain is sudden or rapidly growing (“vascular-like”), unusual, intense and requiring opioids. Other clinical and biological associated signs (vomiting, diarrhea, gastrointestinal hemorrhage, hyperleukocytosis, lactic acidosis) are inconstant or too late in the course of the disease and have no diagnostic value (4). In our retrospective experience of a cohort of 221 patients peritoneal signs, organ failure, and serum lactate elevation were initially

lacking in 85%, 77%, 57% of the cases, respectively (92). When unrecognized at this stage, the diagnosis was carried out later at the stage of necrosis and complications, explaining why 184/221 (83%) of the patients required intestinal resection, resulting in short bowel syndrome in 148/184 (80%).

Improving the prognosis of AMI requires the discovery of early, sensitive and specific diagnostic biomarkers. In the last decade, some potential biomarkers have emerged from the literature. Some of these markers have been studied with particular interest, because of their higher presumed enterocyte specificity.

This review aims to describe the past and present potential biomarkers associated with AMI and to introduce some other promising tracks that emerge from the literature. We focused in the first part on human clinical studies, whereas we choose to present pre-clinical research in the second part.

2.2. Non-specific biomarkers

2.2.1 Common blood biomarkers

Variations in common biological blood parameters (base deficit, lactate dehydrogenase, aspartate aminotransferase, creatinine phosphokinase, alkaline phosphatase, phosphate, amylase) have been frequently observed (93, 94). A metabolic acidosis is commonly observed as presented earlier.

Regarding hematological parameters, attention has been given to platelets indices, and particularly to platelets volume, but also to the various combination of neutrophils, lymphocytes and platelets ratios (95). Budak et al. proposed in a systematic review that low platelets volume could be used in the diagnosis, but considered that high platelets volume would be a poor prognosis indicator (96). As a whole, the use of such indices appears to be difficult to translate into clinical practice.

2.2.2 Biological markers of Thrombosis

D-dimer, an enzymatic degradation product of fibrin has been found to be the most consistent highly sensitive early marker, with however low specificity. Moreover, D-Dimers are usually increased either in arterial or venous occlusive forms (A Nuzzo and O Corcos, personal communication), although they remain in the normal range in non-occlusive acute AMI (97).

2.2.3. Biological markers of Hypoxia and oxidative stress

L-Lactate is a ubiquitous product of glycolysis in the context of anaerobiosis. In 1994, Lange et al. qualified L-lactate as the best marker of intestinal ischemia, regarding its negative predictive value (98). However, as expected, L-lactate elevation in plasma could not differentiate intestinal ischemia out of other etiology of abdominal emergencies or intensive care diseases (99-101). Actually, its elevation better reflects the late stage of the disease, with extensive transmural necrosis, anaerobic metabolism due to systemic hypoperfusion (10). Hence, it should not be used anymore as an early diagnostic marker of AMI (102).

Glutathione S-transferases (GST) are enzymes involved in the detoxification of a wide variety of endo- and xeno-biotics, conjugating them to glutathione. These enzymes are sensitive biomarkers of cytolysis, with a very short half-life, and are currently used in the diagnosis of hepatic cytolysis (103). Alpha-GST showed a significant increase in 50% of acute AMI patients, as compared with 12 other unclear acute abdominal pain suspected for being ischemic, with a negative predictive value of 100 percent (104). In the analysis published by Evennett et al., the pooled estimate of sensitivity was 68%, the pooled estimate of specificity was 85% (94). However, Alpha-GST also increases in non-specific hypotensive patients with multiple organ failure (105).

During acute ischemic conditions, albumin's metal binding capacity is reduced, leading to the apparition of a metabolic variant known as Ischemia Modified Albumin (IMA). It is a sensitive

but non-specific marker of myocardial and muscle ischemia, pulmonary embolism and stroke (106). IMA is usually measured in the plasma or serum by ELISA or using an assay based on a spectrophotometric method that measures altered cobalt-human serum albumin binding. Significantly increased plasmatic concentrations were found at admission time of seven patients with acute AMI, as compared to healthy controls (107). Another small study reported 100% sensitivity and 86% specificity in the detection of 12 intestinal ischemia in preoperative plasmas of 26 patients scheduled for exploratory laparotomy of mesenteric ischemia suspicion (108).

2.2.4. Biomarkers of inflammation

The C-Reactive Protein is commonly increased in acute AMI. Acute inflammatory mediators such as interleukin-2-6 and tumor necrosis factor are non-specific of intestinal injury, although IL-6 has been proposed to be both sensitive and specific in a small cohort of 10 AMI (109).

2.2.5. Biomarker of infection

Acute AMI is usually associated with an increase of leukocytosis that can exceed 20 G/L (110). Procalcitonin (PCT) has been proposed for the diagnosis of AMI. This precursor of the calcitonin is currently used in clinical practice for the differential diagnosis of infection of bacterial origin. According to a systematic review by Cossé et al., PCT's positive and negative predictive values for the diagnosis of acute AMI ranged between 27% and 90%, and 81% and 100%, respectively (111). As underlined by the authors, PCT is usually elevated in the sepsis, in specific bacterial infection, and in various type of ischemia. As a whole, the use of PCT for the diagnosis of acute AMI lacks specificity.

In a meta-analysis published in 2013, Cudnik et al. evidenced in 23 studies that L-lactate and D-dimers exhibited a good pooled sensitivity, although both were not specific enough to be

used as diagnosis markers (112). The inclusion of both occlusive and non-occlusive forms of AMI, as discussed in the larger paper of Matsumoto et al. (97), could lead however to a high heterogeneity in patients.

Finally, none of the parameters cited above show enough clinical accuracy as a diagnosis marker of early, limited and reversible AMI, which is mandatory to prevent the occurrence of intestinal necrosis and reduce the mortality rate. Therefore, the markers cited above, which can be assessed through current laboratory assays, show acceptable sensitivities, but none of them is specific enough to be used as a diagnosis marker.

2.3. Promising candidate biomarkers

In a candidate approach, some markers related to the pathophysiology of ischemia, intestinal insufficiency, and gut barrier failure would be of interest in the biological diagnosis. Selected clinical studies concerning these biomarkers are presented in Table 4.

2.3.1. D-Lactate, a biomarker of gut barrier dysfunction

D-Lactate, the second stereoisomer of lactate, is a byproduct of bacterial fermentation, only tiny produced by human cells (113). It can be found in the circulation after ischemic injury, increased intestinal permeability, or bacterial overgrowth. AMI is associated with an overgrowth of the resident bacterial flora that releases D-lactate into the portal and systemic circulation. The analysis of D-Lactate concentration requires strict preanalytical conditions, which are comparable to the one needed for the assessment of L-Lactate concentration. D-Lactate is usually assayed using an enzymatic UV spectrophotometric method on deproteinized plasma (114). This latter method could be automated (115). In the last decades, few studies were focused on the use of seric D-Lactate in the biological diagnosis of AMI. In 2006, in a

prospective study, Collange et al. compared D-Lactate concentrations in 29 surgical abdominal aortic aneurysms patients (AAA), which may be associated with AMI. D-Lactate levels were increased in AAA patients for whom the inferior mesenteric artery was hypoperfused during the surgery (n=6, 0.13 mmol/L), as compared with AAA patients without hypoperfusion (n=23, 0.03 mmol/L, p=0.007) (116). In 2015, Shi et al. showed that serum D-Lactate levels were increased in AMI patients (52.73 ± 26.46 $\mu\text{g/mL}$ in AMI vs. 15.58 ± 5.17 $\mu\text{g/mL}$ in non-intestinal ischemia) and could participate in the diagnosis (117). As a whole, according to the chosen threshold, the sensitivity of this marker either in plasma or serum ranged between 67 to 90%, whereas the specificity reached 87%. The design of the studies and their definition of intestinal ischemia were, however, heterogeneous. Therefore, there is a need for larger studies with well-characterized populations to confirm the potential use of D-Lactate. This could be a promising track since the assay could be easily automated.

2.3.2. Fatty acid binding proteins (FABP), biomarkers of villi injury

FABP are cytosolic proteins involved in the uptake and intracellular transport of fatty acids. The mature enterocyte expresses three isoforms: Intestinal FABP (I-FABP), Ileal Bile Acid Binding Protein (I-BABP) and Liver FABP (L-FABP).

The intestine, liver, and kidneys express L-FABP, whereas I-BABP is specific of the ileum. I-FABP is a 15-kD soluble protein expressed by enterocytes located at the tips of the intestinal mucosal villi, the anatomical region that is first affected by ischemic injuries. In physiological conditions, I-FABP is not detected in peripheral circulation and is urinary cleared (118, 119). After mucosal tissue injury, and especially enterocyte necrosis, the protein is quickly released into the bloodstream (120). The half-life of the molecule is very short (11 minutes), underlying its potential interest as a very early diagnosis marker (120).

Many studies have reported a relationship between blood I-FABP concentration and small intestinal diseases, either in acute AMI, in critically ill patients, or in post cardiac surgery, in which AMI represents roughly 1% of common complications (121).

Several body fluids could be used for I-FABP measurement using ELISA research assays. In clinical settings, I-FABP concentrations measured in peritoneal fluid, plasma, and urine are significantly higher in patients with AMI than in healthy controls and patients with other causes of the acute abdomen (122-124). In peritoneal fluid, which presence reveals late and severe disease, high levels of I-FABP were detected in patients with intestinal diseases (124).

In the study by Kanda et al., high levels of serum I-FABP upon admission at the hospital were associated with 2/8 patients presenting with strangulated bowel obstruction and 5/5 patients suffering from acute AMI, whereas ranges were normal for healthy subjects (n=35) and patients with abdominal pain of other etiologies (n=48) (123).

Cronk et al. found a 100% sensitivity, 83% and 78% specificity of both urine and plasma I-FABP, respectively, for prediction of ischemic necrosis in 3 out of 21 strangulated bowel obstruction (125). Thuijls et al. confirmed these preliminary findings, showing that plasma and urine I-FABP, L-FABP and urine I-BABP concentrations allowed the differential diagnosis of AMI from other abdominal emergencies in 50 patients with high suspicion of acute AMI (99). Although I-BABP was more specific of ileal ischemia, combining these markers did not improve diagnostic accuracy,

Shi et al. found that the concentration of serum I-FABP was increased in 39 patients with proven AMI (113.8 +/- 46.3 ng/mL), as compared with patients with non-ischemic acute abdomen condition (n=233; 33.9 +/- 12.6 ng/mL) and with healthy controls (n=37; 5.47 +/- 3.64 ng/mL) (117). In a human experimental model of ischemia-reperfusion, Schellekens et al. evidenced a correlation between the duration of ischemia and the increase of serum I-FABP (126). Kittaka et al. found that serum I-FABP concentrations were significantly increased in

patients with strangulated bowel obstruction (n=21; 18.5 ng/mL), as compared with patients with simple obstruction (n=16; 1.6 ng/mL) (127).

Salim et al. recently showed that urine I-FABP could be a biomarker with high specificity and sensitivity (area under receiver operating curve= 0.88) for the diagnosis of acute AMI in 18 patients with suspected AMI, whereas their results were not significant for serum I-FABP (128).

Large variations are observed among studies regarding mean values and ranges. The comparison between seric and plasmatic values has not yet been published. Moreover, different ELISA kits are used participating to the variability of the results. However, a recent meta-analysis evidenced a pooled sensitivity of 80% for serum I-FABP, a pooled specificity of 85%, and an Area Under the ROC Curve of 0.86 in the diagnosis of acute AMI (129).

In conclusion, so far, I-FABP is the protein that is the most assessed in the literature in the diagnosis of acute AMI and to date, it thus seems to be the most relevant marker in urines, serum, and plasma.

However, extensive efforts are still needed to design studies with increased population size, well- characterized in term of phenotypic groups, and with standardized preanalytical and analytical conditions.

2.3.3. Citrulline, a biomarker of enterocyte mass and intestinal failure

Citrulline is a non-proteinogenic amino acid synthesized from glutamine, by small bowel enterocytes. This amino acid is a precursor of nitrogen oxide and participates in the transformation of ammonia into urea, and in the synthesis of arginine. Its plasmatic concentration depends on the gut synthesis and renal elimination, decreasing in short bowel conditions and thus known as a functional marker of enterocyte mass, correlated with remnant small bowel length and home parenteral nutrition dependence (130). Citrulline is usually

measured in plasma or serum, using either ELISA methods, High-Performance Liquid Chromatography or Mass spectrometry. Critically ill patients with shock may have an acute non-occlusive AMI resulting in reduction of enterocyte mass and related citrulline synthesis, leading to low plasma citrulline concentrations (131). In a study by Piton et al., plasmatic citrulline concentration was shown to decrease in the first hours of shock in 24/55 critically ill patients and was correlated with 28 days mortality (131).

In 2016, Kulu et al. found that the concentration of plasmatic citrulline was significantly decreased in 23 patients with acute abdominal findings preoperatively attributed to AMI (Mean: 0.72 mmol/L; range 0.57-0.84), as compared to those in patients with other acute abdominal conditions (132). Acute renal failure induces high plasmatic citrulline concentrations by decreasing renal clearance and citrulline transformation into arginine (133), which may complicate the interpretation of the results in severe patients with multiorgan failures. Moreover, post prandial samples are associated with a 10 to 20% decrease in blood concentration (133). Moreover, some inter-ethnic variations have been described in reference ranges.

These results suggest that citrulline is probably more promising as a prognostic than diagnostic marker of AMI. Moreover, the interpretation of a ratio between plasmatic citrulline and creatinine could help to minimize the effect of acute renal failure leading to possible false negative results.

2.4 Conclusion on current scientific knowledge on AMI biomarkers

AMI is a highly severe condition, with a high mortality rate and major anatomical and functional consequences in case of survival. AMI represents a gut and life-threatening emergency for which the main identified prognosis factor is the precocity of the diagnosis and treatment.

A large number of molecules have been proposed as potential biomarkers of AMI. However, conflicting findings came from different studies with very unsatisfactory results. We mainly chose to focus our review on the three promising biomarkers: I-FABP, D-Lactate and citrulline. Even though these molecules are interesting candidates, the reported findings highlighted many limitations. In the clinical setting, most studies were performed in small populations, with high pre-test probability of high suspicion of intestinal ischemia, and at a late stage. Studies in early and less severe disease are still lacking. As observed in numerous diseases, the combination of several biomarkers rather than the use of a single marker is probably a better paradigm to explore. Moreover, the use of omics studies on well-phenotyped patients could be another promising track. Indeed, several works that used high scale proteomics and metabolomics studies have emerged in the last decade. The advantage of such a technique is to allow the identification of completely novel candidates that haven't been hypothesized yet on the basis of physiopathology. Nevertheless, in 2021, the need for early and robust biomarkers for AMI diagnosis remains.

Table 4: Selected Clinical studies about Citrulline, I-FABP, and D-Lactate as biomarkers of AMI.

First author, year	n	Clinical condition	Biomarker	Tissue	Mean Value	Range	Out-off Value	Sensitivity (%)	Specificity (%)	+LR	-LR	+PV	-PV	Experimental procedure
Murray et al., 1994	41	31 patients undergoing laparotomy for an acute abdominal emergency, including patients with acute mesenteric ischemia (n=9), with Small Bowel Obstruction (n=5) or patient with Small Bowel Obstruction (n=7). 10 healthy controls were also included.	D-Lactate	Plasma	32.37 µg/L	11.71 µg/L - 48.66 µg/L	—	90	87	—	—	70	96	Spectrophotometric method on protein free plasma
Pozze et al., 1998	45	Ruptured Abdominal Aortic Aneurysm (AAA) patients with (n=11) or without (n=13) ischemic complication were compared with controls (n=21).	D-Lactate	Serum	—	—	0.20 mmol/L	82	77	—	—	83	75	Spectrophotometric method on protein free plasma.
Thijls et al., 2011	46	46 patients suspected for intestinal ischemia, including 23 patients diagnosed with intestinal ischemia and 23 patients that were diagnosed with other diseases.	I-FABP I-FABP	Plasma Urine	0.653 ng/mL 3.377 ng/mL	0.04 ng/mL - 74.711 ng/mL 0.142 ng/mL - 442.795 ng/mL	0.268 ng/mL 0.551 ng/mL	68 90	71 89	23.4 81.8	45 11	— —	— —	ELISA (Hycoult Biotech, Uden, The Netherlands).
Güzel et al., 2014	77	30 patients diagnosed for intestinal ischemia were compared with 27 patients with other types of acute abdomen and with 20 healthy controls.	I-FABP	Serum	0.421 ng/mL	0.04 ng/mL - 5 ng/mL	0.09 ng/mL	90	100	—	—	100	87	ELISA (Hycoult Biotech, Uden, The Netherlands).
Kitnaka et al., 2014	37	37 patients diagnosed with Small Bowel Obstruction (SBO) and 21 patients were diagnosed with Strangulated SBO and 16 patients that were diagnosed with Simple SBO.	I-FABP	Serum	18.5 ng/mL	—	6.5 ng/mL	71.4	93.8	—	—	93.8	71.4	ELISA
Matsumoto et al., 2014	208	208 patients, including 24 patients with vascular, intestinal ischemia, 62 patients with non-vascular ischemia and 122 with the non-ischemic disease.	I-FABP	Serum	31 ng/mL	1.1 ng/mL - 498.4 ng/mL	9.1 ng/mL	83.3	89.1	—	—	97.6	50	ELISA
Schellekens et al., 2014	32	Human ischemia-reperfusion model	I-FABP	Arteriovenous difference	463 ng/mL	—	1.3 ng/mL	90	100	—	—	—	—	ELISA
Shi et al., 2015	309	309 patients, including 39 patients with ischemic Acute Abdomen, 233 patients with non-ischemic Acute Abdomen and 37 Healthy controls.	I-FABP D-Lactate	Serum Serum	149.74 ng/mL 52.73 µg/mL	— —	93.07 ng/mL 34.28 µg/mL	76.2 66.7	74.8 85.9	3.25 2.82	0.24 0.31	32.1 86.3	96.3 72.6	ELISA
Kulu et al., 2016	48	48 patients suspected for intestinal ischemia, including 23 that were diagnosed with intestinal ischemia and 25 patients that were diagnosed with other diseases.	Citrulline	Plasma	21.7 mmol/L	—	15.82	39.13	100	—	0.61	100	64.1	ion-exchange chromatography method on plasma. Citrulline analysis was performed by Acik LC-MS/MS Analysis Klin Biyolojik, Ziyak, Technologies, Istanbul, Turkey)
Sahin et al., 2016	18	18 patients suspected AMI among whom 13 were diagnosed with AMI. The five left were used as controls.	I-FABP I-FABP	Serum Urine	9 ng/mL 7 ng/mL	— —	0.69 ng/mL 2.52 µg/mL	92.3 91.7	40 80	1.54 4.58	0.19 0.1	— —	— —	ELISA (R&D Systems, DuSot, Minneapolis, MN)

For simplification, the units were harmonized. +LR : LP: +PV : positive predictive value; -PV : negative predictive value.n : number of patients

3. THE PROMISE OF INTESTINAL STROKE CENTERS AND METABOLOMICS IN ACUTE MESENTERIC ISCHEMIA

3.1. The promise of intestinal stroke centers

The need for an emergency multidisciplinary management of AMI led to the creation of a first of its kind Intestinal Stroke Center (Structure for Intestinal Vascular Emergencies - SURVI) in January 2016 at Beaujon University Hospital, Paris, France. This was a national and international first. (134) This innovation is based on a promising prospective clinical experience having demonstrated that multimodal, multidisciplinary management including early revascularization and targeting intestinal viability allowed 89% 2-year survival without requiring bowel resection in 61% of cases. (53) SURVI thus aims to improve the prognosis of AMI by offering to all patients an expert, multidisciplinary and 24/7 coordinated emergency care. (135). In addition, it enabled the patient recruitment, the standardization of care and the collection of biological samples under standardized conditions. Since the creation of this center in 2016, our team have prospectively enrolled patients who undergo a contrast-enhanced CT scan for acute abdominal pain as part of the SURVIBIO diagnostic study (clinicaltrials.com, NCT03518099). Blood samples from patients with AMI and controls with abdominal pain of another origin were collected on admission and stored in a biobank for further biomarker analysis and research. As detailed in the present, our team has used the admission plasma samples from the biobank to identify novel biomarkers for diagnosis of AMI using targeted approaches and untargeted GC-MS and ¹H-NMR metabolomics.

3.2. The promise of omics strategies in AMI

The lack of suitably validated markers for the diagnosis of AMI pushes the scientific community to continue their investigations on the search for the molecule that will help for the early diagnostic of AMI and that will prevent the complications and the need for surgery. The emerging use of Omics in the discovery of biomarkers open new ways. Regarding genomic data, no clue arises from genetic studies in AMI. However, no large genetic study has been published in this disease. The genetic variants predisposing to venous thromboembolism (*i.e.*, Factor V and II Leiden, *MTHFR* variants) could participate in the pathophysiological process of the disease, although descriptions are rare and related to private patients (136). Transcriptomic studies in models of AMI are scarce. In a pig model with induced proximal arterial occlusion of the superior mesenteric artery, Block et al. identified a panel of up- and down- regulated mRNA (respectively 157 and 57 transcripts). Up-regulated mRNA included noticeably monocyte chemoattractant protein 1 (MCP1) and acyl CoA synthetase long-chain family member 4 (137). In a preclinical study in swine, a microRNA signature was identified in an AMI model related to hypothermic circulatory arrest associated with the gut barrier dysfunction. This signature included noticeably a decrease of miR-31 which interact with the Hypoxia-inducible factor HIF function (138). Proteomic studies investigate the pool of full-length, truncated and post-translationally modified proteins and peptides, whereas metabolomics explores the whole metabolic process of either peptides, saccharides and lipids. Both approaches have been increasingly used for biomarker discovery in the last decade, noticeably thanks to the development of mass spectrometry and bioinformatics. Experimental studies for AMI biomarkers search using both proteomic and metabolomics approaches are summarized in Table 5. Only five studies were reported, using heterogeneous techniques and models, and no common marker was evidenced.

Table 5. Experimental animal studies using omics

First author, year	Omic	Tissue	Molecule	Results	Model	Species	Procedure
Solligard et al., 2005	Metabolomic	Gut lumen?	Glycerol	Increase	Mesenteric ischemia-reperfusion model (ligature of the superior mesenteric artery for 60 or 120 minutes, followed by 3 hours of reperfusion).	Pig	Microdialysis
			Phosphoglycerate mutase 1 Cytochrome b-c1 complex Pyruvate kinase Cytoplasmic aconitate hydratase Glutamate dehydrogenase Enoyl coenzyme A hydratase 1 Isocitrate dehydrogenase Glyceraldehyde 3 Phosphate dehydrogenase Aldose reductase Aldehyde dehydrogenase Protein disulfide isomerase A3 Tubulin α -1B chain Intelectin 1 Retinol binding protein 2 Albumin precursor Annexin A2	Decrease Decrease Decrease Decrease Decrease Decrease Decrease Decrease Decrease Decrease Increase Decrease Decrease Increase Increase			
Li et al., 2010	Proteomic/Metabolomic	Intestinal Mucosa	Glucose Lactate	Decrease Increase	Ischemia/reperfusion model	Rat	2D Gel electrophoresis coupled with MALDI-TOF
Birke-Sorensen et al., 2010	Metabolomic	Intestinal segments	Arabinose Xylose Glucose Ribose Stearic acid Urea Threonic acid Inorganic phosphate	Decrease Decrease Decrease Decrease Decrease Increase Increase Increase	Mesenteric ischemia-reperfusion model (ligature of the superior mesenteric artery for 60 minutes, followed by 2 hours of reperfusion).	Pig	Microdialysis
Fahmer et al., 2012	Metabolomic	Serum			Mesenteric ischemia model (ligature of the superior mesenteric artery for 2 to 4 hours).	Mouse	GC/MS
Steelman et al., 2014	Metabolomic	Serum	Citrulline	Decrease	Horse laminitis model	Horse	GC-LC/MS

Steelman et al. confirmed in the horse the interest of Citrulline as a biomarker (139). None of the proteomic studies identified in either pig or rodent FABP proteins. However, this could be due noticeably to imperfect animal models and to interspecies variability. Regarding metabolomic studies, the only common observation was a decrease in glucose observed in two rodent models.

3.3. The promise of metabolomics in AMI

Gastro-intestinal diseases are among the most complex conditions diagnose and treat as they are highly influenced by environmental factors such as diet and the gut microbiota. Acute mesenteric ischemia (AMI) is a life-threatening digestive and vascular emergency. The pathophysiological processes involved lead to an early severe phenotype including sepsis and acute organ dysfunctions. Moreover, the pathophysiology of AMI involves complex crosstalk between different metabolic pathways influenced by exogenous factors such as gut microflora which to dot affect the genome. Genomics, transcriptomics and proteomics provide extensive information regarding the genotype but convey limited information about the phenotype. Gene expression and protein data do not always reflect the effective physiological processes as several downstream metabolic mechanisms are involved. Thus, as compared to other “omics”, metabolic profiling integrates the effects of gene regulation, post-transcriptional regulation and pathway interactions. This makes metabolites direct molecular readouts of cell status that reflect a meaningful physiological phenotype, taking into account not only endogenous but also exogenous metabolites such as xenometabolites (from microflora) and xenobiotics (drugs, environmental chemicals).(140-142) As a result, metabolic profiling has shown promise in several published studies on gastro-intestinal diseases. The study of metabolites has enabled the characterization in the form of molecular signatures of numerous pathological processes. (141, 143-146). The power of this strategy is illustrated by the establishment of a predictive

diagnostic model of Crohn's disease with 100% sensitivity and 88% specificity by metabolome analysis of 26 patients and 26 healthy subjects. (147). The plasma metabolome represents all the metabolites of low molecular weight (<1kDa) contained in the plasma. (148, 149) Given the acute clinical impact of AMI on organ functions and the high early mortality, we expect the plasma metabolome of AMI patients to be highly disturbed, very early in the course of the disease. In this emergency clinical setting, blood samples constitute the ideal sample for research on new diagnostic biomarkers and the ideal diagnostic tool given the low volumes (< 1ml) required and the ease and acceptability of repeated clinical collection. For all these reasons, we hypothesized that the study of the plasma metabolome would open new avenues in clinical research on AMI and have the potential to discover new biomarkers that could directly affect patient care.

4. METABOLOMICS

As genomics, transcriptomics and proteomics techniques could not explain the metabolic modulations that occur, a relatively new technology, metabolomics, has emerged that highlights the whole system, providing a distinct perspective on exploring the pathophysiological pathways involved in diseases. It is a promising approach for determining the pathogenesis of a disease and the diagnostic and prognostic evaluation through identifying possible biomarkers of the disease. The metabolome refers to low-molecular metabolites (< 1000 Daltons) measured in a range of concentration that vary over nine orders of magnitude (pM-mM). Metabolites are the end products of cell regulatory processes and provide a characteristic phenotypic metabolite profile as a response to the interaction between the host-genome and the environmental influences.(150) These metabolites consist of a variety of

chemically diverse compounds (carbohydrates, lipids, amino acids) which are usually characterized in biofluids such as urine and plasma but also in tissue extracts. The metabolomics analysis can be divided in untargeted or targeted. In the untargeted analysis, the metabolic profiling of the total component of a sample is measured. In the targeted analysis, only selected metabolites are quantified. The methods essentially use nuclear magnetic resonance spectrometry (NMR) and mass spectrometry (MS), whether or not coupled with separation by liquid chromatography (LC-MS) or gas (GC-MS). Whereas MS measures the ratio of mass to charge of ionized particles, NMR takes advantage of the magnetic properties of certain nuclei, such as ^1H , to identify small molecules within a sample. As no single analytical platform is able to perform a complete quantification, combination of these techniques is a common practise in order to obtain a broad perspective of the metabolome.(151) In the present work, we used a combination of ^1H -NMR and GC-MS to profile the plasma metabolome of AMI patients.

4.1 Nuclear magnetic resonance (NMR) spectroscopy

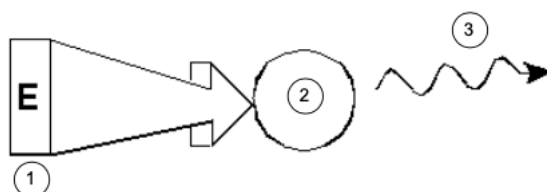
Nuclear magnetic resonance (NMR) was one of the first technologies used for metabolic profiling.(152) Some atomic nuclei possess a magnetic moment (nuclear spin I), which gives rise to different energy levels and emission of resonance frequencies in a magnetic field. All nuclei with non-zero spin ($I > 0$), are observable by NMR, with the most important and abundant one (>99%) in biology being ^1H with $I=1/2$.(153) When a biological sample is placed in a constant magnetic field (B_0), the nuclei align themselves parallel (or antiparallel) to the magnetic field (polarization).(Figure 9) The application of a specific weak oscillating magnetic field (radiofrequency pulse) will excite the nuclei in the sample by perturbing this alignment of the spins which will flip perpendicular to the magnetic field. Once the pulse has stopped, the system returns to equilibrium (relaxation) by emitting an electromagnetic signal (resonance

frequency) which is detected and recorded. The NMR signal is detected as a Free Induction Decay (FID) and subsequently transformed into an interpretable NMR spectrum by a mathematical operation called a Fourier Transform. (Figure 10) The immediate chemical environment of a ^1H nucleus in a particular compound (the atoms it is bonded to and surrounded by, bond lengths, bond angles) affects its resonance frequency. Therefore, the emitted frequency acts as a label which gives analysts qualitative information on the local atomic neighborhood in which a proton is located. The difference in precise resonance frequency is referred to as the chemical shift (δ). The resonance frequency is shifted by the effect of neighboring atoms and the extent of magnetic shielding from local electrons. The size of the chemical shift is usually measured in parts per million (ppm) relative to an external standard, the tetramethylsilane (TMS), whose peak is referenced to 0 ppm. Moreover, nuclei close to each other in a molecule interact with each other through spin-spin coupling or J-coupling which results in resonance lines or multiplet peaks. Together, the shape of the peak and the chemical shift, give each metabolite a unique NMR fingerprint. Tables of chemical shift values are readily available allowing chemists rapid identification of detected nuclei in NMR spectroscopy experiments. Depending on the pulse-sequence applied, different experiments and spectra can be acquired (such as Nuclear Overhauser Effect Spectroscopy (NOESY), Carr-Purcell-Meiboom-Gill sequence (CPMG) and J-resolved (JRES) acquired in this thesis work).

^1H -NMR is the most widely used and the most sensitive of NMR for metabolomics application. (Figure 11) This is due to the strong presence of hydrogen atoms in biomolecules and the fact that the ^1H form represents > 99% of hydrogen atoms. ^1H -NMR has been extensively used for metabolic profiling as it is a high reproducible and robust quantitative technique. Moreover, it is a non-destructive analysis which requires few sample preparation steps. On the other hand,

the main limitation of NMR is its lack of sensitivity compared to mass spectrometry. Therefore, most NMR studies focus on finding biomarkers rather than profiling the whole metabolome.

Figure 9: Basics of the NMR experiments (adapted from Bruker Beginners guide)



The NMR experiment consists of three steps. (1) Place the sample in a static magnetic field. (2) Excite nuclei in the sample with a radio frequency pulse. (3) Measure the frequency of the signals emitted by the sample. From the emitted frequencies analysts can deduce information about the bonding and arrangement of the atoms in the sample and identify chemical compounds.

Figure 10: The NMR Spectrum (adapted from Bruker Beginners guide)

NMR signals are usually plotted as spectra (after Fourier transformation) and analyzed with respect to two features, intensity (1) and frequency (2). It is conventional in NMR to plot resonance frequency as a chemical shift measured in ppm on the x axis and increasing towards the left relatively to an external standard referenced to 0 ppm.

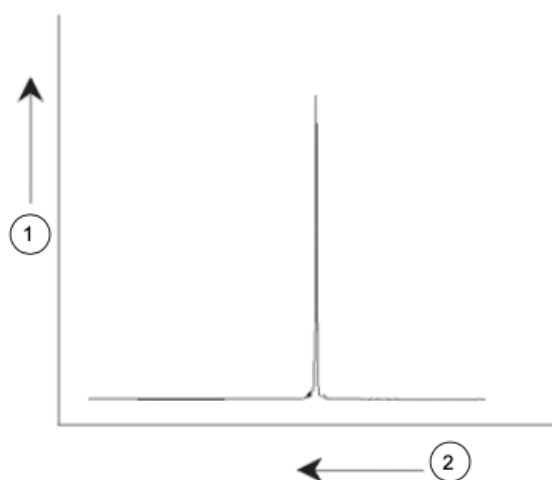
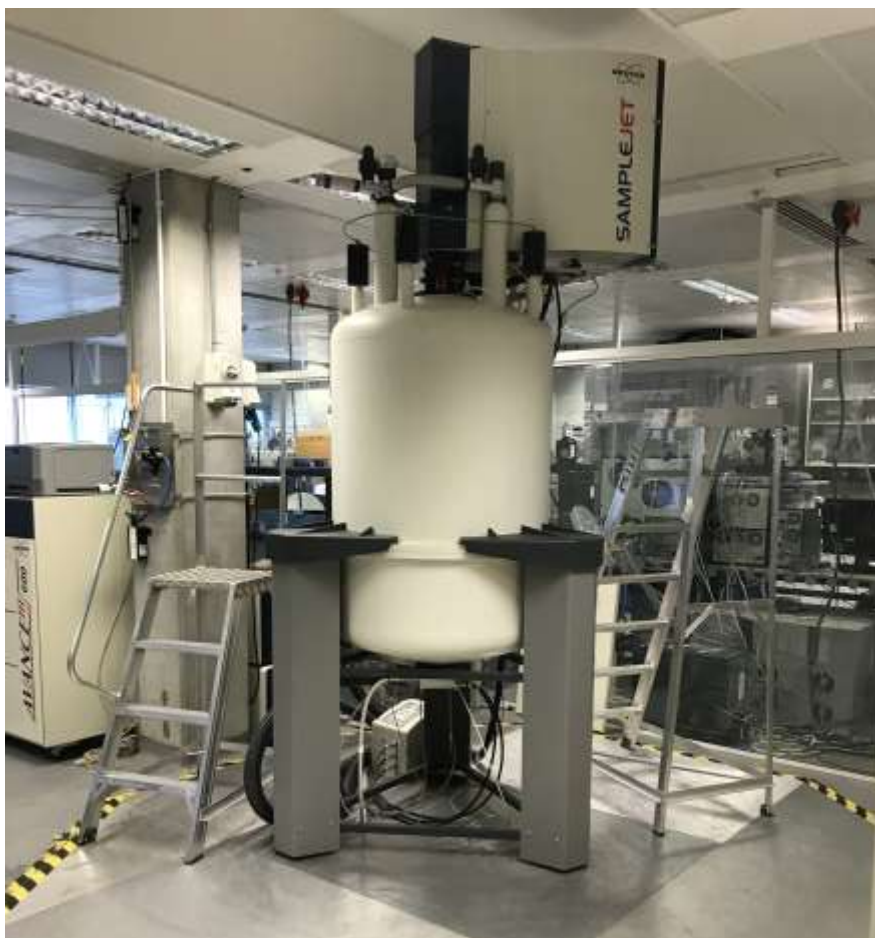


Figure 11: The NMR Bruker spectrometer

Bruker Avance III 600 spectrometer



4.2 Mass spectrometry

Mass spectrometry (MS) is more recent technology that has emerged providing sensitive results in endogenous metabolite research. Mass spectrometry is based on the principle that charged molecules can be deflected from electric and magnetic fields. The amount of deflection corresponds to the mass of the molecule and its charge, thus allowing to calculate the mass-to-charge ratio (m/z) and the abundance of its ion. Two conditions have to be met: 1) the ions produced have to be in gas phase and 2) a high-level vacuum is required to provide low pressure in order to prevent deviation of the ions from collision with other gaseous molecules (air). Generally, a mass spectrometer contains the following elements (Figures 12 and 13): an ion

source, which produces ions from the sample; a mass analyser, which sorts and fragments the ions according to their masses by applying electromagnetic fields; and a mass detector, which detects the ions and their abundance and converts the ions into electrical signals. Different types of ion sources are used depending on the nature of the sample and the type of analysis to be performed. The analyzer separates and fragments the different ions formed at the source. Here again, different types of analyzers exist with their own characteristics. The detector depends on the type of analyzer used and is used to record the ion signal. Signal processing is performed using acquisition software generally provided by the instrument manufacturer.

As the biological matrices analysed with mass spectrometry have an elevated content of metabolites and produce elevated levels of fragments and complex spectra, an extra step is usually employed prior to detection with MS. The analytical technique employed is chromatography which provides the separation of complex mixtures by deconvoluting the compounds included over time, thus making the downstream detection more accurate with improved ionisation. In chromatography, the sample is dissolved in a mobile phase which is forced through a stationary phase, fixed in a column or a solid surface. Depending on the chosen phases, analytes are either retained by the stationary phase or weakly held, thus moving in different rates with the flow of the mobile phase which leads in separation of the compounds and elution from column in different times, known as the retention time (RT). Gas chromatography and liquid chromatography were widely employed prior to MS.

Gas-liquid chromatography (GC) is used to analyse volatile compounds in the gas phase. As not all compounds are volatile, compounds can be chemically modified through derivatization to become suitable for GC analysis. In GC, samples are dissolved in solvent and injected to a sample port, where they vaporize before being introduced to the column. The mobile phase is an inert gas (most often helium) regulated to specific pressure which carries the vaporized samples from the injector to the column and does not interact with the molecules of the

analytes. The column itself contains a liquid stationary phase which is adsorbed onto a solid inert packing or coated on the capillary tubing walls and is located in the ‘‘thermostated oven’’. Retention of the compounds occurs by differential partitioning in the stationary phase which depends on the affinity of the compounds for the stationary phase and the compounds’ vapour pressure that is controlled by the temperature of the oven. As the flow of the gaseous mobile phase transports the sample through the column, temperature is increased in steps in order to separate all the components with a wide range of boiling points. With the initial lower temperature, more volatile compounds that have lower boiling components will be resolved and whilst the temperature rises, less volatile compounds will be eluted. When GC is coupled with MS, the eluents from the column pass into the MS and the compounds can be identified as mentioned above.

Mass spectrometry has two main advantages over NMR. MS offers a higher sensitivity. Indeed, where NMR is limited to the detection of metabolites having a concentration in the micromolar range,⁽¹⁵⁴⁾ MS can detect compounds at concentrations below the nanomolar.⁽¹⁵⁵⁾ This higher sensitivity increases the range of metabolites detected ⁽¹⁵⁶⁾ and makes of MS the technique of choice for untargeted metabolomics analyzes. In addition, it is possible to process reduced quantity of sample (from ten microliters, compared to milliliters in NMR). The second advantage is the speed of acquisition of a mass spectrum which varies between ten milliseconds and a few seconds. This is to be compared with the acquisition time of the NMR spectra which is of the order of a minute in 1D NMR and of the order of a quarter of an hour in 2D NMR.⁽¹⁵⁷⁾ The main limitation that mass spectrometry can present is the need to ionize compounds in order to be able to detect them. While different types of ionization sources exist, some non-ionizable compounds cannot be detected by this technique. In addition, the ionization step can be difficult to control, which makes MS less reproducible than NMR can be and in particular limits the comparisons of results between different laboratories.

Figure 12: Basic diagram for a mass spectrometer (158)

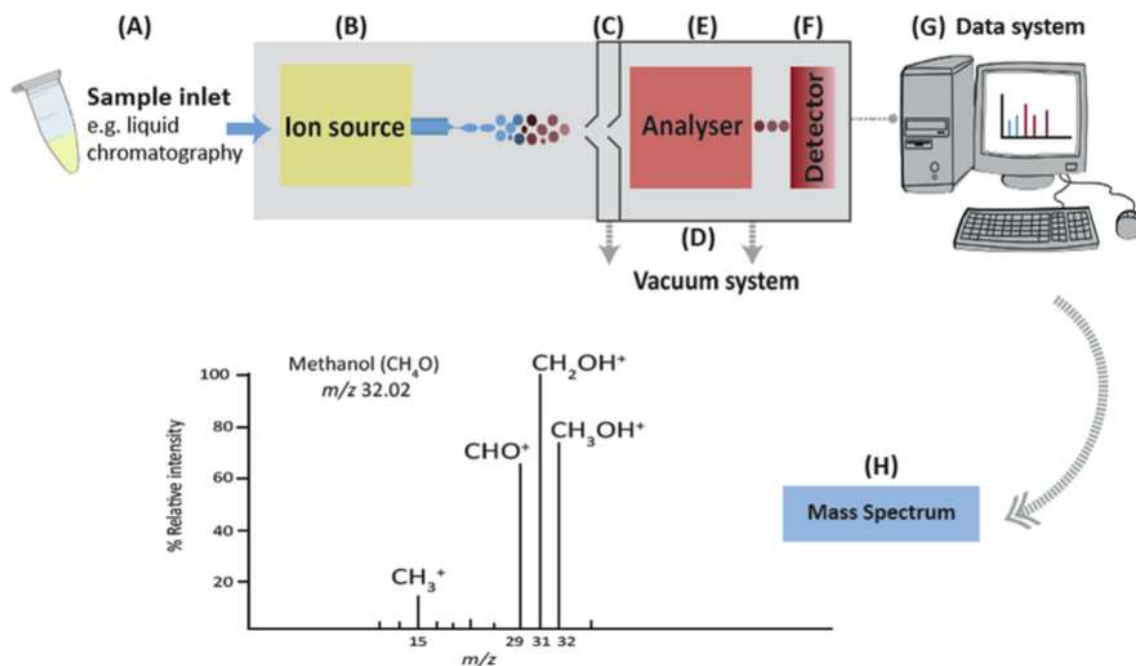


Figure 13: The GC-MS platform used at Imperial College London

(The Agilent 7890B/5977A Series Gas Chromatograph/Mass Selective Detector)



4.3 General workflow of metabolomics analyses

The general analysis workflow of metabolic profiling studies is summarized in figure 14. Briefly, patient biofluid samples are measured using a variety of spectroscopic platforms and after specific sample preparation. The spectra generated contain many thousands of data-points and require computational preprocessing and modeling to identify chemical structure within the spectral data. Specific sample preparation and spectral and data pre-processing for GC-MS and ¹H-NMR experiments used in our work are detailed in the Chapter 5.

NMR pre-processing steps include:(159)

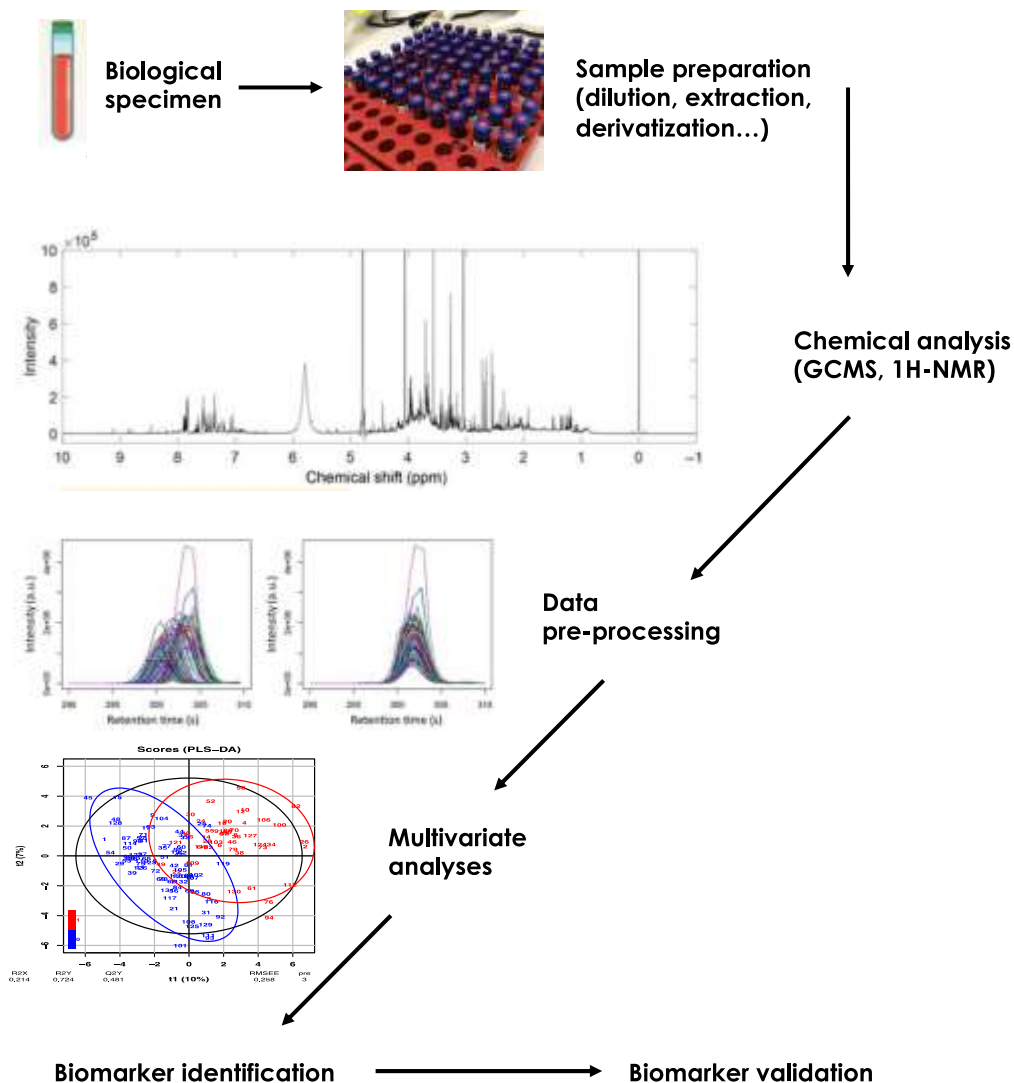
- apodisation, FFT, phase and baseline correction which are generally implemented in the instrument vendor software and in most cases can be applied automatically to a set of spectra
- removal of uninteresting/outlying regions (water, urea) that may influence the normalisation of the spectra;
- spectral peak alignment, binning and deconvolution
- data normalisation
- identification of outliers on principal component analysis.

GC-MS pre-processing steps include (Kalaman):

- peak detection and integration;
- retention and migration time alignment;
- batch effect correction;
- drift intensity correction using quality control (QC) samples and internal standards,
- signal normalization;
- feature filtering by retention time trimming, and calculation of coefficient of variation of QC samples;
- identification of outliers on principal component analysis.

After the pre-processing of raw data files, a data table of features ready for statistical analysis is obtained. Then pathology-related metabolic signatures are extracted from the statistical multivariate models and bioinformatics approaches applied to enhance molecular identification and to highlight discriminatory biomarkers. Finally, validation study will have to validate candidate biomarkers from the primary development study. Mapping the metabolic signature obtained to biochemical networks can also improve mechanistic knowledge of pathological processes.

Figure 14: General workflow of metabolomic analyses for biomarker discovery



4.4 Statistical analyses in metabolomics

The metabolomics analytical techniques detailed above imply several samples' analysis, leading to simultaneous measurement of thousands of variables for each sample, with a usual overlap of important metabolic information and noise. The fact that the number of variables usually exceeds the number of samples creates the need to reduce the data dimensionality and transform the raw data obtained to a more easily interpreted set of variables. This is achieved by multivariate statistics that manage to extract information from complex datasets. Univariate statistics that focus on evaluating single metabolites remain important in metabolomics, especially when investigation diagnostic biomarkers.

4.4.1 Univariate statistics

Univariate statistics provide an overview of potentially significant metabolites and help to validate data obtained with multivariate models. Student's t-test is a parametric test can examine a null hypothesis of the difference of the mean of two groups and requires the variables to follow a normal distribution. Mann-Whitney U test is a non-parametric alternative to t-test. In all cases, the p value is obtained, indicating the rejection or acceptance of the initial hypothesis based on the hypothesis level defined. As a test can be repeated several times due to the high number of variables in metabolomics dataset, false positive or negative results can appear. To account for that, a multiple testing correction (corrected p-values, or q-values) is applied by calculation of the false discovery rate (FDR) with the Benjamini-Hochberg procedure. (160)

4.4.2 Multivariate statistics

Another approach for statistical interpretation is the use of multivariate analysis tools. These tools treat the entire dataset as a single item and allow more immediate visualization of the samples' dispersion. These approaches are based on the use of transient variables, the principal components (PCs), which are calculated as linear combinations of the original variables and

make it possible to highlight the interactions existing between these different variables. There are two types of multivariate statistical analyses: unsupervised and supervised methods. Unsupervised techniques describe how variables relate to each other without any a priori knowledge of classification and whereas supervised techniques focus on predicting the outcome of a set of variables when the phenotype is known.(161)

PCA is a well-established unsupervised projection method, providing an overview of large datasets, visualizing similarities and differences between observations and highlighting information about the metabolite features responsible for the observed patterns. It is a mathematical algorithm that reduces dimensionality by extracting principal components (PCs) that account for most of the variance in the original data. Each PC is a linear combination of the original variables and in a decreasing order, explains the maximum amount of variance possible, not accounted for by the previous PCs. As PCA only discriminates by variance explained, confounding factors can dominate the results in cases where more variance is found there than between groups of interest. This conversion of the data into PCs results in a scores matrix and a loadings matrix. Scores are the derived coordinates of the samples in the PCA model and loadings are the variances of the original variables to form the scores. The directions in both scores and loadings spaces are the same and can be interpreted by looking at the corresponding direction in the loadings space. In the scores plot, each point represents one sample from the study and thus can identify metabolic separation if differences between metabolic profiles exist. Moreover, PCA is useful to identify outliers as situated outside of the 95% confidence interval of the modelled variation, the Hotelling's T² region.(162) The optimal number of components for each model is determined based on the variation explained by each component (R²X) which shows the 'goodness of fit' and with consideration of the predictive value (Q²X) that estimates the variation of the model in case of new data entered into the existing model.

Supervised multivariate statistical analysis methods seek to highlight a hierarchy of the data set based on predefined classification phenotype groups.(161) They seek to highlight the metabolites which participate the most in the separation of samples according to their groups. Orthogonal Partial Least Squares- Discriminant Analysis (OPLS-DA) is a supervised pattern recognition technique which is a projection tool for modelling a relationship between dependent and independent variables. The matrix is separated into two parts: the predictive component which models the variation in the set of variables and is related to the response (class information) and the non-predictive which is not correlated, but orthogonal to this information. This enhances the variation of interest whereas it minimizes uncorrelated variation.(162) Similar to PCA, the statistical model produces a scores plot that can be investigated. As the model can be subjected to over-fitting, optimization of the model requires cross-validation with permutation testing.

4.4.3 Machine learning

Machine learning has recently been proposed as a useful tool in diagnostic and prognostic accuracy studies in many fields of Medicine to facilitate clinicians diagnostic or therapeutic algorithms. Conversely to conventional statistical models which are constrained by strong assumptions (e.g., logistic regression), machine learning models do not make assumptions about the data distribution and focus mainly on predictive accuracy.(163) The machine learning approach is characterized by the widespread use of cross-validation, able to reduce the risk of overfitting more than common hold-out methods. Metabolomic studies may open new avenues of clinical research, but also bring challenges related to the high-dimensionality of datasets (or high feature-to-sample ratio). Since many of the variables may be irrelevant or redundant to the clinical question to address, the use of machine learning and data mining may be very efficient to select diagnostic variables.

Existing feature selection methods are traditionally categorized as filter methods (where variables are scored and ranked, e.g. t-tests, Pearson correlation, principal component analysis) or wrapper methods (where best models performance is selected by backward elimination or forward selection).(164, 165) The most popular learning algorithm used in wrapper methods is the support vector machines (SVM) Nevertheless, SVMs are sensitive to noisy training data and are prone to over-fitting and poor generalization.

Fuzzy set theory allows an individual to partially and simultaneously belong to a class with a certain degree of membership between 0 and 1. In a machine learning framework, an approach is defined as “fuzzy” if we consider that an individual belongs to each class with a certain degree of membership. (164, 165) LAMDA is a fuzzy classification method which is based on finding the global membership degree of a sample to an existing class, considering all the contributions of each variable. This contribution is called the marginal adequacy degree (MAD). The MADs are calculated by means of a membership function and they are then combined in order to obtain the global adequacy degree (GAD) of an element to a class. Finally a patient will be assigned to the class for which its GAD is the highest.

Hedjazi et al. proposed a new feature selection algorithm, referred to as MEMBAS for Membership Margin Based Attribute Selection based on fuzzy logic concepts.(164, 165) The algorithm selects the most pertinent biomarkers based on feature weighting according to the maximization of a membership margin. The combined fuzzy machine learning model MEMBAS/LAMBDA used for both feature selection and classification outperformed other classifiers such as k-NN or SVM when applied to several datasets with mixed-type and high-dimensional data.(164, 165) This algorithm is used in the present work described in Chapter 5 to select the optimal subset of new diagnostic biomarkers of AMI.

5. OBJECTIVES OF THE WORK

The main objective of the present study was to identify plasma diagnostic biomarkers of acute mesenteric ischemia from a cross-sectional human study: the SURVIBIO study. (ClinicalTrials.gov Identifier: NCT03518099)

In Chapter 1, we reviewed the state-of-the-art knowledge on pathophysiology and management of acute mesenteric ischemia, while highlighting the critical clinical unmet diagnostic need. We summarized the current knowledge on biomarkers of acute mesenteric ischemia, underlining the need for validation of existing candidate biomarkers and for new biomarkers. While introducing the principles of metabolomics studies, we hypothesize that the plasma metabolome profiling of patients with AMI would have the potential to discover new biomarkers that could directly affect patient care.

In Chapter 2, we will describe the SURVIBIO study design and population of patients with AMI and abdominal pain controls studied in the present PhD work, and assess the diagnostic value of common clinical and biological factors collected in routine clinical practice.

In Chapter 3, we will test the accuracy of three candidate biomarkers identified by conventional targeted studies from the scientific literature – citrulline, D-lactate and intestinal fatty acid-binding protein (I-FABP) – in the diagnosis of acute mesenteric ischemia.

In Chapter 4, we will eventually use a combined analytical strategy comprising ¹H-NMR and GC-MS metabolomics to identify new circulating diagnostic metabolites in blood samples obtained from patients with AMI.

In Chapter 5, we will discuss the perspectives opened by the results of this work and the major remaining steps we need to accomplish in order to translate these findings to clinical practice and to the patients clinical benefit.

Chapter 2

The SURVIBIO study: presentation and analysis of the clinical and biological of the cohort

This work has been presented as an oral communication in 2019 at the congresses of the French Gastroenterology Society and the French Emergency Society.

Original article

Title: Improving clinical recognition of acute mesenteric ischemia among acute abdominal pain patients: a cross-sectional study from an intestinal stroke center.

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INTRODUCTION

Acute mesenteric ischemia (AMI) is a life-threatening vascular and digestive emergency.(1) Early diagnosis and treatment before the occurrence of intestinal infarction are the most important physician-dependent factors to improve survival.(1, 37, 53, 166) The rate of intestinal necrosis increases from 0% when AMI is diagnosed within the first 12 hours of presentation, to 44% and 82% after 12 and 24 hours, respectively.(166, 167) However, diagnosis at an early, potentially reversible stage, is currently the most challenging clinical issue of AMI given no clinical sign nor biomarker has proven to be sensitive or specific enough.(1, 2) As a result, diagnosis of AMI still requires a high index of clinical and radiological suspicion and confirmation on a contrast-enhanced CT scan. However, even though CT-scan is widely available in the emergency setting and reported having excellent accuracy for AMI diagnosis, timely clinical suspicion and selection of patients remains challenging. Moreover, lower sensitivities of the CT-scan have been observed in the real-life clinical setting when the CT-scan was performed during both arterial and venous phases (83%) or venous phase only (72%). This is consistent with prior studies where the clinical suspicion

was not always mentioned to the radiologist (42), resulting in either an inappropriate IV contrast protocol, and/or an analysis that did not focus on the mesenteric vessels.(43)

Following the results of a pilot study showing an improvement in survival and lower resection rates(53), we created an intestinal stroke center in our hospital that provides 24/7 standardized multimodal and multidisciplinary care to AMI patients referred from all hospitals in the Paris region. Since the creation of this center in 2016, we have prospectively enrolled patients who undergo a contrast-enhanced CT-scan for acute abdominal pain as part of the SURVIBIO diagnostic study. The aim of this study was to identify readily available routine clinical and biological factors associated to the diagnosis of AMI in compare to control patients with acute abdominal pain of another origin.

METHODS

Study design and setting

This was an observational cross-sectional study enrolling patients with acute abdominal pain requiring a contrast-enhanced C.T. scan from January 2016 to March 2018. Patients with AMI were admitted to the intestinal stroke center, whereas those in whom the diagnosis was ruled out (controls) were admitted to the emergency room (see patient flowchart, Figure 15). The study was approved by the Ethics Committee of Paris-Nord Val de Seine University Hospitals. The study complies with the Standards for Reporting Diagnostic Accuracy (STARD) guidelines.(168) Informed consent was obtained from all patients.

Patients and controls

AMI was defined by the association of 1) acute clinical, biological and/or contrast-enhanced C.T. features of bowel injury, 2) vascular insufficiency (occlusive or non-occlusive) of the celiac trunk and/or the superior mesentery artery and/or superior mesenteric vein, and 3) the

absence of an alternative diagnosis.(54) The diagnosis was confirmed by histology following intestinal resection.(37) All the AMI patients were managed by a standardized multimodal and multidisciplinary approach in our intestinal stroke center, as previously described.(53) Briefly, the patients were systematically administered oral antibiotics, and antithrombotics(53, 54), and emergency endovascular revascularization of arterial AMI was performed whenever technically feasible. Alternatively, open surgical revascularization was performed. Bowel viability was evaluated by laparotomy, decided based on published risk factors for irreversible transmural intestinal necrosis (occurrence of organ failure, elevated serum lactate concentrations, small bowel dilatation or perforation on C.T.).(37) Irreversible transmural intestinal necrosis was confirmed upon pathological assessment.

The diagnosis of AMI was ruled in or out by the C.T. scan, and alternative final diagnoses were based on clinical, biologic, and C.T. findings. Finally, all the included patients underwent a contrast-enhanced abdominal C.T. scan and routine biologic work-up. Any patients presenting with a diagnosis of left-sided colon ischemia without small bowel injury, chronic mesenteric ischemia without acute injury, vascular lesions with no small bowel injury, or strangulated bowel obstruction were not included (see patient flowchart, Figure 15). The primary outcome was the diagnosis of AMI. All the patients' clinical records, CT-scans, and pathologic specimens were reviewed in a multidisciplinary meeting including gastroenterologists, radiologists, digestive and vascular surgeons and intensivists, all experts in digestive vascular diseases, to avoid diagnostic misclassification.

Data collection and processing

Routine baseline clinical and biological characteristics were prospectively collected upon admission for all patients: age, gender, body mass index (BMI), history of cardiovascular disease, atherosclerosis risk factors (i.e. tobacco consumption, high blood pressure, diabetes mellitus, elevated cholesterol or triglycerides), history of venous thromboembolism, history of

chronic kidney disease, cirrhosis, ischemic colitis, chronic mesenteric ischemia, abdominal surgery, cocaine abuse. The following data regarding AMI was collected too: presenting clinical signs (abdominal pain, sudden onset, gastrointestinal bleeding, diarrhea, vomiting, constipation, abdominal guarding, organ failure [defined as a SOFA score ≥ 2], and presenting laboratory test values (White Blood Cell count (WBC), platelets count, plasma levels of C-reactive protein, lactate, blood urea nitrogen, creatinine, aspartate aminotransferase, lactate dehydrogenase, creatinine kinase). The admission prescription of opioid analgesics was recorded but left at the discretion of the on-duty physician. The origin of AMI (arterial – thrombotic or embolic – venous, or non-occlusive) was specified based on the patient’s records, CT-scan, and pathologic review.

Statistical analysis

We reported quantitative data as medians and interquartile ranges (IQR). Qualitative data was reported as the number and percentages of patients. Univariate analyses used the Chi-square, Fisher and Mann-Whitney U tests as appropriate to assess the association between clinical and biological presentation and the diagnosis of AMI. All variables with a p -value < 0.10 and those considered as clinically relevant (age) were included in a multivariate logistic regression model. We reported adjusted odds ratio (OR) and their 95% confidence interval (CI). All tests were two sided. A p value < 0.05 was considered to be significant. Multicollinearity between selected variables was assessed using the variance inflation factor (VIF). Variables were considered as suspicious for collinearity when the VIF was higher than 5.(169) All analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Mac OSX software (version 23.0, Chicago, Illinois, USA). This study was performed following the ethical standards of the Committee on Human Experimentation of our institution (Institutional Review Board N°00006477, approval 15-062) and reported according to the

Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines.(170)

RESULTS

Characteristics of the SURVIBIO population

Between January 4, 2016, and March 5, 2018, 179 patients with acute abdominal pain requiring a contrast-enhanced CT-scan were assessed for eligibility (Figure 15). We enrolled 137 patients, including 52 admitted to our intestinal stroke center for AMI and 85 admitted to the emergency room for non-AMI acute abdominal pain (see Flowchart, Figure 15). The baseline characteristics of both populations are summarized in Table 6. The final diagnosis of the controls is presented in Table 8. Patients with AMI [median age: 65 years (55-74), 37 % of women] included arterial and venous causes in respectively 65% and 35% of cases. None of the patients included had non-occlusive AMI. AMI occurred in seven patients exhibiting signs of chronic mesenteric ischemia. The control population of patients admitted for acute abdominal pain requiring CT-scan investigation included the following diagnoses: infectious disease (n=20 ; 10 diverticulitis, 5 appendicitis, 5 others), abdominal inflammatory diseases (n=15 ; 8 intra-abdominal neoplasms, 7 IBD flares), intestinal mechanical obstruction (n=13), functional GI disease (n=13), pancreatic or biliary syndromes (n=12), urological or genital causes (n=11) and one patient with abdominal pain related to an invasive meningococemia. After admission in the intestinal stroke center, AMI patients received antiplatelet therapy (n=34, 100% arterial AMI), anticoagulants (n=51, 98%), oral antibiotics (n=51, 98%), and intravenous antibiotics (n=21, 40%). Emergency revascularization was performed in 30 patients (88% of arterial AMI patients) within a median delay of 14 hours.

Factors associated to the diagnosis of AMI

When compared to patients with abdominal pain of another origin, patients with AMI were significantly older, had a higher BMI, and were more likely to have risk factors or a history of cardiovascular disease than controls (Table 6). AMI patients were also more likely to present hematochezia, guarding, and organ dysfunction (as measured by a total SOFA score ≥ 2) and a higher white blood cell count and C-reactive protein at baseline. Other baseline clinical and laboratory characteristics, including L-Lactate, did not differ significantly (Table 7). AMI patients had normal plasma lactate levels (< 2 mmol/L) upon admission in 69% of cases (n=36). On multivariate analysis, three independent factors were associated to the diagnosis of AMI: the sudden-onset (OR=13, IC95% : 3.3 – 52, $p<0.001$) and opioid-requiring nature of the acute abdominal pain (OR=7.6, IC95% : 2 – 29, $p =0.003$), and plasma C-reactive protein levels > 20 mg/L (OR=12.2, IC95% : 2.6 – 58, $p=0.002$). All AMI patients had at least one of the three independent factors.

DISCUSSION

In this cross-sectional study of patients with contemporary AMI compared to acute abdomen controls, we identified three independent clinical and biological factors associated with a diagnosis of AMI. Interestingly, these clinical and biological factors are readily available to clinical practice upon admission. In the current absence of accurate diagnostic tool or biomarker, we believe these factors may help emergency physicians, gastroenterologists, and surgeons in the earlier suspicion of AMI. Most importantly, all AMI patients from this study had at least one of the three independent factors, thus suggesting a high negative predictive value in the absence of all three factors.

Despite constant improvements in diagnostic, interventional and surgical techniques, AMI remains a life-threatening emergency with high mortality rates. The time to diagnosis is the

most important predictor of patients' outcome. Therefore, prompt diagnosis is essential to reduce treatment delay and mortality in AMI patients. To our knowledge, no contemporary prospective human study has investigated clinical and common laboratory test differences between AMI patients and other cause of acute abdomen.

First, as expected, we observed significantly higher rates of cardiovascular history and risk factors in AMI patients as compared to controls. However, these epidemiological factors were no longer associated to the diagnosis of AMI in multivariate analysis. Although AMI patients were two times more likely to have cardiovascular risk factors and three times more likely to have prior cardiovascular history, they were also significantly older than controls. Most importantly, one third of AMI patients did not have prior history of cardiovascular disease whatsoever. These observations are consistent with those reported by Adaba et al. in a UK retrospective cohort.⁽³⁹⁾ As previously described, ^(40, 56, 81, 171) acute abdominal pain of AMI patients was commonly out of proportion to physical examination as more than 65% of patients reported a sudden onset and required opioid. Nevertheless, AMI patients presented without severe signs in most of cases, with no report of abdominal guarding in 67% of cases, and no organ failure in 94% of cases. Similarly, in the study by Koungias et al., peritonitis and shock were reported on admission in only 36% and 6% of cases.⁽⁴⁰⁾ Taken together, these results are another reminder that AMI patients may commonly present at an early and potentially reversible stage, while elevated lactate and organ failures are late findings associated with intestinal necrosis.⁽³⁷⁾ Noteworthy, plasma lactate levels were initially within normal range in 69% of AMI patients of our cohort, thus confirming again their diagnostic inutility during the early stages of AMI. Worse, in a retrospective cohort study of survivors from mesenteric infarction, a delayed diagnosis was more likely when initial plasma lactate levels were < 2 mmol/L suggesting that physicians might be misguided by unremarkable plasma lactate levels.⁽³⁵⁾ Interestingly in the present study, C-reactive protein (CRP) may be

a sensitive biomarker of AMI with an interesting negative predictive value, although being totally nonspecific. As a matter of fact, none of AMI patients had CRP < 20mg/dL on admission.

Finally, based on our comparative study of clinical and biological presentation of AMI and other causes of acute abdominal pain, we suggest that suspicion of an AMI diagnosis should be raised in front of any acute abdominal pain especially when a sudden onset is reported, when opioids are required, and when CRP is elevated > 20mg/dL. As the discriminative weight of epidemiological factors was insufficient, we suggest the diagnosis be evoked irrespective of the patients age and prior cardiovascular history, and lead to urgent investigation by a dedicated abdominal CT angiography mentioning the ischemic suspicion.(42) In the absence of a validated available diagnostic biomarker of AMI, timely clinical suspicion is currently the only way to achieve an earlier diagnosis and better outcomes. (1, 56)

Some limitations of our study should be discussed. First, as this study was designed to collect biological samples for further biomarker assessment, we did not include patients with strangulated bowel obstruction, left-sided colon ischemia, and mesenteric vessel occlusion without evidence of acute small bowel injury in both patients and control groups. Although these conditions may share pathophysiological processes comparable with that of AMI, they are different disease with different prognoses and could introduce noise in the comparison of AMI patients and controls. However, this might decrease the generalizability of our clinical findings to the discrimination of AMI from these other conditions not included in the study. Nonetheless, our work represent one of the largest population of well-characterized and homogeneous AMI patients, included at an early stage in the course of the disease. This is crucial as the early diagnosis of AMI amongst emergency patients admitted for acute abdominal pain remains a critical clinical challenge, and our findings indicate three factors associated with the diagnosis which may potentially be discriminant. However, one can argue

that the sudden onset feature and opioid requirement may be partially subjective as no strict definition was used and opioid therapy decision left at the physician's discretion. Finally, as a referring hospital for AMI, the prevalence of AMI was high in this cross-sectional study and a diagnostic score with predictive values could not be calculated.

In conclusion, this cross-sectional study identified three independent clinical and biological features associated to the diagnosis of AMI, which may help physicians in suspecting the disease among patients with acute abdominal pain of another origin. As a timely diagnostic remain the most important determinant of patient outcomes, our result suggests suspecting AMI in any patients presenting with acute abdominal pain especially of sudden onset, requiring opioid and associated with elevated C-reactive protein. Further studies are required to confirm the diagnostic value of these factors, and to improve objective means to achieve an earlier diagnosis.

Figure 15. Flowchart of AMI patients and controls: screening and selection

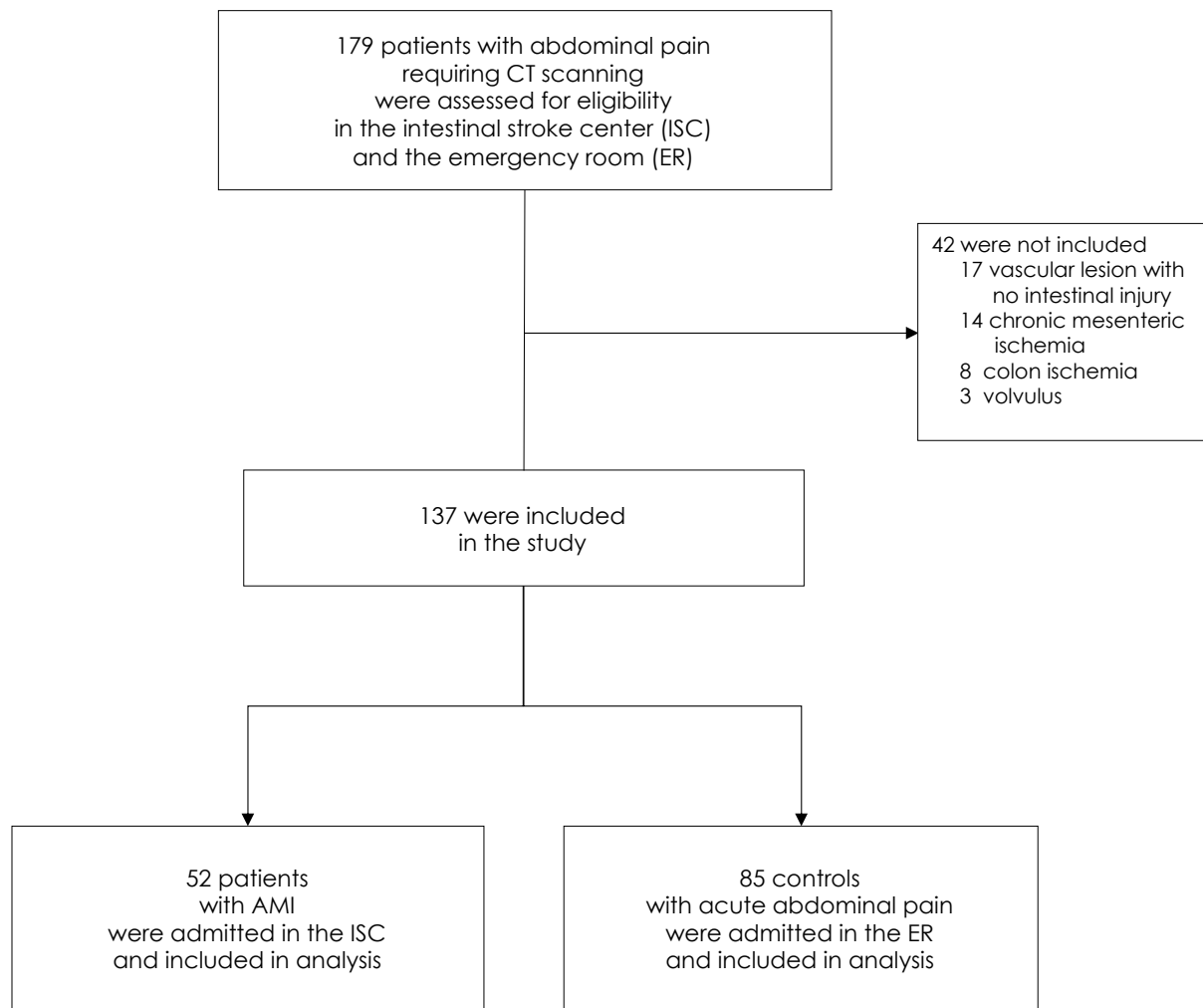


Table 6. Baseline characteristics of AMI and controls

	AMI patients n=52 (%)	Controls n=85 (%)	<i>p-value</i> ¹
Age, years ²	65 (55-74)	48 (35-70)	< 0.001
Female	19 (37)	34 (40)	0.69
<i>Atherosclerosis risk factors</i>	42 (81)	38 (45)	
Tobacco use	24 (46)	17 (20)	0.001
Arterial hypertension	29 (56)	23 (27)	0.001
Dyslipidemia	20 (39)	12 (14)	0.001
Diabetes mellitus	12 (23)	5 (6)	0.003
<i>Cardiovascular history</i>	34 (65)	20 (24)	
Myocardial ischemia	10 (19)	5 (6)	0.02
Stroke	6 (12)	5 (6)	0.33
Limb ischemia	9 (17)	2 (2)	0.003
Atrial fibrillation	11 (21)	4 (5)	0.003
Heart surgery	3 (6)	1 (1)	0.15
Vascular surgery	13 (25)	1 (1)	< 0.001
Deep vein thrombosis	4 (8)	4 (5)	0.48
Pulmonary embolism	6 (12)	4 (5)	0.18
<i>Other comorbidities</i>			
Chronic kidney disease	1 (2)	2 (2)	1.00
Colon ischemia	0 (0)	1 (1)	1.00
Cirrhosis	4 (8)	4 (5)	0.48
Abdominal surgery	29 (56)	40 (47)	0.32
<i>Clinical features</i>			
Temperature	37.0 (36.3-37.1)	36.8 (36.5-37.5)	0.52
Mean arterial pressure	99.8 (89.1-110.1)	96.0 (84.3-107.2)	0.35
Heart rate	88 (76-104)	86 (71-104)	0.35
Sudden onset abdominal pain	31 (71)	9 (11)	< 0.001
Opioid-requiring abdominal pain	33 (64)	19 (22)	< 0.001
Ileus	8 (15)	14 (17)	0.87
Vomiting	21 (40)	41 (48)	0.37
Diarrhea	12 (23)	13 (15)	0.25
Hematochezia	8 (15)	3 (4)	0.02
Guarding	17 (33)	16 (19)	0.07
Organ failure (SOFA>2)	3 (6)	3 (4)	0.67
<i>Biological features</i>			
White blood cells count, G/L ^{2,3}	12 (9-14.8)	9.5 (7.5-17)	0.52
Platelets count, G/L	266 (171-363)	271 (-319)	0.46
Hemoglobin, g/dL	12.5 (11.6-15.1)	13.6 (12.3-14.9)	0.21
C-reactive protein, mg/L	116 (29-206)	21 (5-98)	< 0.001
Lactate levels, mmol/L	1.5 (1.0-2.2)	1.3 (0.8-2.4)	0.68
Creatinine, μmol/L	72 (63-100)	70 (63-89)	0.76
ASAT, UI/L	25 (19-38)	27 (22-40)	0.23

¹ Log-rank test² Median (interquartile range)³ White blood cell count was available in 64 patients⁴ Lactate levels was available in 62 patients⁵ Antiplatelet and revascularization variables were expressed and analyzed within the subgroup of arterial acute mesenteric ischemia patients (n=35)

Abbreviations: BMI: Body Mass Index

Table 7. Multivariate analysis of factors association with the diagnosis of acute mesenteric ischemia

	Logistic regression model		
	<i>p-value</i>	OR	(95% CI)
<i>Epidemiological factors</i>			
Age	0.95	-	-
Sex (Female)	0.41	-	-
Tobacco use	0.32	-	-
Arterial hypertension	0.71	-	-
Dyslipidemia	0.61	-	-
Diabetes mellitus	0.09	-	-
Myocardial ischemia	0.95	-	-
Limb ischemia	0.82	-	-
Atrial fibrillation	0.26	-	-
Vascular surgery	0.11	-	-
<i>Clinical and biological features</i>			
Sudden onset abdominal pain	< 0.001	13.0	(3.3 – 51.7)
Opioid-requiring abdominal pain	0.003	7.6	(2.0 – 29.1)
Hematochezia	0.25	-	-
Guarding	0.83	-	-
C-reactive protein > 20 mg/L	0.002	12.2	(2.6 – 58.1)

Multivariate model included 123 complete cases

Abbreviations: OR: Odd Ratio; CI: Confidence Interval; AMI: acute mesenteric ischemia

Table 8. Diagnosis of abdominal pain controls

Diagnosis of abdominal pain controls	n=85 (%)
Bowel obstruction (non-strangulated)	13 (15)
Acute GI functional disorders (reflux, constipation...)	13 (15)
Diverticulitis	10 (12)
Cancer related abdominal pain	8 (9)
IBD flares	7 (8)
Pancreatitis	7 (8)
Urinary tract related	6 (7)
Biliary related	5 (6)
Peritonitis/abcess	5 (6)
Genital tract related	5 (6)
Appendicitis	5 (6)
Invasive Meningococemia	1 (1)

¹ Median (interquartile range); Abbreviations: AMI: acute mesenteric ischemia; CMI: chronic mesenteric ischemia; SBS: short bowel syndrome (i.e. remnant bowel length in continuity < 200cm)

Chapter 3

Candidate Diagnostic Biomarkers of Acute Mesenteric Ischemia

This article has been submitted to an international scientific peer-reviewed journal.

Title: Accuracy of Citrulline, IFABP and D-Lactate in The Diagnosis of Acute Mesenteric Ischemia

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ABSTRACT

Diagnosis of acute mesenteric ischemia (AMI) remains a clinical challenge and no biomarker is currently validated. In a cross-sectional diagnostic study, we aimed to assess the accuracy of three promising plasma biomarkers for the diagnosis of AMI – citrulline, intestinal fatty acid binding protein (IFABP), and D-lactate. We included 129 patients – 50 AMI and 79 controls. Plasma citrulline concentrations were significantly lower in AMI patients compared to the controls [15.3 $\mu\text{mol/L}$ (12.0-26.0) vs. 23.3 $\mu\text{mol/L}$ (18.3-29.8), $p=0.001$]. However, the area under the receiver operating curve (AUROC) for the diagnosis of AMI by Citrulline was low: 0.68 (95% confidence interval= 0.58-0.78). No statistical difference was found in plasma I-FABP and D-lactate levels between the AMI and control groups, with an AUROC of 0.44 and 0.40. Thus, citrulline, IFABP and D-lactate failed to differentiate AMI patients from patients with acute abdominal pain of another origin. Further research is needed to identify new biomarkers.

KEYWORDS: Mesenteric infarction, intestinal ischemia, ischemic colitis, colon ischemia, intestinal necrosis, I-FABP, IFABP, biomarkers, diagnosis, recognition

INTRODUCTION

Background

Early management of acute mesenteric ischemia (AMI) can avoid fatal outcome and prevent related short bowel syndrome.(1, 53, 91, 172) To this end, there is a dire need for tools to establish an immediate diagnosis. Mortality and intestinal resection rates have remained unchanged for decades despite the progress made in radiology, endovascular procedures, and intensive care medicine. However, recent reports suggest improved outcomes for patients if diagnosis and standardized multidisciplinary expert care are provided at an early stage.(53, 54, 173) Indeed, early AMI is a fully reversible condition, as opposed to “advanced” AMI with irreversible transmural necrosis.(1, 37) However, AMI patients present unspecific acute abdominal pain which renders clinical suspicion and identification challenging, and can often lead to missed or delayed diagnosis and care.(1, 4) Moreover, when the suspicion is not evoked, AMI may be underdiagnosed on contrast-enhanced computed tomography (C.T.) of the acute abdomen.(42)

While biologic abnormalities – such as leucocytosis or lactic acidosis – have been documented in patients with AMI, their performance to establish the early diagnosis is poor.(91, 174) The high complexity of the layered intestinal wall structure increases the diversity of the proteins and metabolites released in AMI. Their hepatic metabolism through the hepatic portal system results in substantial overlap with liver proteins and metabolites. These factors, along with the heterogeneity of the disease, explain why identifying clinically reliable biological early markers of AMI has been unsuccessful so far.(1, 4, 91)

Three blood biomarkers have gained attention over the past decades: citrulline, a marker of enterocyte function; I-FABP, a marker of enterocyte damage; and D-lactate, a marker of intestinal barrier dysfunction and microbial translocation.(91, 94, 129, 175) As a result, these tests are increasingly used in basic and clinical research as indirect markers of an ischemic

intestinal injury in a broad range of emergency clinical settings. However, to date, their alleged diagnostic performances have only been assessed in small heterogeneous cohorts. Besides, conflicting results have been reported, and most of the studies consist of preoperative data in late-stage necrotic AMI patients.(172) As a consequence, the accuracy of these three biomarkers in identifying early-stage AMI remains to be conclusively tested.

Following the results of a pilot study showing an improvement in survival and lower resection rates(53), we created an intestinal stroke center in our hospital that provides 24/7 standardized multimodal and multidisciplinary care to AMI patients referred from all hospitals in the Paris region. Since the creation of this center in 2016, we have prospectively enrolled patients who undergo a contrast-enhanced CT-scan for acute abdominal pain as part of the SURVIBIO diagnostic study. The patients' blood samples are collected on admission and stored in a biobank for further biomarker analysis and research. We used the blood samples from the biobank to assess the performance of three circulating candidate biomarkers — citrulline, I-FABP, and D-lactate — in the diagnosis of AMI. This was a cross-sectional diagnostic study enrolling patients with acute abdominal pain requiring a contrast-enhanced C.T. scan from January 2016 to March 2018. Patients with AMI were admitted to the intestinal stroke center, whereas those in whom the diagnosis was ruled out (controls) were admitted to the emergency room (see patient flowchart, Figure 16).

METHODS

Study design and setting

This was a cross-sectional diagnostic study enrolling patients with acute abdominal pain requiring a contrast-enhanced C.T. scan from January 2016 to March 2018. Patients with AMI were admitted to the intestinal stroke center, whereas those in whom the diagnosis was ruled

out (controls) were admitted to the emergency room (see patient flowchart, Figure 16). Blood samples were collected from all the patients on admission and stored for further biomarker analysis. The study was approved by the Ethics Committee of Paris-Nord Val de Seine University Hospitals. The study complies with the Standards for Reporting Diagnostic Accuracy (STARD) guidelines.(168) Written informed consent was obtained from all patients.

Patients and controls

AMI was defined by the association of 1) acute clinical, biological and/or contrast-enhanced C.T. features of bowel injury, 2) vascular insufficiency (occlusive or non-occlusive) of the celiac trunk and/or the superior mesentery artery and/or superior mesenteric vein, and 3) the absence of an alternative diagnosis.(54) The diagnosis was confirmed by histology following intestinal resection.(37) All the AMI patients were managed by a standardized multimodal and multidisciplinary approach in our intestinal stroke center, as previously described.(53) Briefly, the patients were systematically administered oral antibiotics, and antithrombotics(53, 54), and emergency endovascular revascularization of arterial AMI was performed whenever technically feasible. Alternatively, open surgical revascularization was performed. Bowel viability was evaluated by laparotomy, decided based on published risk factors for irreversible transmural intestinal necrosis (occurrence of organ failure, elevated serum lactate concentrations, small bowel dilatation or perforation on C.T.).(37) Irreversible transmural intestinal necrosis was confirmed upon pathological assessment.

The diagnosis of AMI was ruled in or out by the C.T. scan, and alternative final diagnoses were based on clinical, biologic, and C.T. findings. Finally, all the included patients underwent a contrast-enhanced abdominal C.T. scan, routine biologic work-up, and blood and urine sampling. Any patients presenting with a diagnosis of left-sided colon ischemia without small bowel injury, chronic mesenteric ischemia without acute injury, vascular lesions with no small bowel injury injury, or strangulated bowel obstruction were not included (see patient

flowchart, Figure 16). All the patients' clinical records, CT-scans, and pathologic specimens were reviewed in a multidisciplinary meeting including gastroenterologists, radiologists, digestive and vascular surgeons and intensivists, all experts in digestive vascular diseases, to avoid diagnostic misclassification before any blood sample analysis.

Data collection and processing

Routine baseline clinical and biological characteristics were collected upon admission for all patients: age, gender, body mass index (BMI), history of cardiovascular disease or risk factors, general and digestive clinical signs and common biologic features. The origin of AMI (arterial – thrombotic or embolic – venous, or non-occlusive) was specified based on the patient's records, CT-scan, and pathologic review. Serum and plasma samples were collected in appropriate tubes before immediate centrifugation at 3,000 rpm for 15 min at room temperature and subsequent storage at -80°C until further analysis. Biochemical assays were performed after the final diagnosis classification had been made in all patients. After randomization of all samples, Citrulline and D-lactate levels were measured on plasma collected in heparin- and oxalate-treated tubes, respectively. I-FABP concentrations were measured from EDTA serum.

I-FABP concentrations were measured in serum using two different ELISA kits [Hycult Biotech, Uden, The Netherlands, HK40602, range: 0 – 3000 ng/L;(99) (176) and R&D Systems, Liey, DY3078, range: 0 – 1000 ng/L(128)] according to the manufacturer's instructions. We used two quality controls (sample pool) – at the beginning and the end of the plates. Briefly, samples and standards were incubated in 96-well microtiter plates coated with antibodies recognizing human I-FABP. The biotinylated secondary antibody was then added to the wells. After washes, streptavidin-peroxidase conjugate, which binds the biotinylated secondary antibody, was added and then reacted with tetramethylbenzidine (TMB) substrate. The absorbance was then measured with a spectrophotometer at 450 nm (Infinite[®] 200 PEO, TECAN).

Citrulline plasma levels were assayed using an ultra-performance liquid chromatography-mass spectrometer (UPLC-MS; Xevo TQS, Waters®). We first conducted a two-step extraction: 100 µL of heparin plasma were added to 300 µL of acetonitrile with 0.05% citrulline C₁₃ (ACN; vWR) and 1% formic acid (F.A., vWR) to precipitate proteins. After 5 min of mechanical agitation, the mixes were centrifuged at 13000 rpm for 5 min at room temperature. Then, 300 µL of the supernatant were loaded onto an Ostro Plate (Waters®) and extracted under positive pressure. The eluate was diluted 1:2 in ACN/H₂O (90:10), and 2 µL were injected in the UPLC-MS. The mobile phase A was water with ammonium formate 10 mM (A.F.; vWR) and 0.15% F.A., and the mobile phase B was A.F. 10 mM in ACN with 0.15% F.A. The retention time was 1.43 min on the Xevo TQS, Waters®. The results are presented as an average of two measurements (duplicate). If a coefficient of variation higher than 10% was observed between two measures, the sample was reanalyzed.

D-lactate plasma concentration was assayed by a kinetic spectrophotometric method using an adapted method of the Biosentec D-Lactic acid kit on a Cobas C111® analyzer (Roche). Briefly, the reaction used an endpoint analysis of the following reaction, catalyzed by the D-lactate dehydrogenase: Lactate+NAD → Pyruvate+NADH+H⁺Pyruvate+Glutamate → Alanine+α-ketoglutarate. NADH was measured at 340 nm. Low-and high-level quality controls were included at the beginning and the end of each batch.

Statistical analysis

For each of the continuous variables, we report the median and the interquartile range (IQR). Categorical variables are expressed as the number of observations and percentages. Normally distributed quantitative data were analyzed with the Student t-test. Mann-Whitney U test was used otherwise. Subgroup analyses were performed with the use of the Kruskal-Wallis tests for skewed distributions. When the result of a global test was significant ($p < 0.05$), post hoc

Bonferroni-corrected pairwise comparisons were performed. Qualitative data were compared with either the Pearson χ^2 test or the Fisher exact test, depending on the sample size. We determined that the enrollment of 50 patients in each group would provide a power of more than 95% for assessing diagnostic tests with $AUC \geq 0.70$.(177) The diagnostic accuracy of each biomarker was evaluated by analyzing the receiver operating curve (ROC) with the calculation of the area under the ROC (AUROC). The maximum value of Youden's index was used as a criterion for selecting the optimal cut-off value of each biomarker, reported with its associated sensitivity, specificity, negative predictive values (NPV), and positive predictive values (PPV). All tests were two-sided. No imputation of missing data was performed. Analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Mac OSX software (version 23.0, Chicago, IL, USA) and the pROC package (178) in R software, version 3.6.2 (R Foundation for Statistical Computing).

RESULTS

Characteristics of study subjects

Between January 4, 2016, and March 5, 2018, 179 patients with acute abdominal pain requiring a contrast-enhanced CT-scan were assessed for eligibility (Figure 16). We included and collected blood samples from 131 patients, including 50 admitted to our intestinal stroke center for AMI and 81 admitted to the emergency room for non-AMI acute abdominal pain (see Flowchart, Figure 16). We excluded 2 patients (controls) with missing samples upon biological analysis. The baseline characteristics of both populations are summarized in Table 9. The final diagnosis of the controls is presented in Table 10. Patients with AMI [median age: 65 years (55-75), 38 % of women] included arterial and venous causes in respectively 66% and 34% of cases. None of the patients included had non-occlusive AMI. AMI occurred in seven patients

exhibiting signs of chronic mesenteric ischemia. Patients with AMI were significantly older, had a higher BMI, and were more likely to have risk factors or a history of cardiovascular disease than controls (Table 9). AMI patients were also more likely to present hematochezia, guarding, and organ dysfunction (as measured by a total SOFA score ≥ 2) and a higher white blood cell count at baseline. Other baselines clinical and laboratory characteristics, including L-Lactate, did not differ significantly (Table 9). After admission in the intestinal stroke center, AMI patients received antiplatelet therapy (n=33, 100% arterial AMI), anticoagulants (n=50, 100%), oral antibiotics (n=49, 98%), and intravenous antibiotics (n=21, 42%). Emergency revascularization was performed in 29 patients (88% of arterial AMI patients).

Main results

Median plasma citrulline concentrations were significantly lower in the AMI patients compared to the controls [15.3 $\mu\text{mol/L}$ (12.0-26.0) vs. 23.3 $\mu\text{mol/L}$ (18.3-29.8), $p=0.001$], although the two distributions broadly overlapped (Figure 17). Serum I-FABP concentrations tended to be lower in the AMI population compared to the controls using Hycult kit [278 ng/L (209-544) vs. 348 ng/L (268-587), $p=0.06$]. We then confirmed a similar trend using R&D kit (Supplementary data Fig1). Results obtained with Hycult kit were further used for calculation. Plasma D-lactate concentrations did not significantly differ between AMI patients and controls [0.042 mmol/L (0.025-0.095) vs. 0.073 mmol/L (0.031-0.115), $p=0.28$]. Performances of the three biomarkers for the diagnosis of AMI are summarized in Table 11.

Arterial versus venous AMI subgroups

Compared to the controls, median plasma citrulline was significantly lower in patients with arterial AMI [14.5 $\mu\text{mol/L}$ (11.1-29.1) vs. 23.3 $\mu\text{mol/L}$ (18.3-29.8), $p=0.01$] and slightly lower in patients with venous AMI [16.6 $\mu\text{mol/L}$ (13.0-21.2) vs. 23.3 $\mu\text{mol/L}$ (18.3-29.8), $p=0.06$]. We did not observe significant differences in the concentration of serum I-FABP in arterial and venous AMI versus control patients [281 ng/L (169-647) and 262 ng/L (248-487) versus 348

ng/L (268-587), respectively, $p=0.21$]. Neither did we observe significant differences in the concentration of plasma D-Lactate concentrations [0.054 mmol/L (0.033-0.107) in arterial AMI and 0.025 mmol/L (0.022-0.088) in venous AMI *versus* 0.073 mmol/L (0.031-0.115) in controls, $p=0.24$] (Supplemental data, Figure 19).

Early *versus* late necrotic AMI subgroups

During the follow-up period, 17 of the AMI patients (34%) required a laparotomy, and 12 (24%) required bowel resection. Irreversible transmural intestinal necrosis was pathologically confirmed in 10 of the 12 resected cases. Four additional patients had undergone open-close procedures (i.e. without performing bowel resection) showing visible extensive dark necrosis and were included in the late necrotic AMI subgroup ($n=14$). Patients with superficial and non-transmural necrosis ($n=2$), as well as those who recovered from AMI with no need for bowel resection ($n=34$) were included in the early AMI subgroup ($n=36$).

As presented in Figure 18, plasma citrulline concentrations were significantly lower in both late necrotic AMI [13.5 (10.3-25.5) $\mu\text{mol/L}$, $p=0.02$] and early AMI patients [17.9 (12.3-26.0) $\mu\text{mol/L}$, $p=0.02$] as compared to controls [23.3 (18.3-29.8) $\mu\text{mol/L}$]. However, the comparison failed to discriminate late necrotic from early AMI patients. We observed a trend of higher serum I-FABP concentrations in late necrotic AMI patients [619 ng/L (212-1632)] compared to early AMI patients [268 ng/L (205-403), $p=0.12$] and controls [348 ng/L (268-587), $p=1.0$]. However, I-FABP levels were significantly lower in early AMI patients compared to controls ($p=0.03$). We did not find any significant difference in plasma D-lactate concentrations between late necrotic AMI patients [0.069 mmol/L (0.036-0.128)] or either early AMI patients or controls [0.035 mmol/L (0.024-0.091) and 0.073 mmol/L (0.031-0.115), respectively, $p=0.26$].

DISCUSSION

This cross-sectional diagnostic study of 129 patients admitted for acute abdominal pain found that the three most promising circulating biomarker candidates for AMI – citrulline, I-FABP, and D-lactate – were neither sensitive nor specific enough for differential diagnosis of AMI: I-FABP and D-lactate displayed low AUROC values, and citrulline assays had low sensitivity. These results contrast with published reports.(91, 94, 129, 175) This could be explained by two critical differences in the experimental designs. Firstly, previous studies enrolled severe AMI patients at a late necrotic surgical stage. This may have created a selection bias leading to an overestimated performance of the studied biomarkers, not generalizable to earlier stages of the disease. Secondly, in most of the studies published so far, only a restricted number of AMI patients were included, and they were merged with heterogeneous pathologic conditions labeled “ischemic bowel diseases”, such as strangulated bowel obstructions or left-side colon ischemia with no small bowel injury. This may have led to an overestimation of the specificity of the studied biomarkers for the diagnosis of genuine vascular-related AMI. For instance, Shi et al. studied 39 patients with “acute intestinal ischemic disease” including 26 with strangulated bowel obstruction, six with colon ischemia with no small bowel injury, and only seven patients with genuine AMI at the infarction stage.(117) Similarly, Kanda et al. studied 52 preoperative patients with “small bowel ischemia,” including 45 cases of strangulated bowel or incarcerated hernia and only three patients with late necrotic AMI.(179) In the present study, we included a large and homogeneous population of 50 patients with confirmed arterial and venous AMI, treated with a standardized care protocol in an intestinal stroke center, enrolled on admission at the time of diagnosis, and at an early “non-transmural” AMI stage in 72% of cases. Therefore, our design yields reliable results on clinically applicable AMI biomarkers in its emergency “real-life” early-stage diagnosis.

I-FABP is a cytosolic protein expressed by mature enterocytes at the tip of the small bowel villi, which is the first region affected by ischemia.(118) In the past decades, a few studies on human samples have identified I-FABP as the most promising biomarker for the diagnosis of AMI. The recent meta-analysis by Sun *et al.* calculated a pooled sensitivity, specificity, and AUROC of 0.80, 0.85, and 0.86, respectively.(129) Nevertheless, it should be noted that only 60 of the 1246 patients studied in this meta-analysis had genuine vascular-related AMI, mostly at the infarction stage (after careful review of seven of the nine English-language studies included).(129) Furthermore, this meta-analysis included studies using various methods for I-FABP measurements and various blood sample types that could be significant confounding factors.

Although we observed higher I-FABP serum concentrations in late necrotic AMI patients, its discriminative diagnostic performance for AMI was not confirmed. This result was supported by another ELISA assay to exclude any analytical bias (see Supplemental Data, Figure 20). We even observed higher I-FABP concentrations in controls in comparison with early-stage AMI patients. This observation is also in line with numerous published studies that observed high I-FABP blood concentrations in various other non-ischemic gastro-intestinal conditions such as Crohn's disease,(180) celiac disease,(181, 182) acute pancreatitis,(183, 184) or abdominal surgery/infection.(185)

D-Lactate is the stereoisomer of L-lactate and is almost exclusively produced by microbial fermentation in the gastro-intestinal tract. As a consequence, animal experiments and a few clinical studies identified this molecule as a marker of intestinal barrier dysfunction and microbial translocation or overgrowth.(186, 187) However, its accuracy has been questioned by studies such as the ones by Van Der Voort *et al.* (188) and Block *et al.* (110) reporting low (23%) specificity, consistent with the present findings.

Citrulline is an amino acid synthesized from glutamine by small bowel enterocytes. It is considered as a functional biomarker of total enterocyte mass and intestinal failure. Low citrulline plasma levels have been found to correlate with remnant small bowel length and intestinal failure in patients with short bowel syndrome (130) and with the severity of villous atrophy in patients with celiac disease.(189) In a prospective single-center observational study that included adults without small bowel disease and chronic renal failure consecutively admitted in an intensive care unit, citrulline plasma concentrations were found to be lower for the 24 hours following the circulatory shock.(131) Concentrations $< 10 \mu\text{mol/L}$ were highlighted as an independent risk factor of mortality, suggesting that low plasma citrulline at 24 h could be a marker of acute intestinal failure in critically ill patients. We observed significantly lower citrulline plasma levels in patients with AMI compared to the controls, consistent with prior reports.(175, 190) As already reported — but here in a larger cohort — we confirm that the sensitivity of citrulline is insufficient in this setting. Our findings are consistent with the results obtained by Kulu *et al.* They found a sensitivity of 39% for citrulline for the identification of AMI in 23 patients, with the best cut-off value at $15.8 \mu\text{mol/L}$.(190) Altogether, this corroborates that citrulline displays a low diagnostic performance for AMI which, regrettably, limits its usefulness: sensitivity is more important than specificity for AMI given the severe consequences of misdiagnosis and given that confirmation by a contrast-enhanced C.T. scan remains mandatory to guide emergency treatment (type of revascularization, need for intestinal resection).

A few limitations derived from the design of the present study deserve to be mentioned. First, we chose not to include patients with strangulated bowel obstruction, left-sided colon ischemia, and mesenteric vessel occlusion without evidence of acute small bowel injury from both patients and control groups. Although these conditions may share pathophysiological processes comparable with that of AMI, they are different disease with different prognoses. Although

this might decrease the generalizability of our findings to these other conditions, we believe this selection is critical to reduce variability and avoid analytical bias which are the main shortcomings of available reports in this field of research.(172) Nonetheless, our results are based on the largest population of well-characterized and homogeneous AMI patients. This is crucial as the diagnosis of AMI amongst emergency patients admitted for acute abdominal pain remains a critical clinical challenge (172), and our findings indicate that the three tested biomarkers do not reach a sufficient diagnostic performance level. Moreover, as compared with previously published studies, we used two different assays for I-FABP, as well as two robust standardized methods for both citrulline and D-lactate measurements.

In conclusion, Citrulline, I-FABP, and D-lactate failed to identify AMI from acute abdominal pain controls in a large cross-sectional diagnostic study from an Intestinal Stroke Center. Early diagnosis remains a critical clinical and research challenge as it would allow early management and consequently improve the dire prognosis of AMI. To meet this challenge, we believe that relevant new biomarkers may be identified using non-targeted multi-omics discovery approaches in large cohorts of early-stage AMI patients admitted in intestinal stroke centers. This objective is of the utmost importance since the introduction of new biomarkers may genuinely alter the epidemiology, diagnosis, management, and outcome of AMI.

Table 9: Baseline characteristics of AMI patients and controls.

	AMI patients n=50 (%)	Controls n=79(%)	<i>p-value</i>
Age, years ¹	65 (55-75)	46 (35-71)	< 0.001
BMI, kg/m ² ¹	27 (21-33)	22 (20-24)	< 0.001
Female	19 (38)	31 (39)	0.89
<i>Atherosclerosis risk factors</i>			
Tobacco use	23 (46)	16 (20)	0.002
Arterial hypertension	28 (56)	20 (25)	< 0.001
Dyslipidemia	19 (38)	12 (15)	0.003
Diabetes mellitus	12 (24)	5 (6)	0.004
<i>Cardiovascular history</i>			
Myocardial ischemia	9 (18)	5 (6)	0.04
Stroke	6 (12)	5 (6)	0.34
Limb ischemia	9 (18)	2 (3)	0.003
Atrial fibrillation	11 (22)	3 (4)	0.001
Deep vein thrombosis	3 (6)	4 (5)	1.00
Pulmonary embolism	5 (10)	4 (5)	0.31
<i>Other comorbidities</i>			
Chronic kidney disease	1 (2)	2 (3)	1.00
Cirrhosis	4 (8)	4 (5)	0.71
Abdominal surgery	27 (54)	37 (47)	0.43
<i>Clinical features</i>			
Temperature ¹	37.0 (36.3-37.1)	36.8 (36.5-37.5)	0.49
Mean arterial pressure ¹	100.3 (90.8-110.3)	96.0 (83.7-105.7)	0.17
Vomiting	21 (42)	39 (49)	0.41
Diarrhea	12 (24)	13 (17)	0.29
Hematochezia	8 (16)	3 (4)	0.02
Guarding	16 (32)	13 (17)	0.04
Organ dysfunction (total SOFA _≥ 2)	15 (30)	8 (10)	0.004
<i>Biological features ¹</i>			
White blood cell count, G/L	12.3 (9.3-18.3)	10.6 (8.0-14.0)	0.02
Platelet count, G/L	268 (177-367)	269 (231-333)	0.74
Hemoglobin, g/dL	12.3 (11.5-15.1)	13.6 (12.4-14.8)	0.14
Lactate levels, mmol/L	1.49 (1.02-2.41)	1.37 (0.83-2.44)	0.56
Creatinine, μmol/L	70 (62-100)	70 (62-84)	0.86
ASAT, UI/L	26 (19-38)	27 (21-41)	0.29

Abbreviations: AMI: acute mesenteric ischemia; BMI: body mass index; SOFA: sequential organ failure assessment; ASAT: aspartate aminotransferase

¹ Median (Inter Quartile Range)

Table 10. Etiological diagnosis of the control group with acute abdominal pain.

Diagnosis	n=79 (%)
Infectious	18 (23)
<i>Diverticulitis</i>	9
<i>Appendicitis</i>	4
<i>Other causes of peritonitis/abdominal abscess</i>	5
Inflammatory	15 (19)
<i>Intra-abdominal neoplasm progression</i>	8
<i>IBD flares</i>	7
Bowel obstruction (non-strangulated)	13 (16)
Functional G.I. disorders (reflux, diarrhea, constipation...)	12 (15)
Biliopancreatic tract	10 (13)
<i>Pancreatitis</i>	7
<i>Biliary complications</i>	3
Urogenital	10 (13)
Invasive meningococcal disease	1 (1)

Abbreviations: IBD: inflammatory bowel disease; G.I.: gastro-intestinal

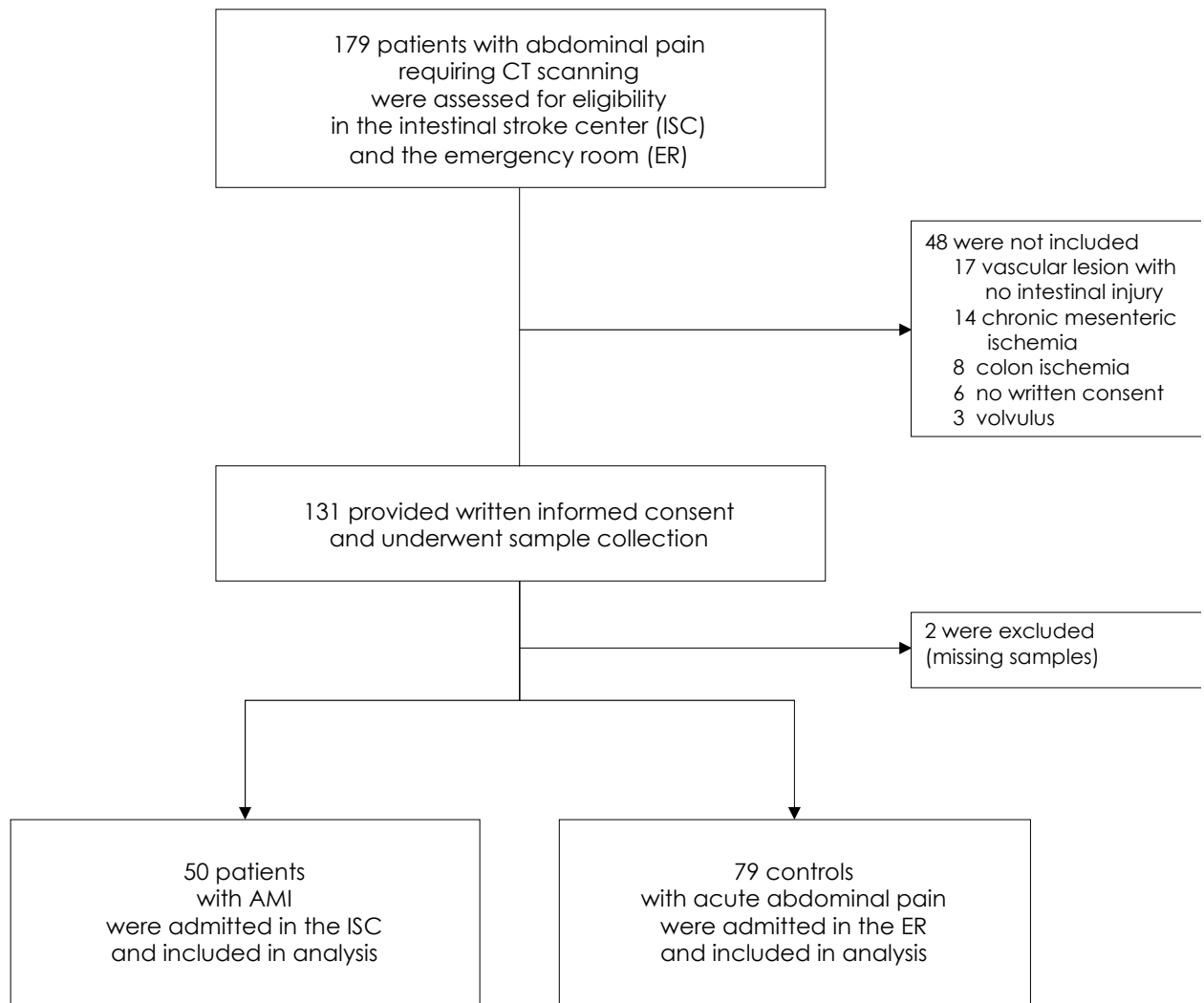
Table 11. Diagnostic performance of citrulline, I-FABP, and D-lactate for the diagnosis of acute mesenteric ischemia

Biomarker	AUROC (95%CI)	Cut-off value	Sensitivity	Specificity	PPV	NPV	Youden index
Citrulline (μmol/L)	0.679 (0.577-0.781)	16.6	0.56	0.84	0.68	0.75	0.40
I-FABP (ng/L)	0.401 (0.291-0.512)	974	0.15	0.95	0.64	0.64	0.10
D-lactate (mmol/L)	0.438 (0.328-0.547)	0.012	0.98	0.17	0.43	0.92	0.14

PPV and NPV were calculated based on a prevalence of AMI = 0.39 in the study population

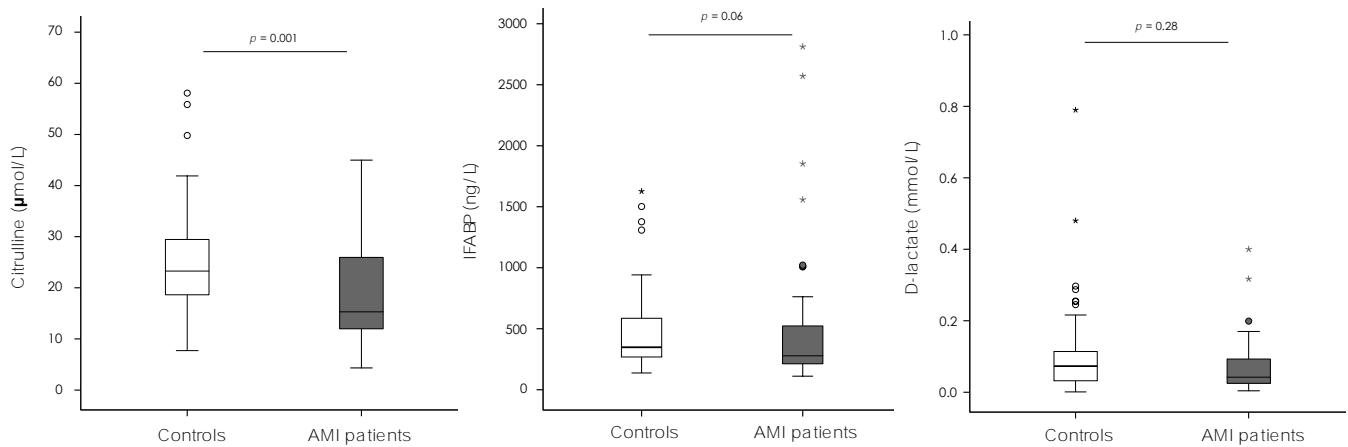
Abbreviations: AUROC: area under the receiver operating curve; PPV: positive predictive value; NPV: negative predictive value; CI: confidence interval; I-FABP: intestinal fatty acid-binding protein

Figure 16. Flowchart of AMI patients and controls: screening and selection



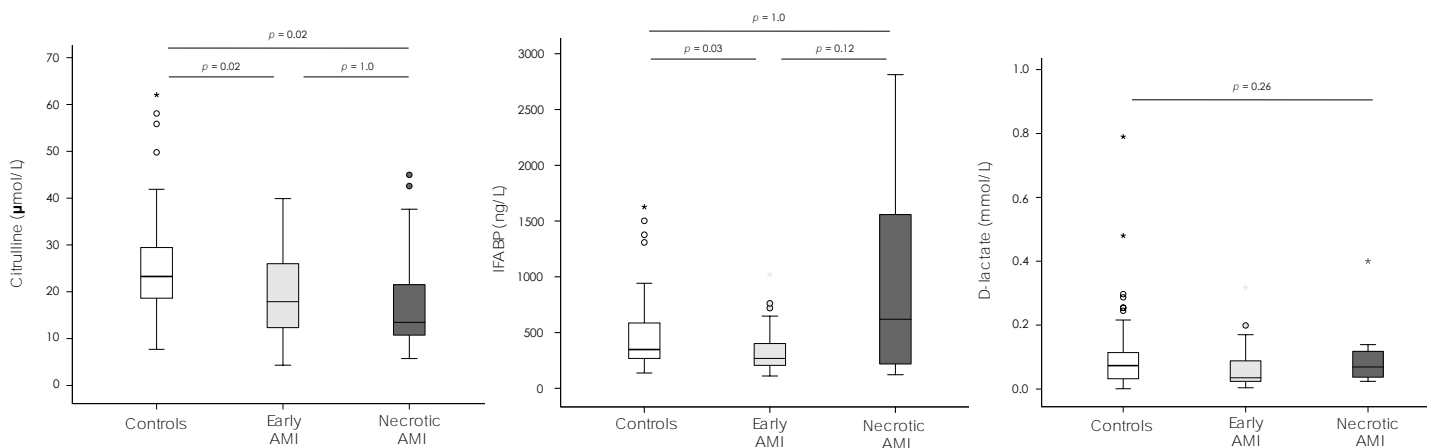
Abbreviations: AMI: acute mesenteric ischemia; CT: contrast-enhanced computed tomography;

Figure 17: Citrulline, I-FABP and D-lactate blood concentrations in AMI patients and controls



Abbreviations: I-FABP: intestinal fatty-acid binding protein; AMI: acute mesenteric ischemia
 The horizontal line in the boxes represents the median, and the bottom and top of the boxes the 25th and 75th percentiles, respectively. I bars represent the upper adjacent value (75th percentile plus 1.5 times the interquartile range) and the lower adjacent value (corresponding formula below the 25th percentile), and the dots/asterisks outliers.

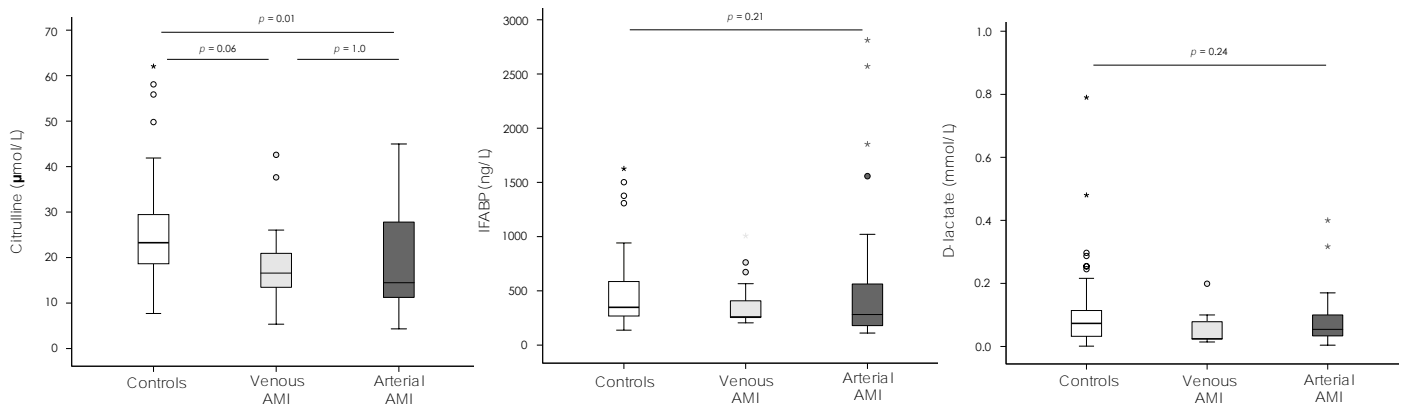
Figure 18. Citrulline, I-FABP, and D-lactate blood concentrations according to the severity of acute mesenteric ischemia (early or late – necrotic – ischemia)



Abbreviations: AMI: acute mesenteric ischemia; I-FABP: intestinal fatty-acid binding protein;
 The horizontal line in the boxes represents the median, and the bottom and top of the boxes, the 25th and 75th percentiles, respectively. I bars represent the upper adjacent value (75th percentile plus 1.5 times the interquartile range) and the lower adjacent value (corresponding formula below the 25th percentile), and the dots/asterisks outliers.

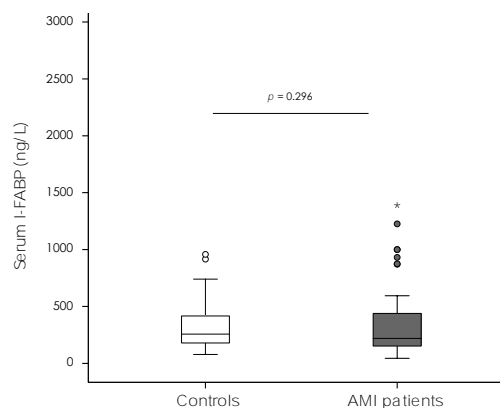
SUPPLEMENTAL DATA:

Figure 19. Citrulline, I-FABP, and D-lactate blood concentrations according to the origin of acute mesenteric ischemia (venous or arterial)



Abbreviations: AMI: acute mesenteric ischemia; I-FABP: intestinal fatty-acid binding protein; The horizontal line in the boxes represents the median, and the bottom and top of the boxes, the 25th and 75th percentiles, respectively. I bars represent the upper adjacent value (75th percentile plus 1.5 times the interquartile range) and the lower adjacent value (corresponding formula below the 25th percentile), and the dots/asterisks outliers.

Figure 20. I-FABP serum concentrations in AMI patients and controls using the R&D Systems ELISA kit



I-FABP concentrations were measured in serum using the ELISA Kits from R&D Systems, Liey, DY3078, (linearity range: 0 – 1000 ng/L). No significant difference was found in serum I-FABP concentrations between AMI patients compared to the controls [220 ng/L (152-445) vs 258 ng/L (178-420), p=0.30]. The area under the receiver operating curve (AUROC) for the diagnosis of AMI by serum I-FABP was 0.44 (95% CI = 0.33 – 0.56)

Chapter 4

The results of this work have led to a patent application submitted on March 3rd 2021 to the European Patent Office (application number EP21305252.5)

Original article

Title: Plasma levels of metabolites and lipid moieties identify acute mesenteric ischemia patients from acute abdominal pain controls: a diagnostic study from an intestinal stroke center

INTRODUCTION

Early management of acute mesenteric ischemia (AMI) can avoid fatal outcome and prevent related short bowel syndrome.(1, 53, 91, 172) Recent reports suggest improved outcomes for patients if diagnosis and standardized multidisciplinary expert care are provided at an early stage.(53, 54, 173) However, such outcomes are achievable only if a timely diagnosis can be provided, which remains a clinical and research challenge. Indeed, AMI patients present unspecific acute abdominal pain which can often lead to missed or delayed diagnosis and care.(1, 4) Biologic abnormalities – such as leucocytosis or lactic acidosis – have very low performance to establish the early diagnosis.(91, 174) Moreover, AMI may be underdiagnosed on contrast-enhanced computed tomography (C.T.) of the acute abdomen in the real life setting, especially when the clinical suspicion was not mentioned to the radiologist, resulting in either

an inappropriate IV contrast protocol and/or an analysis that did not focus on the mesenteric vessels.(35, 42) Therefore, except for recent intestinal stroke center experience which provide early management to early diagnosed AMI patients, mortality and intestinal resection rates have remained unchanged for decades.(1, 37)

A large number of molecules have been proposed as potential biomarkers of AMI, the three most promising being: citrulline, a marker of enterocyte function; I-FABP, a marker of enterocyte damage; and D-lactate, a marker of intestinal barrier dysfunction and microbial translocation.(91, 94, 129, 175) However, in a preliminary analysis of this SURVIBIO study, Citrulline, I-FABP, and D-lactate failed to identify AMI from acute abdominal pain controls, consistent with conflicting results from the scientific literature.(172) As a consequence, the accuracy of these three biomarkers in identifying early-stage AMI remains to be conclusively tested. (65, 191) As a result, there is today a critical unmet need for new biomarkers to establish an immediate diagnosis.

Systems medicine provides a compelling opportunity to develop novel diagnostic strategies based on the measurement of many thousands of genes, proteins or metabolites. The metabolic phenotype (metabotyping) of individuals can be measured from the composition of accessible biofluids sampled in the clinic. Metatypes vary extensively between individuals and populations and result from the complex interplay of host genes, lifestyle, diet and gut microbes. Metabolic phenotyping may provide novel diagnostic strategies for GI diseases such as AMI.(192)

Following the results of a pilot study showing an improvement in survival and lower resection rates(53), we created an intestinal stroke center in our hospital that provides 24/7 standardized multimodal and multidisciplinary care to AMI patients referred from all hospitals in the Paris region. Since the creation of this center in 2016, we have prospectively enrolled patients who undergo a contrast-enhanced CT-scan for acute abdominal pain as part of the SURVIBIO

diagnostic study (Clinicaltrials.org - NCT03518099). The patients' blood samples are collected on admission and stored in a biobank for further biomarker analysis and research. We used the admission plasma samples from the biobank to identify novel biomarkers for diagnosis of AMI using untargeted GC-MS and ¹H-NMR metabolomics.

MATERIALS AND METHODS

Study design and setting

This was a cross-sectional diagnostic study enrolling patients with acute abdominal pain requiring a contrast-enhanced C.T. scan from January 2016 to March 2018. Patients with AMI were admitted to the intestinal stroke center, whereas those in whom the diagnosis was ruled out (controls) were admitted to the emergency room (see patient flowchart, Figure 21). Blood samples were collected from all the patients on admission and stored for further biomarker analysis. The study was approved by the Ethics Committee of Paris-Nord Val de Seine University Hospitals. The study complies with the Standards for Reporting Diagnostic Accuracy (STARD) guidelines.(168) Written informed consent was obtained from all patients.

Patients and controls

AMI was defined by the association of 1) acute clinical, biological and/or contrast-enhanced C.T. features of bowel injury, 2) vascular insufficiency (occlusive or non-occlusive) of the celiac trunk and/or the superior mesentery artery and/or superior mesenteric vein, and 3) the absence of an alternative diagnosis.(54) The diagnosis was confirmed by histology following intestinal resection.(37) All the AMI patients were managed by a standardized multimodal and multidisciplinary approach in our intestinal stroke center, as previously described.(53) Briefly, the patients were systematically administered oral antibiotics, and antithrombotics(53, 54), and emergency endovascular revascularization of arterial AMI was performed whenever technically feasible. Alternatively, open surgical revascularization was performed. Bowel

viability was evaluated by laparotomy, decided based on published risk factors for irreversible transmural intestinal necrosis (occurrence of organ failure, elevated serum lactate concentrations, small bowel dilatation or perforation on C.T.).(37) Irreversible transmural intestinal necrosis was confirmed upon pathological assessment.

The diagnosis of AMI was ruled in or out by the C.T. scan, and alternative final diagnoses were based on clinical, biologic, and C.T. findings. Finally, all the included patients underwent a contrast-enhanced abdominal C.T. scan, routine biologic work-up, and blood and urine sampling. Any patients presenting with a diagnosis of left-sided colon ischemia, chronic mesenteric ischemia without acute injury, vascular lesions with no intestinal injury, or strangulated bowel obstruction were excluded (see patient flowchart, Figure 21). All the patients' clinical records, CT-scans, and pathologic specimens were reviewed in a multidisciplinary meeting including gastroenterologists, radiologists, digestive and vascular surgeons and intensivists, all experts in digestive vascular diseases, to avoid diagnostic misclassification before any blood sample analyses.

Data collection and processing

Routine baseline clinical and biological characteristics were collected upon admission for all patients: age, gender, body mass index (BMI), history of cardiovascular disease or risk factors, general and digestive clinical signs and common biologic features. The origin of AMI (arterial – thrombotic or embolic – venous, or non-occlusive) was specified based on the patient's records, CT-scan, and pathologic review. Plasma samples were collected in heparin-treated tubes before immediate centrifugation at 3,000 rpm for 15 min at room temperature and subsequent storage at -80°C until further analysis. Metabolomic analyses were performed after the final diagnosis classification had been made in all patients.

Experimental rat model of intestinal ischemia and reperfusion

We developed a standardized rat model of AMI. (193) Details of the procedure are described in the Supplemental data section. This animal experimentation aimed at analyzing the kinetics of the biomarkers identified during ischemia and reperfusion, under perfectly homogeneous conditions (animal age, nutrition) limiting inter-individual variability, which was unfeasible in the human cohort. Wistar rats (n=8) were operated on and AMI was induced by selective clamping of the superior mesenteric artery for 1 hour after medial laparotomy, followed by a period of reperfusion of 1 hour. In the sham group (n=7), the superior mesenteric artery was dissected, exposed but not ligated (sham operated). Plasma samples were collected at four time points: at baseline (T0), after 30min and 60min of ischemia, and after 1 hour of reperfusion. Samples were stored at -80°C until metabolomic analyses. At the end of the procedure, every rat was sacrificed under general anesthesia and small intestine was removed for histological qualitative and quantitative analysis (transversal and longitudinal sections).

Metabolomics profiling

Analyses were performed using GC-MS and 1H-NMR within the Department of Surgery and Cancer, South Kensington campus, Imperial College London.

GC-MS profiling

Materials

Methanol (HPLC gradient grade) was obtained from Fisher Scientific. Methoxamine reagent (MOX) and N-Methyl-N-trimethylsilyltrifluoroacetamide + 1% -trimethylchlorosilane (MSTFA + 1% TMCS) silylation reagent were purchased from VWR. Heptane (Chromasol-V), methyl stearate (analytical standard) and Fatty acid methyl esters mix (FAME Supelco 37 component) were purchased from Sigma-Aldrich. GC-MS analysis was performed on an Agilent 7890B/5977A Series Gas Chromatograph/Mass Selective Detector. The consumables for the GC-MS run were obtained from Agilent Technologies: silanized vials (part number 5183-2072), PTFE caps (5185-5820), glass inserts (5183-2072).

Sample preparation

Plasma samples were prepared by transferring 100 μL to eppendorf tubes after thawing and brief vortexing. Then, 10 μL of myristic acid d27 3 mg/ml internal standard were added to each sample. Proteins were precipitated by addition of 850 μL of cold methanol and samples were centrifuged for 20 min at 4°C at 13.000 g. 750 μL of the supernatant were transferred to silanized glass vials and dried at speed vacuum evaporator at 45°C for 1.45 hours. To the dried extracts, 50 μL of MOX in pyridine (2% w/v) were added in order for MOX to react with carbonyl groups of ketones and aldehydes, thus protecting the functional groups of metabolites. Samples were then left overnight at room temperature for the reaction to be completed. Subsequently, 100 μL of MSTFA with 1% (v/v) TMCS were added in order to increase the volatility and thermal stability of the samples and after vortexing, samples were incubated at 60°C for 1 hour. In the end, samples were transferred to a clean glass vial containing a glass insert for GC-MS analysis.

Workflow of GC-MS experiment

An analytical standard (FAME mix) was analyzed in the beginning and end of the analysis as it contains 37 fatty acid methyl esters with known retention index that would help in subsequent identification of compounds by comparison of the expected retention times to the experimental ones. In addition, in order to check the overall instrument sensitivity as well as the compliance of the injector system and to monitor the performance of data acquisition, Quality Control (QC) samples were used. Biological QCs were prepared by pooling aliquots of all plasma samples in a QC master sample in order to provide true representation of the range of metabolites present in the sample set and with similar matrix composition. Subsequently, the resultant pooled sample was separated into 100 μL -aliquot QC samples that were subjected to the above sample preparation and then injected between every 17 plasma samples during the GC-MS run.

One blank sample was also employed at the start of the run, consisting only of methanol that have been prepared following the procedure mentioned in the previous section.

GC-MS analysis

Derivatised samples were analysed by GC-MS with an Agilent 7890B/5977A Series Gas Chromatograph/Mass Selective Detector. 2 μ L of each sample were injected into the instrument and the injector temperature was set at 250°C with a split ratio of 1:10. For the chromatographic separation, helium was used as a carrier gas through the column at a constant flow rate of 1.1 mL/min. The temperature of the oven started at 60°C and rise at the rate of 10°C per minute to reach a final temperature of 325°C. For the detection with the mass spectrometer, the transfer line temperature was set at 290°C and ions were generated by an electron impact of 70 eV. The source temperature was maintained at 250°C whereas the temperatures of the quadrupole at 150°C. A solvent delay of 5.90 min was set up and the total run time was 37.50 min per sample.

Pre-processing of GC-MS data

Acquired raw data were subjected to pre-processing steps. For the retention index correction for GC-MS data, a calibration file was produced with the retention index of the FAME standards obtained during the experiment. The Automated Mass Spectrometry Deconvolution and Identification System (AMDIS_32) was firstly used for deconvolution and automatic peak detection according to the calibration file and the Fiehn mass spectral library. The retention time alignment and filtering of peaks were performed with the use of the Agilent Mass Profiler Professional software 12 integrated with GeneSpring GX12. After exporting a file with identified compounds, raw spectral data were re-examined in order to identify that the metabolites found exist in the QCs and they are not result of contamination if they were found present in blank samples. In the case of existing metabolites, a new target ion that would be more selective (if thought to be necessary) and the actual retention time observed in our experiment were reported. A new library was built in AMDIS, which included the identified

metabolites with the experimental retention time and a more abundant target ion. Following this, data were once again processed in AMDIS with the new library and then put through GeneSpring GX12 for final alignment and re-filtering of peaks. Resultant values were reported as peak intensity (area). Data were normalized with the use of the IS, by dividing each sample with the corresponding value of IS. Coefficients of variation (CV %) were then calculated for all metabolites in QCs and identified compounds with a CV% greater than 30% were removed prior to statistical analysis. The final dataset was then processed with univariate and multivariate statistics. Peak picking was performed with Gavin3 package in Matlab to ensure that all the targeted metabolites were accurately integrated. Reproducibility, blank contamination, instrument drift, run order and batch effects were evaluated with the use of supervised and unsupervised multivariate statistics and corrected accordingly, with the aim to control and minimise the analytical variability and focus exclusively in the biological variability.

1H-NMR profiling

Materials

All reagents were of analytical grade and purchased from Sigma-Aldrich: Disodium Hydrogen Phosphate Na_2HPO_4 (7558-79-4), Deuterium Oxide D_2O (7789-20-0), Sodium Azide NaN_3 (26628-22-8), 3-(Trimethyl-silyl) propionic acid-d4 sodium salt TSP (24493-21-8), Potassium Hydroxide Solution KOH 45% w/w (1310-58-3), Hydrochloric Acid HCl 37% w/w (7647-01-0), Potassium Dihydrogen Phosphate KH_2PO_4 was purchased from VWR International(7778-77-0). NMR analysis was performed using a Bruker Avance III 600 spectrometer.

Sample preparation

Plasma samples were prepared by transferring 300 μL to eppendorf tubes after thawing and brief vortexing. Samples were then mixed with 300 μL of phosphate buffer solution in H_2O (0.142 M of NaHPO_4 , pH 7.4) containing 2mM of NaN_3 as a bacteriostatic agent, 10% (v/v)

D₂O providing a lock signal for the magnetic field and 0.08% (v/v) TSP as an internal chemical shift reference. After centrifugation with 12000 x g at 4°C for 5 min, 580 µL of sample were transferred in 5mm NMR tubes and loaded onto a Sample Jet robot, maintained at 4°C awaiting NMR analysis.

Workflow of metabolomic experiment

All samples included were randomized prior to sample preparation and NMR analysis. Biological QC samples were also generated from a composite of all samples and analyzed every 30 samples.

Acquisition of ¹H NMR spectra

Proton (¹H) NMR spectra were acquired using a Bruker Avance III 600 spectrometer. Two monodimensional and one bidimensional experiments were acquired in automation using the standard pulse sequences previously described.(194) These experiments consisted of the 1D-¹H- Nuclear Overhauser Effect Spectroscopy (NOESY), the 1D-Carr-Purcell-Meiboom-Gill (CPMG), and the 2D J-RESolved (J-RES)-presat spectra.

Pre-processing of ¹H NMR spectra

All spectra were calibrated, phased and baseline corrected automatically using TopSpin (v3.2, Bruker BioSpin, Rheinstetten, Germany) and later again phased, baseline corrected and referenced to the α-glucose resonance (δH 5.225 (d)) for plasma using in-house software developed by Dr. R. Cavill, in MATLAB environment (R2013a, The MathWorks, Inc.). The 32K data points produced (δH -1 – 10) were further reduced by exclusion of the water resonance (4.5 - 4.9 ppm) and the TSP region (-0.2 - 0.2 ppm). After spectral exclusion, spectra were aligned. (195) Samples regions were further subjected to normalization using probabilistic quotient normalization (PQN) and dimensionality of data was reduced using SRV (Statistical Recoupling of Variables).(196, 197)

Statistical analyses

For each of the continuous variables, we report the median and the interquartile range (IQR). Categorical variables are expressed as the number of observations and percentages. Normally distributed quantitative data were analyzed with the Student t-test. Mann-Whitney U test was used otherwise. Qualitative data were compared with either the Pearson χ^2 test or the Fisher exact test, depending on the sample size. We determined that the enrollment of 50 patients in each group would provide a power of more than 95% for assessing diagnostic tests with AUC ≥ 0.70 .(177)

Unsupervised Principal component analysis (PCA) models, which do not include knowledge of the results of the reference standard, were produced to investigate whether there were any hard outliers, due to either analytical error or biological deviation. PCA is a multivariate projection method, used to extract and display systematic variation in a data matrix. The scores plots of the PCA models display correlations between the participants metabolic profiles, with points closer together representing more similar profiles, allowing groups and trends to be revealed. Metabolomics data analyzed pertains to all metabolites including amino acids, carbohydrates, lipids, nucleotides, microbiota metabolism, energy, cofactors & vitamins, xenobiotics, and novel metabolites. To identify biochemicals that differed significantly between groups, metabolites were compared by univariate and multivariate logistic regression adjusting for age and sex. An estimate of the false discovery rate (FDR) was calculated to consider the multiple comparisons that normally occur in metabolomic-based studies. The univariate and multivariate tables were ranked according to p-values and q-values (false discovery rates adjustment). Then a machine learning analysis using a fuzzy feature learning and selection algorithm (Learning Algorithm for Multivariate Data Analysis – LAMBDA – and Membership Margin Based Attribute Selection – MEMBAS)(164, 165) was performed to identify a panel of diagnostic biomarkers with an optimal AUC/number of metabolites ratio.

The algorithm was applied on a training set and validation set consisting of 85% and 15% of patients, respectively.

The diagnostic value of the panel of biomarkers identified was evaluated by analyzing the receiver operating curve (ROC) with the calculation of the area under the ROC (AUROC). All tests were two-sided. No imputation of missing data was performed. Analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Mac OSX software (version 23.0, Chicago, IL, USA) and in R software, version 3.6.2 (R Foundation for Statistical Computing).

RESULTS

Characteristics of study subjects

Between January 4, 2016, and March 5, 2018, 179 patients with acute abdominal pain requiring a contrast-enhanced CT-scan were assessed for eligibility (Figure 21). We included and collected blood samples from 131 patients, including 50 admitted to our intestinal stroke center for AMI and 81 admitted to the emergency room for non-AMI acute abdominal pain (see Flowchart, Figure 21). We excluded 4 patients (2 AMI patients and 2 controls) with missing samples upon biological analysis. After PCA analyses of metabolomics spectra, we excluded one AMI patient with a hard outlier metabolic signature on all PCA plots (see supplemental data, Figure 28), also corresponding to an extreme late clinical presentation of AMI. Eventually, 47 AMI patients and 79 abdominal pain controls were included in metabolome analyses. The baseline characteristics of both populations are summarized in Table 12. The final diagnosis of the controls is presented in Table 13.

Patients with AMI [median age: 65 years (55-75), 38 % of women] included arterial and venous causes in respectively 66% and 34% of cases. None of the patients included had non-occlusive

AMI. AMI occurred in seven patients exhibiting signs of chronic mesenteric ischemia. Patients with AMI were significantly older, had a higher BMI, and were more likely to have risk factors or a history of cardiovascular disease than controls (Table 12). AMI patients were also more likely to present hematochezia, guarding, and organ dysfunction (as measured by a total SOFA score ≥ 2) and a higher white blood cell count at baseline. Other baselines clinical and laboratory characteristics, including L-Lactate, did not differ significantly (Table 12). After admission in the intestinal stroke center, AMI patients received antiplatelet therapy (n=33, 100% arterial AMI), anticoagulants (n=50, 100%), oral antibiotics (n=49, 98%), and intravenous antibiotics (n=21, 42%). Emergency revascularization was performed in 29 patients (88% of arterial AMI patients).

Metabolomics

218 different metabolites were identified, including 97 and 127 through GC-MS and $^1\text{H-NMR}$ profiling, respectively. Some metabolites, such as glycerol, were identified through both GC-MS and $^1\text{H-NMR}$ profiling. The metabolites the most positively and negatively associated with a diagnosis of AMI in logistic regression (adjusted for age and sex) are shown in Table 14. A machine learning algorithm (fuzzy feature selection algorithm) including the 218 identified metabolites and age and gender was implemented in order to identify the metabolites, and panels thereof, that could best discriminate patients with AMI from control patients.

A panel of 13 metabolites (glutamine, phenylalanine, isomaltose, threose, 1-monoolein, TPA2, L3TG, glycerol, L1PN, L1TG, H4A2, L1AB, and L1PL) discriminating patients with AMI from control patients was thus selected by fuzzy logic machine learning algorithm applied on a training set (85% of the cohort). 8 of the 13 metabolites (glutamine, phenylalanine, isomaltose, threose, 1-monoolein, TPA2, glycerol, and H4A2) are among the metabolites the

most positively and negatively associated with a diagnosis of AMI in logistic regression presented in Table 14.

Individual comparisons for each of the 13 metabolites (Figure 22) show that the levels of glutamine (**2A**), threose (**2D**), TPA2 (**2F**) and H4A2 (**2K**) were lower in AMI patients than in control patients, while the levels of phenylalanine (**2B**), isomaltose (**2C**), 1-monoolein (**2E**), L3TG (**2G**), glycerol (**2H**), L1PN (**2I**), L1TG (**2J**), L1AB (**2L**) and L1PL (**2M**) were higher in AMI patients than in control patients.

The combination of 13 metabolites allowed the identification of patients with AMI with an AUC = 0.89 (Figure 23) in the training cohort (n=108), indicating an excellent diagnostic performance. Furthermore, Figure 23 shows that a lesser number of metabolites could actually be used to identify AMI patients with a very good diagnostic performance. For example, 1-monoolein alone allowed the identification of patients with AMI with an AUC = 0.8 in the training set (n=118). On the whole cohort (n=126), 1-monoolein, isomaltose and glutamine alone allowed the identification of patients with AMI with an AUC of 0.86 (95%CI=0.78 – 0.93), 0.85 (95%CI= 0.77 – 0.93) and 0.78 (0.70 – 0.86), respectively. After internal validation (cross-validation) performed on the 15% of the cohort (n=18), all cases of AMI were identified with an error rate of 0% (AUC = 1.00) (Figure 24). The unsupervised validation of the results on the data reduced to 13 biomarkers by principal component analysis and hierarchical clustering shows a clear separation of the two groups of patients (Figure 24B & C).

Early and late necrotic AMI subgroups

During the follow-up period, 16 of the AMI patients (34%) required a laparotomy, and 11 (23%) required bowel resection. Irreversible transmural intestinal necrosis was pathologically confirmed in 10 of the 11 resected cases. Three additional patients had undergone open-close procedures (i.e. without performing bowel resection) showing visible extensive dark necrosis

and were included in the late necrotic AMI subgroup (n=13). Patients with superficial and non-transmural necrosis (n=1), as well as those who recovered from AMI with no need for bowel resection (n=33) were included in the early AMI subgroup (n=34). As presented in Figure 25, plasma monoolein and isomaltose concentrations were significantly higher and plasma glutamine was significantly lower in both early and late necrotic AMI as compared to controls.

Experimental rat model of AMI

Histological results

No significant difference was found between Sham and AMI groups in terms of weight (395 g [371 - 415] vs. 405g [368 - 410]) and of small bowel length (96 cm [92 – 105] vs. 98 cm [94 – 105]). No significant correlation was found between weight, small bowel length and measured epithelial thickness and area.

The experimental design including 1 hour of ischemia and 1 hour of reperfusion led to significant but heterogeneous intestinal injury (Figure 26). The Chiu's score was significantly higher in the AMI group, and the highest on the ileum transverse sections (mean 2.9 ± 1.4 vs. 0.43 ± 0.5 in the Sham group) (Figure 30). Accordingly, we found lower epithelial surface and thickness on transverse and longitudinal histology quantitative analysis.

Metabolomics results

We focused the analysis on the three most discriminant metabolites identified from the human metabolome: monoolein, isomaltose and glutamine. Isomaltose was not detected in the plasma of both sham and AMI rats. No significant difference was found in monoolein and glutamine plasma concentrations in rats during ischemia and reperfusion and between sham and AMI groups. (Figure 27)

DISCUSSION

Using a combined analytical strategy comprising ¹H-NMR and GC-MS, clear metabolic differences in blood samples obtained from patients with AMI and controls with abdominal pain of another origin were identified based on amino-acid, carbohydrates and lipid metabolism. After multivariate and integrated machine learning analyses, we identified a combination of 13 plasma metabolites allowing the identification of AMI among patients with acute abdominal pain with an AUC of 0.89. Internal cross-validation confirmed excellent diagnostic accuracy.

A number of studies have sought to identify biomarkers specifically associated with AMI, with the aim of developing a reliable AMI diagnosis test for patients presenting with acute abdominal pain. However, the lack of specificity and sensitivity of available laboratory tests, the complexity of the small bowel metabolome involving endogenous and exogenous diet or microbiota-derived, their liver metabolism through the portal system, and the heterogeneity and relative rarity of the disease, explain why identifying clinically reliable biomarkers of early AMI has remained unsuccessful so far. In a preliminary analysis of the SURVIBIO cohort, we did not confirm the accuracy of the most promising candidate biomarkers of AMI highlighted so far by conventional targeted approaches – Citrulline, I-FABP, and D-lactate. This result strongly supported the use of non-targeted omics discovery approaches to identify new biomarkers. To our knowledge, this is the first human study providing a metabolomic signature of AMI. Interestingly, we highlighted three main biomarkers with excellent diagnostic accuracy as single tests –monoolein, a monoglyceride, glutamine, an amino-acid, and isomaltose, a disaccharide.

Monoolein plasma concentrations were significantly higher in AMI patients. Monoolein, or glyceryl monooleate, is a monoglyceride, an end product of the lipid digestion which can enter by simple diffusion in the enterocyte. Under physiological conditions, monoglycerides are

packaged into triglycerides and chylomicrons, and transported in lymph before reaching the systemic blood.(198) In the ischemic small bowel, experimental studies have shown that even a brief occlusion of the SMA led to a marked decrease in the lipid transport in lymph not explained by an impaired lymph flow or lipid absorption.(199) Instead, a marked increase in lipid absorption was observed through the portal blood. As a result of ischemia-reperfusion injury, increased epithelial permeability and impairment of the re-esterification of monoglycerides and packaging into chylomicrons may explain the increase in monoolein as well as free glycerol concentrations in plasma of AMI patients. Indeed, glycerol was the second main lipid significantly increased in AMI patients of our study. This is consistent with micro dialysis studies of animal intestinal ischemia-reperfusion model that observed early and specific rise of glycerol concentrations in gut lumen, gut wall and in the peritoneum fluid after mesenteric ischemia.(200-202)

Isomaltose is a disaccharide consisting of two glucose, similar to maltose, which is not absorbed in itself by enterocytes. In order to be absorbed, isomaltose must be split into its monosaccharide, glucose, by the brush border disaccharidases. As the activity of maltase/isomaltase is 10 times higher than that of lactase, widespread mucosal impairment is needed to cause decreased enzymatic activity, which has been shown in experimental mesenteric ischemia-reperfusion studies(203, 204) In a feline AMI model, the loss of maltase occurred considerably faster than the loss of some cytoplasmic enzymes.(205) Impaired maltase activity was proposed as an early indicator of small intestinal allograft rejection, before histological alteration in an animal model, and associated with an early decrease and delayed absorption of glucose.(206) In humans, unexpected large amounts of isomaltose were described in severely injured surgical patients, not correlated to glucose and other sugars.(207) Also, isomaltase gene expression was decreased in human intestinal epithelial cells by intestinal inflammatory cytokines.(208) In an experimental study of 26 patients undergoing a

cardiopulmonary bypass, significant intestinal hypoperfusion and associated malabsorption of monosaccharides and increased urine concentrations of non-absorbable disaccharide lactulose were observed. (209) Therefore, the increase in isomaltose plasma concentrations in AMI patients may be explained by an early brush-border enzymes activity loss leading to monosaccharides malabsorption and increased isomaltose portal blood absorption as a result of increased epithelial permeability. This is also consistent with our observation of a decrease in monosaccharides plasma levels such as threose.

Glutamine is the most abundant amino acid in the body and the amino acid most affected by catabolic diseases. Low plasma glutamine concentrations have been observed in patients with pancreatitis, burns, sepsis and multiple organ failure and attributed to intense glutamine depletion.(210) In critically ill patients, low plasma glutamine is an independent risk factor for mortality, associated with shock and older patients.(210) As a result, glutamine has been considered an essential amino acid in critically ill patients and glutamine supplementation a beneficial intervention.(211) During experimental intestinal ischemia-reperfusion, a reduction of enterocytes uptake of glutamine and lower plasma levels have been observed. (212, 213) as Also, glutamine is the substrate for gut synthesis of citrulline, a validated marker for gut mass and function in short bowel syndrome.(189) Accordingly, low plasma Citrulline concentrations have been observed in critically ill patients and AMI patients, but with low sensitivity in the diagnosis of AMI.(131, 190) Interestingly, in a preliminary analysis of the present human cohort, we found significantly lower plasma citrulline concentrations in AMI patients, with insufficient AUROC value however. We suggest here an enhanced diagnostic value of low plasma glutamine in the diagnosis of AMI.

Several potential limitations of this study should be discussed. Although promising, our results were obtained in a small single-center sample of AMI patients and an external validation study is required. However, our cohort represents an important sample of prospective and well-

defined AMI patients, with an important proportion of patients included at an early stage thus allowing for the discovery of early biomarkers of AMI. In this study, we were unable to assess the kinetics of the diagnostic metabolites identified given patients were collected a single timepoint (admission), and given our findings were not confirmed on the experimental rat model. However, human findings are obviously more generalizable than animal models and our experimental rat design, although standardized, was not comparable to the human study (sham controls, duration of ischemia...). This is consistent with the few metabolome studies of intestinal ischemia performed in animals, which did not highlight the same combination of discriminant metabolites as ours, but which did not include an acute abdomen control group thereby neglecting to assign specific metabolic features to AMI.(139, 214-216) Besides, independent studies of the same disease in different patient groups do not always reveal the exact same biomarker patterns.

In conclusion, we identified a combination of 13 plasma metabolites from ¹H-NMR and GCMS metabolomics, allowing the identification of AMI among patients with acute abdominal pain with excellent diagnostic accuracy. Although it seems clear that metabolic profiling can identify distinct differences between patients with AMI and patients with abdominal pain of another origin, strong validation study is required.

Table 12: Baseline characteristics of AMI patients and controls.

	AMI patients n=47 (%)	Controls n=79 (%)	<i>p-value</i>
Age, years ¹	65 (55-74)	46 (35-71)	< 0.001
BMI, kg/m ² ¹	27 (22-33)	22 (20-24)	< 0.001
Female	18 (38)	31 (39)	0.92
<i>Atherosclerosis risk factors</i>			
Tobacco use	21 (45)	16 (20)	0.004
Arterial hypertension	27 (57)	20 (25)	0.001
Dyslipidemia	17 (36)	12 (15)	0.007
Diabetes mellitus	12 (26)	5 (6)	0.002
<i>Cardiovascular history</i>			
Myocardial ischemia	9 (19)	5 (6)	0.03
Stroke	6 (13)	5 (6)	0.33
Limb ischemia	9 (19)	2 (3)	0.002
Atrial fibrillation	10 (21)	3 (4)	0.004
Deep vein thrombosis	3 (6)	4 (5)	1.00
Pulmonary embolism	5 (10)	4 (5)	0.29
<i>Other comorbidities</i>			
Chronic kidney disease	1 (2)	2 (3)	1.00
Cirrhosis	4 (8)	4 (5)	0.47
Abdominal surgery	24 (51)	37 (47)	0.65
<i>Clinical features</i>			
Temperature ¹	37.0 (36.3-37.2)	36.8 (36.5-37.5)	0.49
Mean arterial pressure ¹	100.3 (91.7-110.2)	95.7 (83.7-105.7)	0.17
Vomiting	19 (40)	39 (49)	0.33
Diarrhea	12 (24)	13 (17)	0.22
Hematochezia	8 (16)	3 (4)	0.02
Guarding	14 (30)	13 (17)	0.08
Organ dysfunction (total SOFA \geq 2)	13 (30)	8 (10)	0.01

Abbreviations: AMI: acute mesenteric ischemia; BMI: body mass index; SOFA: sequential organ failure assessment; ASAT: aspartate aminotransferase

¹ Median (Inter Quartile Range)

Table 13. Etiological diagnosis of the control group with acute abdominal pain.

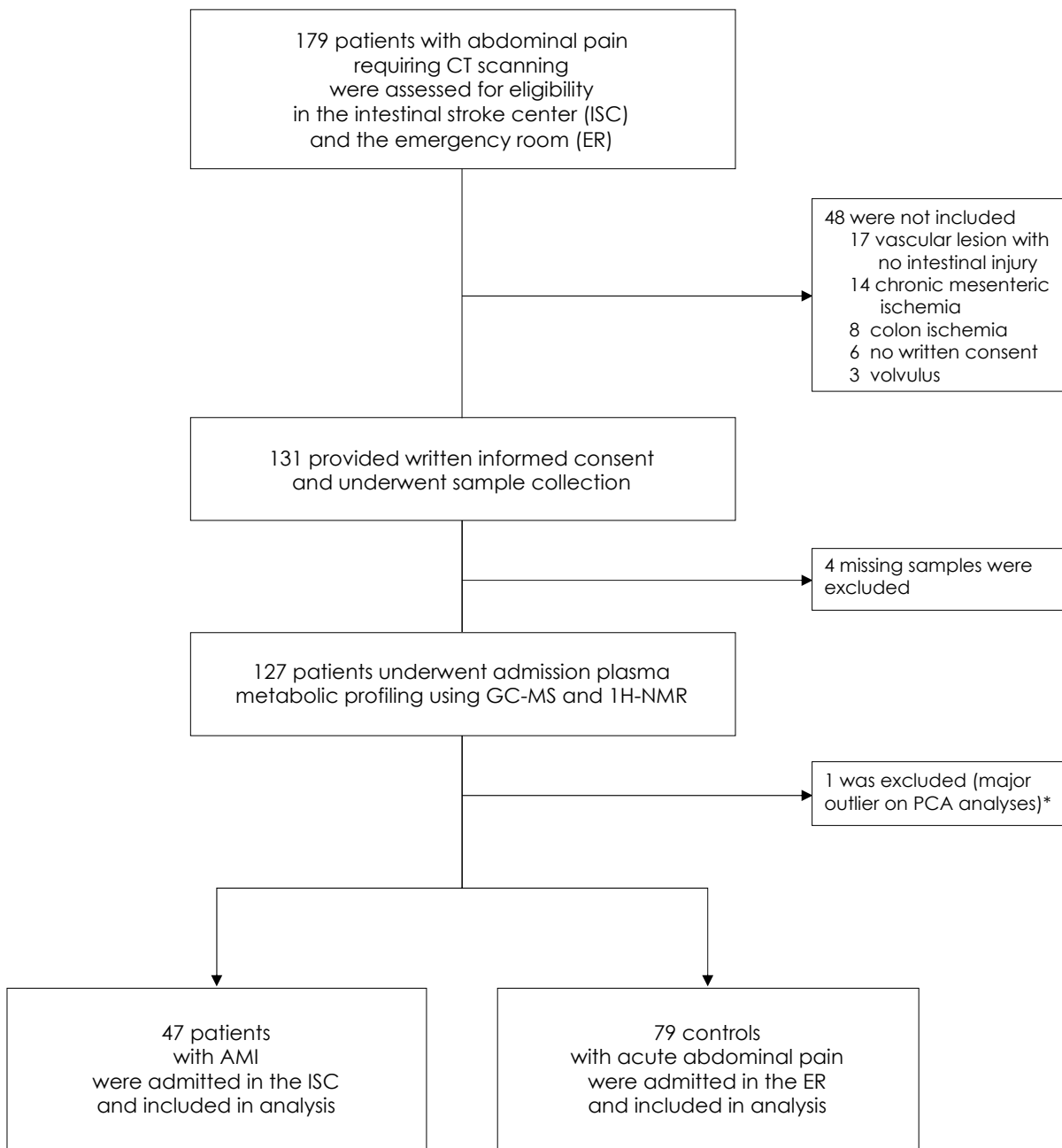
Diagnosis	n=79 (%)
Infectious	18 (23)
<i>Diverticulitis</i>	9
<i>Appendicitis</i>	4
<i>Other causes of peritonitis/abdominal abscess</i>	5
Inflammatory	15 (19)
<i>Intra-abdominal neoplasm progression</i>	8
<i>IBD flares</i>	7
Bowel obstruction (non-strangulated)	13 (16)
Functional G.I. disorders (reflux, diarrhea, constipation...)	12 (15)
Biliopancreatic tract	10 (13)
<i>Pancreatitis</i>	7
<i>Biliary complications</i>	3
Urogenital	10 (13)
Invasive meningococcal disease	1 (1)

Abbreviations: IBD: inflammatory bowel disease; G.I.: gastro-intestinal

Table 14. Acute mesenteric ischemia related plasma metabolites based on multivariate logistic regression (age and sex adjusted)

Compound	Estimates	p-values	adjusted p-values
GC-MS metabolites			
1-monoolein	4.70389	1.33 e-06	1.08 e-04
alpha-tocopherol	-2.5361	1.41 e-05	4.54 e-04
isomaltose	1.640267	3.98 e-05	8.06 e-04
2-hydroxybutyric acid	1.818458	6.24 e-05	1.01 e-03
threose	-2.50963	0.000140173	1.80 e-03
D-allose	1.669573	0.000155207	1.80 e-03
2-ethylhexanoic acid	-1.70034	0.000560675	5.68 e-03
threonic acid	-1.20665	0.001608285	1.45 e-02
palmitic acid	-1.667887	0.001943953	1.57 e-02
succinic acid	2.02738	0.003143423	2.12 e-02
oleic acid	1.273331	0.002962736	2.12 e-02
quinic acid	-0.62265	0.003841036	2.39 e-02
pyruvic acid	-1.23496	0.00472627	2.56 e-02
phosphoric acid	-1.9044	0.004748942	2.56 e-02
cholesterol	-2.83442	0.005787982	2.76 e-02
2-ketoisocaproic acid	-1.56142	0.006613241	2.86 e-02
glyceric acid	-1.40387	0.009348605	3.79 e-02
¹H-NMR metabolites			
glutamine	-7.42315	1.75 e-05	6.67 e-04
glycerol	6.66567	1.36 e-05	6.67 e-04
H4A2 (ApoA2-HDL4)	-0.1968	2.56 e-05	6.67 e-04
H4PL (Phospholipids-HDL4)	-0.16603	2.90 e-05	6.67 e-04
HDA2 (ApoA2-HDL)	-0.1898	2.28 e-05	6.67 e-04
TPA2 (ApoA2)	-0.17824	1.45 e-05	6.67 e-04
H4A1(ApoA1-HDL4)	-0.05513	5.03 e-05	9.75 e-04
H4CH (Cholesterol-HDL4)	-0.16939	5.65 e-05	9.75 e-04
H3FC (Free cholesterol-HDL3)	-1.11921	0.000184702	2.83 e-03
H4FC (Free cholesterol-HDL3)	-0.6547	0.000296495	3.72 e-03
HDA1 (ApoA1-HDL)	-0.02764	0.000475334	5.05 e-03
TPA1 (ApoA1)	-0.02888	0.000442939	5.05 e-03
L6FC (Free cholesterol-LDL6)	-0.31397	0.000637088	6.28 e-03
L5PL (Phospholipids-LDL5)	-0.19109	0.00069921	6.43 e-03
L5CH (Cholesterol-LDL5)	-0.10191	0.000781915	6.74 e-03
H3A2 (ApoA2-HDL3)	-0.58864	0.000967893	7.81 e-03
L5FC (Free cholesterol-LDL5)	-0.36079	0.001018216	7.81 e-03
H3PL (Phospholipids-HDL3)	-0.26557	0.001592942	1.06 e-02
L5AB (ApoB-LDL5)	-0.13923	0.001620641	1.06 e-02
L5PN (LDL5 particule number)	-0.00766	0.001618221	1.06 e-02
L6PL (Phospholipids-LDL6)	-0.15294	0.001870843	1.17 e-02
acetic acid	18.27703	0.002741432	1.40 e-02
phenylalanine	19.97602	0.002342867	1.40 e-02

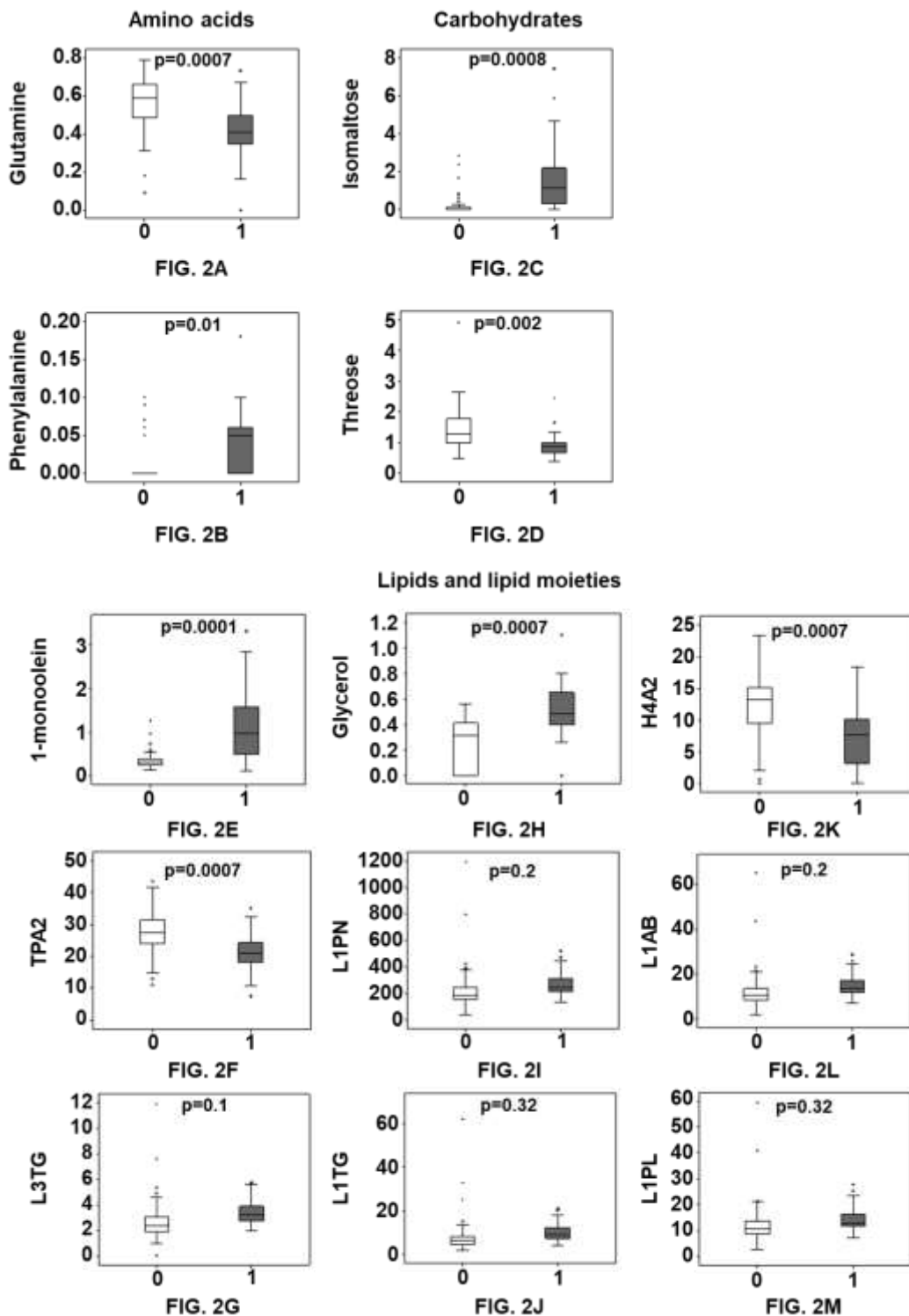
Figure 21. Flowchart of AMI patients and controls in the metabolomics study: screening and selection



Abbreviations: AMI: acute mesenteric ischemia; CT: contrast-enhanced computed tomography; GC-MS: gas chromatography-mass spectrometry; ¹H-NMR: hydrogen-1 nuclear magnetic resonance spectroscopy; PCA: principal component analysis

*One AMI patient was excluded due to a hard outlier metabolic signature on all PCA plots (see supplemental data) corresponding to an extreme and late clinical presentation of AMI. The patient died the day after admission.

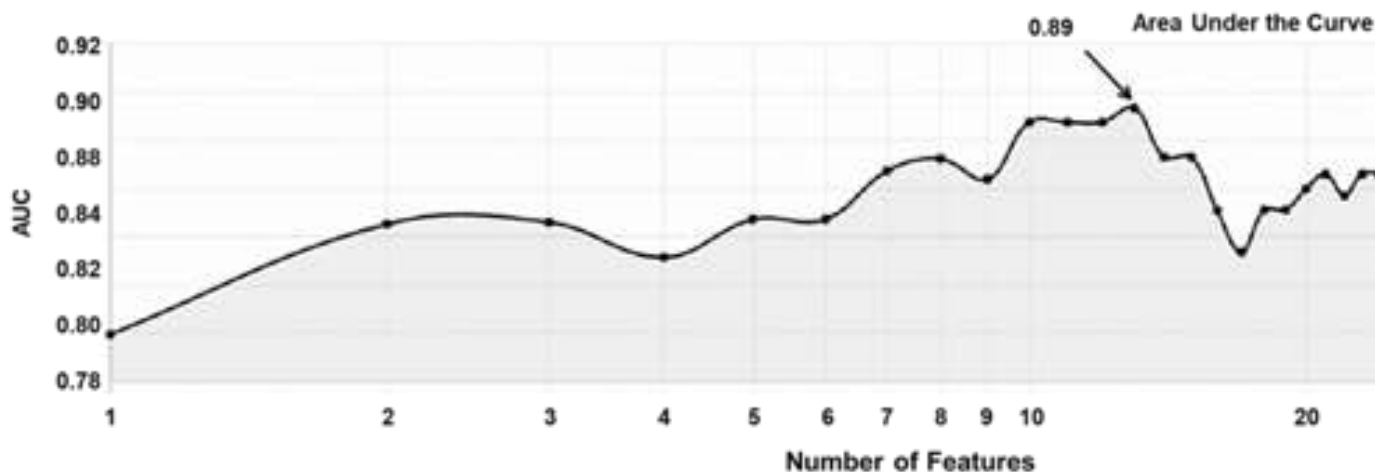
Figure 22: Acute mesenteric ischemia related plasma metabolites



0= controls; 1=acute mesenteric ischemia patients. P-values are adjusted on age and sex, and corrected for false discovery rate (Benjamini-Hochberg). The horizontal line in the boxes represents the median, and the bottom and top of the boxes the 25th and 75th percentiles, respectively. I bars represent the upper adjacent value (75th percentile plus 1.5 times the interquartile range) and the lower adjacent value (corresponding formula below the 25th percentile), and the dots/asterisks outliers.

Figure 23: Model accuracies according to the number of metabolites on LAMDA integrative analysis

Graph showing the model accuracies according to the number of metabolites on LAMDA integrative machine learning analysis applied on the 85% of the cohort (n=108). Best AUC / number of features ratio was obtained with the 13 metabolites and their corresponding weight listed in the table.



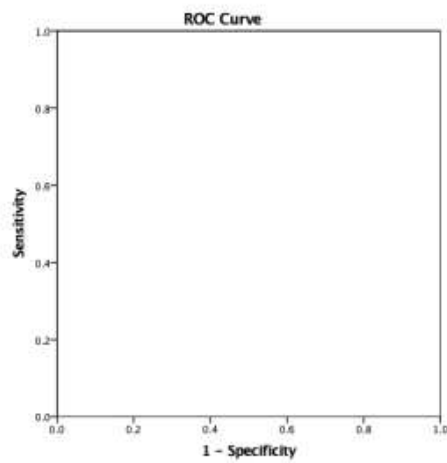
Rank	Metabolites	Weight in the machine learning model
1	Monoolein	1.00
2	Isomaltose	0.72
3	Glutamine	0.66
4	Phenylalanine	0.57
5	Glycerol	0.57
6	L1PN (LDL1-particule number)	0.56
7	L1AB (ApoB-LDLA)	0.56
8	L3TG (Triglycerides-LDL3)	0.55
9	Threose	0.55
10	H4A2 (ApoA2-HDL4)	0.53
11	L1TG (Triglycerides-LDL1)	0.53
12	TPA2 (ApoA2)	0.51
13	L1PL (Phospholipids-LDL1)	0.50

Figure 24: Validation studies of the diagnostic model reduced to 13 metabolites

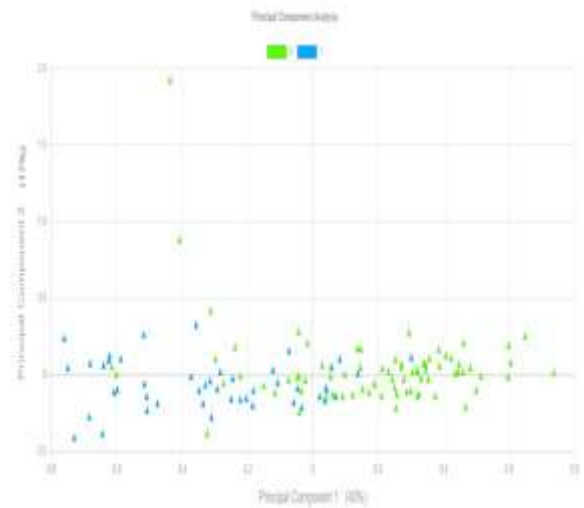
On the validation cohort (n=18) : AUROC (A)

On the whole cohort (n=126) : Principal component analysis (B) and Hierarchical clustering (C)

A



B



C

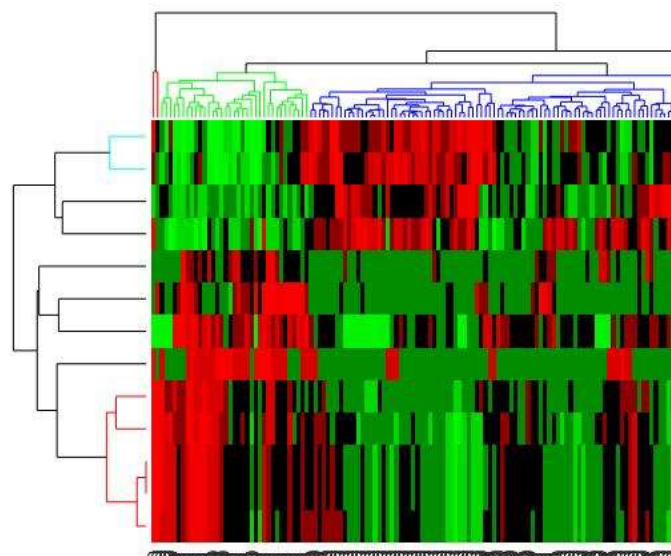
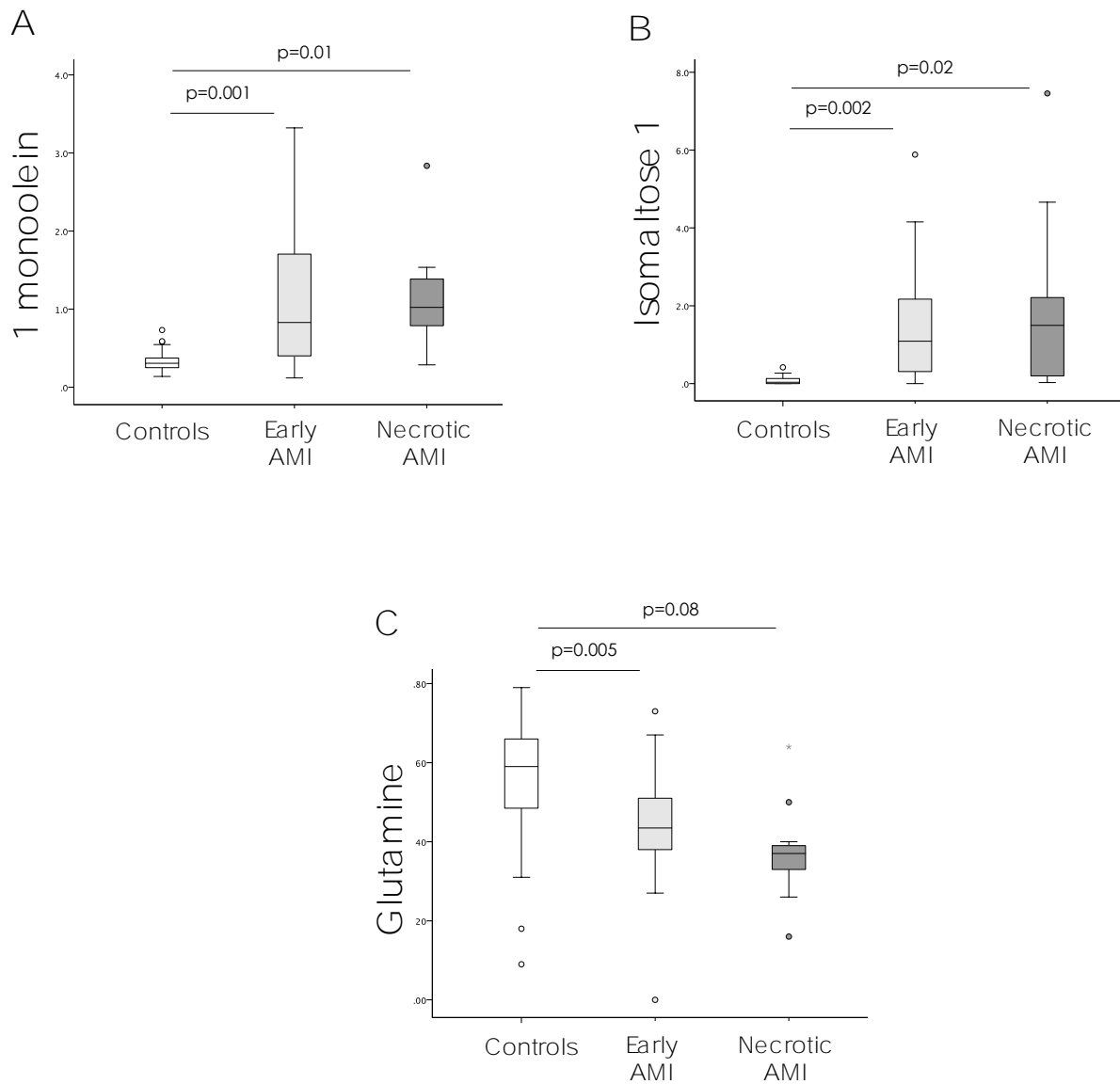
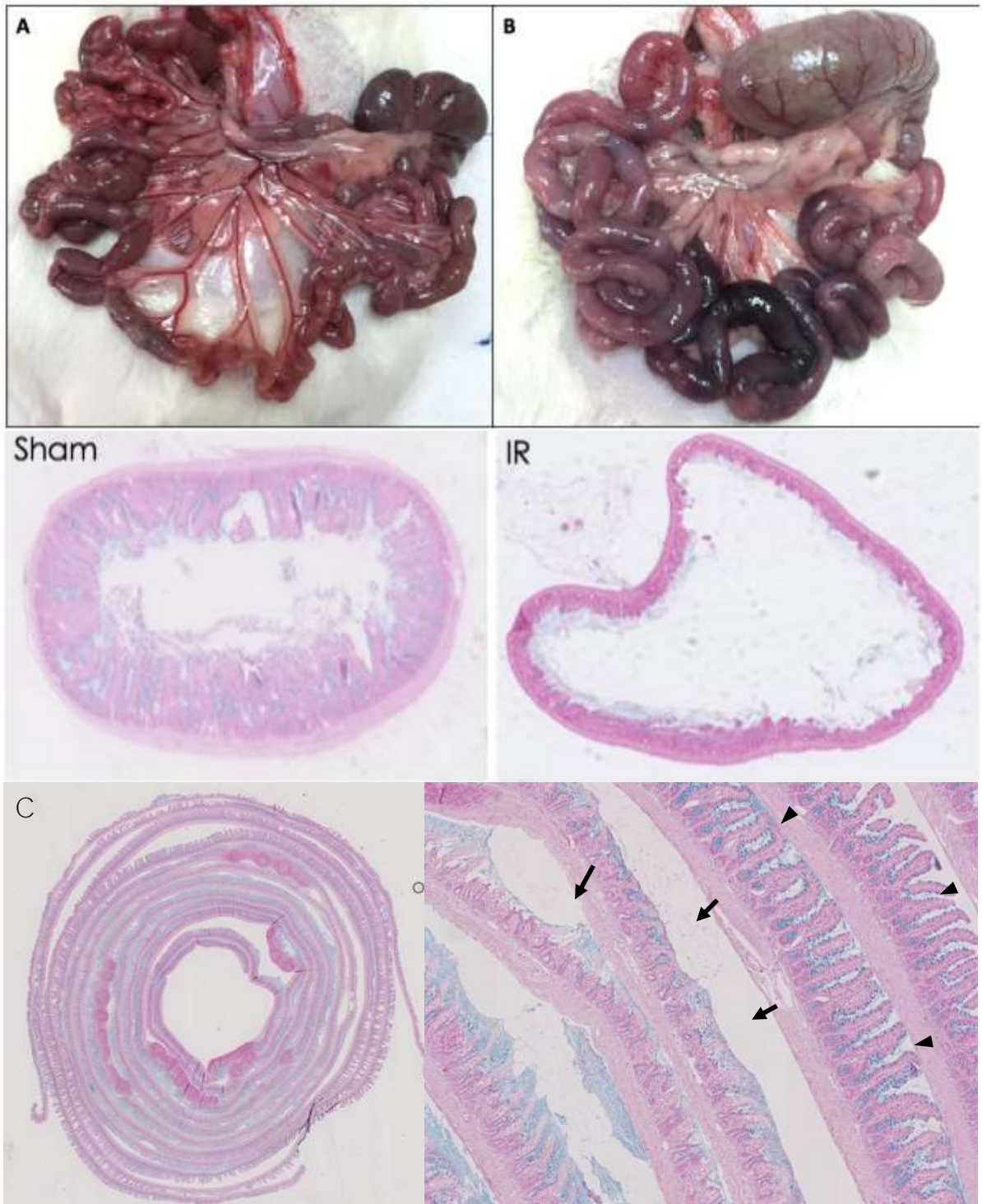


Figure 25. Plasma metabolites concentrations according to the severity of acute mesenteric ischemia: early (n=34) or late necrotic (n=13)



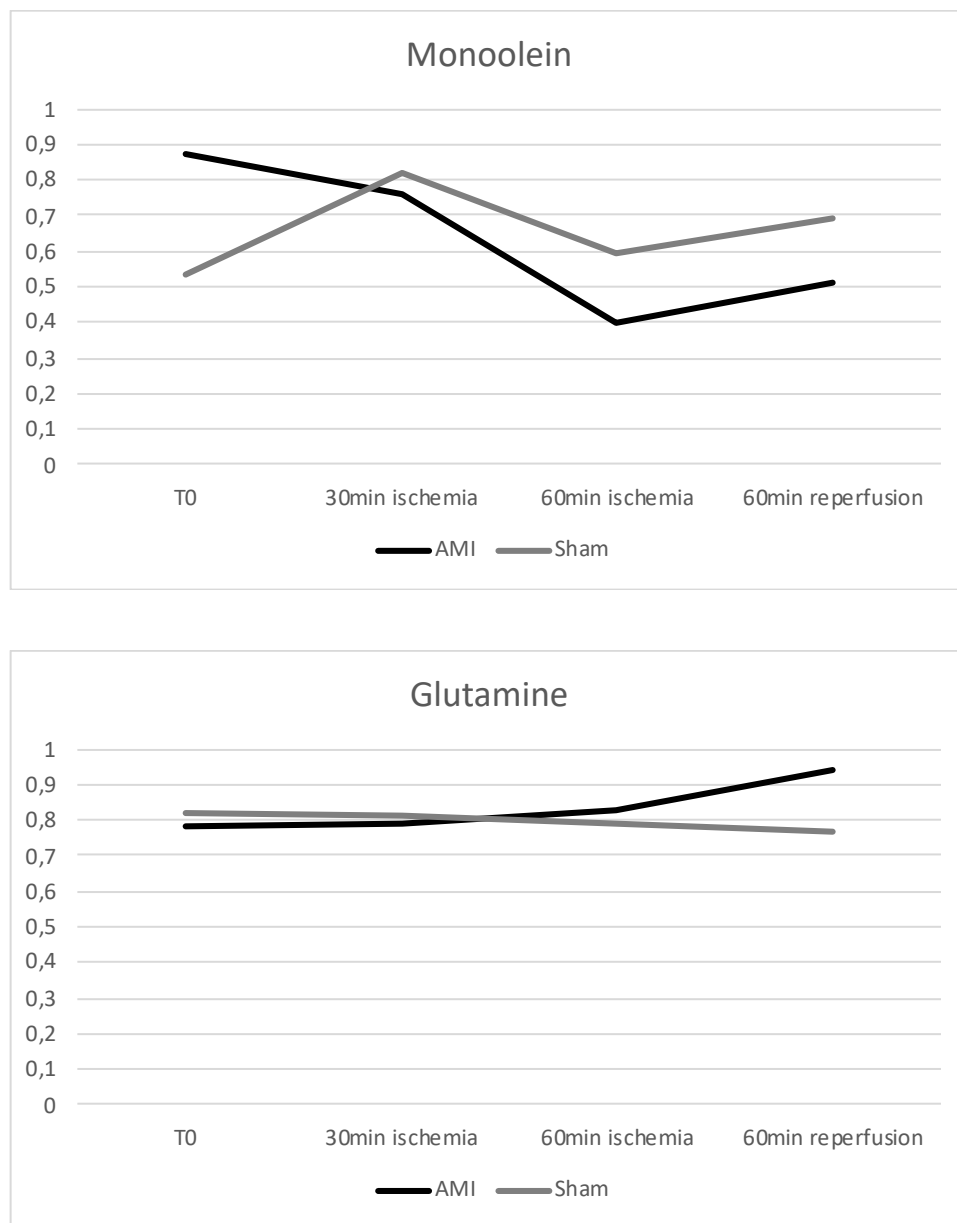
Abbreviations: AMI: acute mesenteric ischemia; P-values are adjusted on age and sex, and corrected for false discovery rate (Benjamini-Hochberg). The horizontal line in the boxes represents the median, and the bottom and top of the boxes, the 25th and 75th percentiles, respectively. I bars represent the upper adjacent value (75th percentile plus 1.5 times the interquartile range) and the lower adjacent value (corresponding formula below the 25th percentile), and the dots/asterisks outliers.

Figure 26. Macroscopic and microscopic evaluation of intestinal damage avec ischemia and reperfusion injury in the rat model.



Macroscopic view of normal small bowel loops (A) and ischemic dilated and necrotic loops (B), confirmed by transverse and longitudinal histological analysis. Longitudinal swiss-roll analysis (C) highlights the heterogeneity of ischemic intestinal injury all along the small bowel length with subnormal histological villi (arrowheads) next to areas of mucosal necrosis (arrows).

Figure 27. Monoolein and glutamine concentrations in the experimental rat model after 1 hour of ischemia and 1 hour of reperfusion



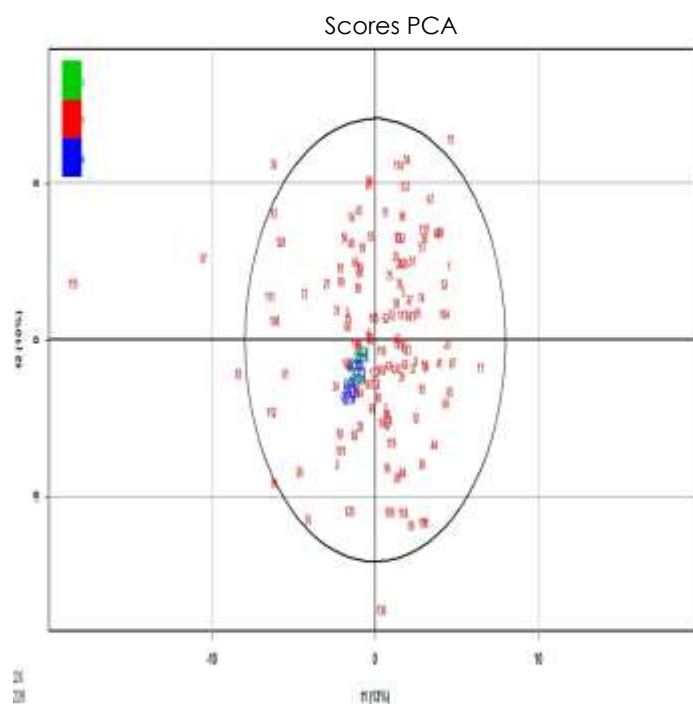
No significant difference in monoolein and glutamine concentrations were found during ischemia and reperfusion and between sham operated and AMI animals. Abbreviations: AMI: acute mesenteric ischemia

SUPPLEMENTAL DATA:

Figure 28 : Principal component analyses on GC-MS (A) and 1H-NMR (B) metabolomics data

Patient #115 on GCMS PCA scores and #210 on NMR NOESY PCA scores correspond to the same AMI patient and major outlier, which was removed from the final analysis. The AMI patient was an 88-year-old woman who presented on admission with shock, acute kidney injury, elevated plasma lactate at 5mmol/L and died from massive intestinal infarction at 48 hours despite superior mesenteric artery stenting.

(A) GC-MS data PCA scores plot



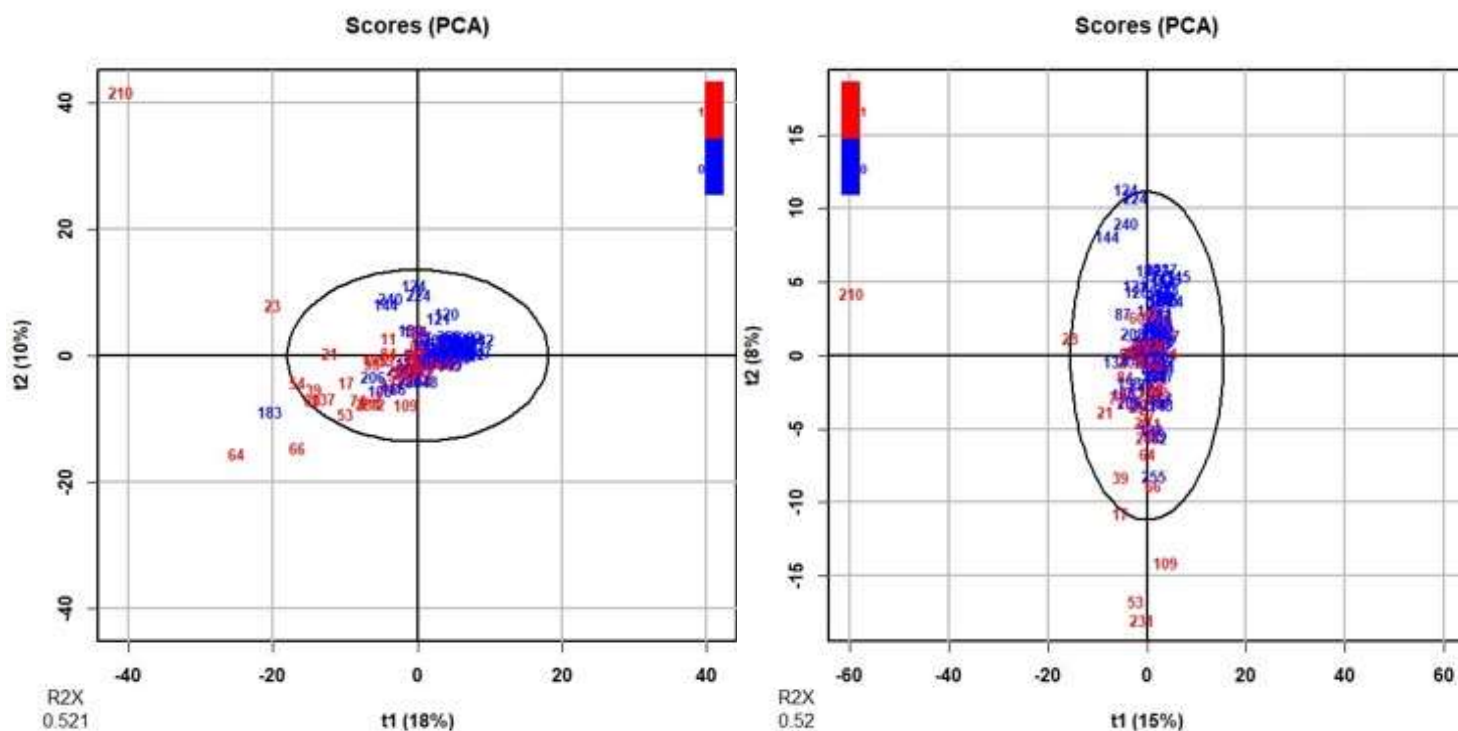
On the GC-MS data PCA scores plot, 1 (red) points correspond to the samples, and the 0 (blue) and 2 (green) points correspond to quality control (QC) samples.

(B) ¹H-NMR data PCA scores plot

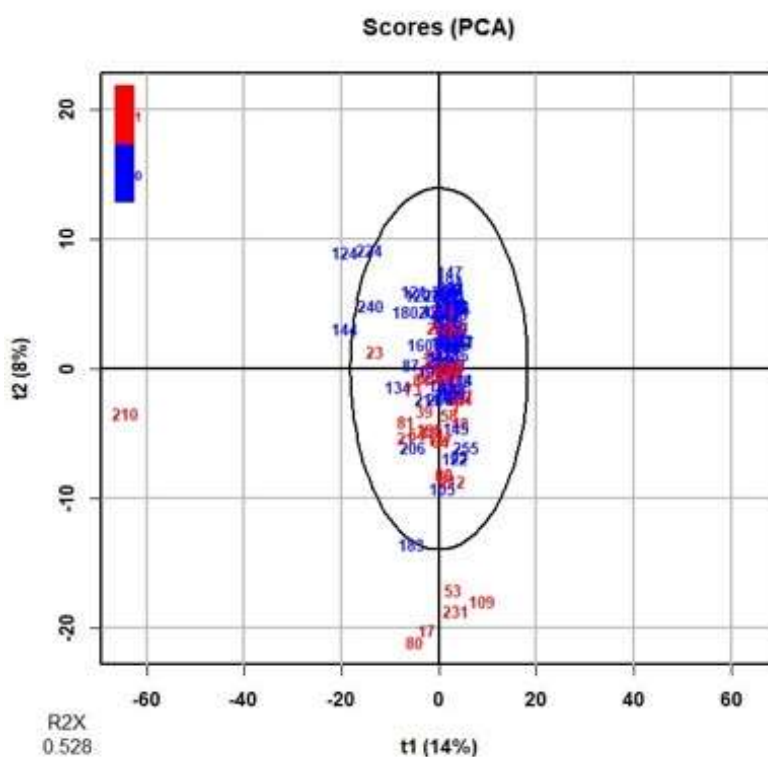
On the NMR data PCA scores plot, 1 (red) points correspond to the AMI patients samples, and the 0 (blue) to controls samples. Data were normalized (PQN) and dimensionality reduced using SRV

NOESY spectra data

CPMG spectra data



J-RES spectra data



Supplemental data: experimental rat model of ischemia and reperfusion

Methods

The procedure and animal care complied with principles formulated by the National Society for Medical Research (animal facility agreement: n° B75-18-03, experimentation authorization n° 75-101, APAFiS#8724). Eight-week-old male Wistar rats were purchased from Janvier laboratory. Rats were randomized into 2 groups: an ischemia/reperfusion group (AMI group, 8 rats) and a sham-operated group (Sham group, 7 rats). Rat body temperature was maintained at 37.5°C by a heated surgical table. Non-fasting rats were anesthetized by intraperitoneal injection of 65 mg/kg of pentothal. A cannulation of the right jugular vein was performed for saline infusion and the collection of blood samples. After a laparotomy, the superior mesenteric artery (SMA) was exposed but not occluded in the sham group. In the AMI group, the SMA was clamped. The clamp was removed after 60 min of ischemia, followed by 1 hour of reperfusion. Blood samples were collected in heparin-treated tubes before the laparotomy, 30 min and 60min after clamping the SMA, and before sacrifice after 1 hour of reperfusion. Samples were centrifuged for 30 min at 16,000 g. Plasma supernatants were aliquoted and stored at -80°C for further metabolomic analyses. All rats were euthanized just before the small intestine was collected, measured in length and fixed in formalin for histological analysis. Three small bowel 1cm-segments were resected for transversal histological analysis (proximal jejunum, mid-gut and distal ileum). The two long remnant segments of small bowel were cut on the mesenteric side and swiss-rolled before fixation in formalin as previously described, (217) in order to allow longitudinal histological analysis. (Figure 29) Highly glycosylated mucins and cores were respectively stained in Alcian blue and in nuclear fast red on paraffin-embedded sections of small intestine. Histological evaluation was performed using the grading system described by Chiu et al. (see Table 15). A morphometric evaluation of histological gut

sections was then performed using QWin software to determine the luminal area, epithelial layers areas and thickness on transverse and longitudinal sections (see table).

Table 15. Modified Park/Chiu grading system of intestinal ischemia/reperfusion injury (218)

Grade	Histological findings
0	Normal
1	Subepithelial edema, partial separation of apical cells
2	Epithelial cell sloughing from tips of villi
3	Progression of sloughing to base of villi
4	Partial mucosal necrosis of lamina propria
5	Total mucosal necrosis

Figure 29. Morphometric analysis of the intestinal wall

Histological gut sections were scanned by a Nanozoomer (A) and analysed using QWin software (B). The luminal area (in blue) was defined by delimiting the space inside the intestinal ring whose external border was limited by the epithelium. The muscular layer (in red) was defined as extending from the basal side of the epithelial layer to the external border of the intestinal ring. The epithelial layer (in green) was determined by drawing the cell layer between the muscular layer and the external border of the luminal surface. The normalized epithelial thicknesses was obtained by dividing the epithelial area by the external border of the intestinal ring.

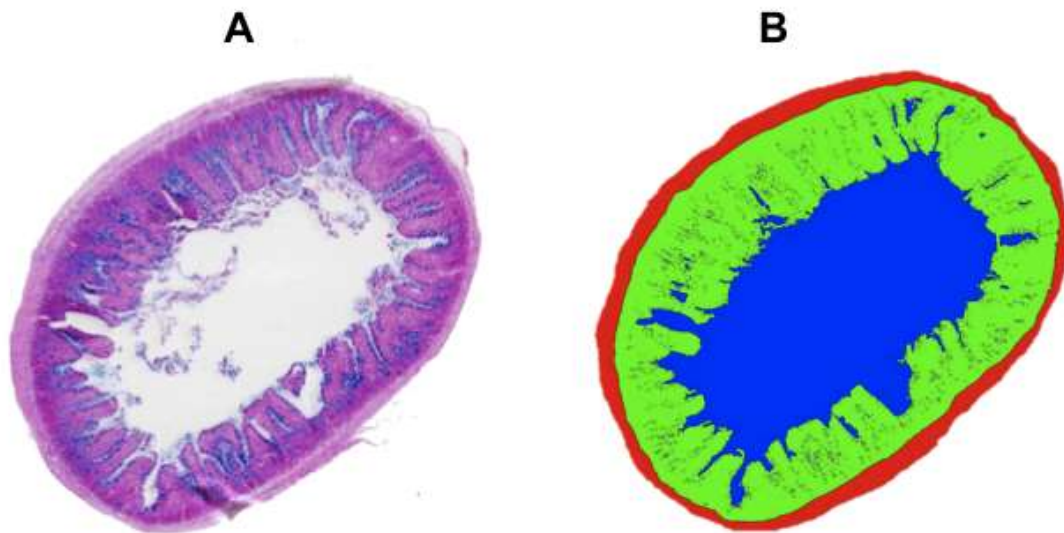
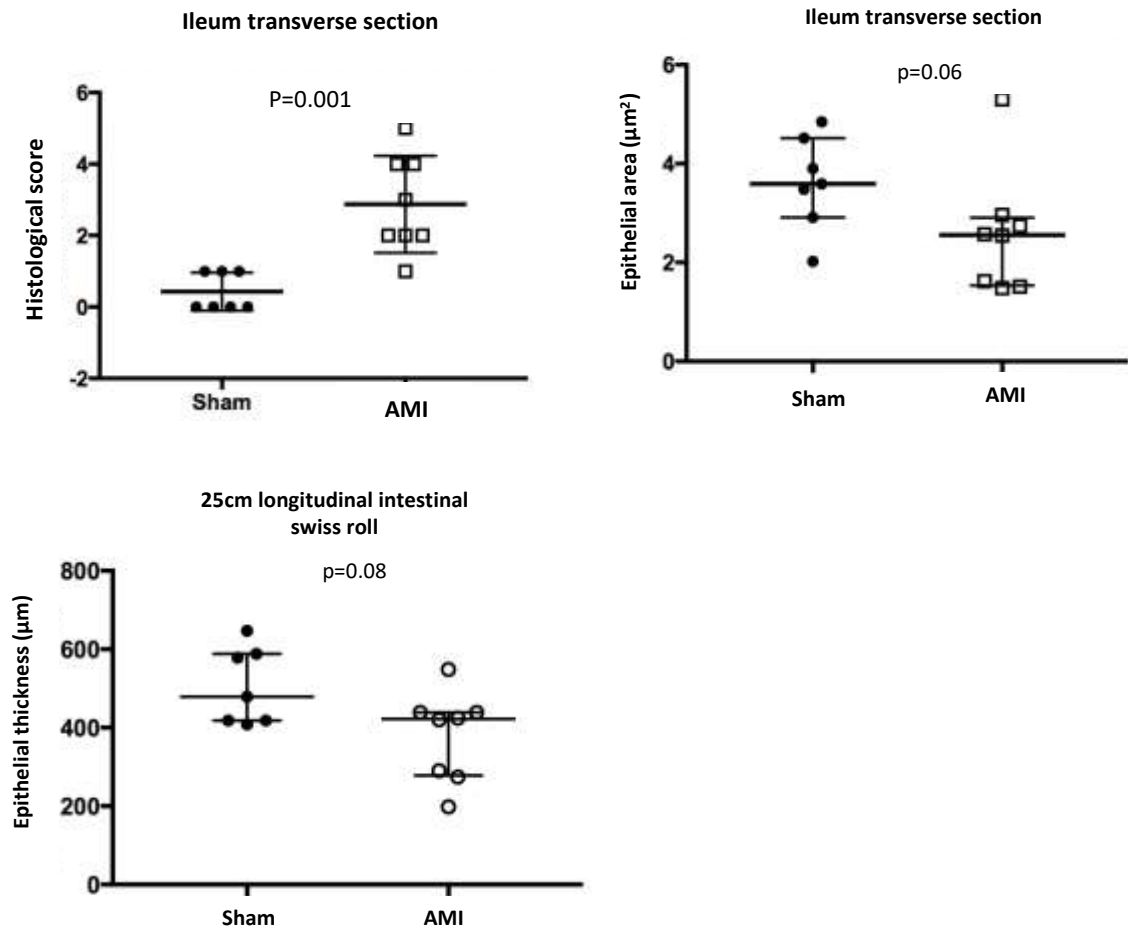


Figure 30. Qualitative and quantitative histological analysis

AMI group had significantly higher intestinal damage as assessed by transverse and longitudinal (swiss-roll) sections histology (Park/Chiu score) and quantitative morphometric analysis (epithelial area and thickness)



Chapter 5

Concluding remarks and thoughts

Among acute abdominal pain patients, there is currently no biological test to guide the diagnosis of AMI and the need for an abdominal CT angiography. Such a test allowing for an early diagnosis, ruling out the diagnosis, or identifying patients requiring an abdominal CT angiography among acute abdominal pain patients would have a major impact on the prognosis of AMI patients. In the present thesis, we made significant steps forward in the search for new biomarkers of AMI. This was possible through a unique multidisciplinary and translational collaboration combining the expertise of 1) metabolomics by the expert leading team of Imperial College London - UK, and 2) acute mesenteric ischemia by the clinical and experimental research teams of the Beaujon-Bichat hospital group (SURVI - Structure d'Urgences Vasculaires Intestinales, INSERM 1148) and the use of their unique biobank from prospective AMI patients and abdominal pain controls recruited in the intestinal stroke unit.

The analysis of prospectively collected metadata on clinical and biological presentation of AMI allowed the identification of three independent features of an AMI-related abdominal pain: its sudden onset, its intensity requiring opioids, and its association to elevated plasma C-reactive proteins levels. These findings may be useful to improve clinical recognition of the disease while awaiting for the validation of new biomarkers. The comparison of admission blood samples from patients and controls allowed to invalidate the use of plasma citrulline, I-FABP and D-lactate as early diagnostic tests for AMI, consistent with decades of conflicting results.(91, 94, 129, 175) Moreover, these negative results further supported our non-targeted metabolomics approach to identify new potential diagnostic biomarkers of the disease. Using a complementary analytical strategy including ¹H-NMR and GC-MS and multivariate and

integrated statistical we identified a panel of plasma amino-acids, carbohydrates and lipid moieties highly associated to the AMI phenotype with excellent diagnostic accuracy. These new biomarkers also open new avenues of research on the pathophysiology and treatment of AMI.

However, these promising results now require strong external validation. However, to our knowledge, no other team is currently prospectively including and collecting biological samples from these patients as the implementation of intestinal stroke centers is very new and unique so far. Meanwhile, we plan to validate our results in the independent temporal cohort of the 167 patients included and plasma collected in our center after the present cohort, i.e., between the 6th March 2018 and 1st October 2020 (71 AMI patients + 96 controls). A new metabolomic analysis targeted on the selected panel of biomarkers will allow to validate and refine the performance of this combination. Clinical translation will then require taking into account the possibility of quickly measuring these biomarkers for emergency medical use, which were measured by GC-MS and ¹H-NMR but may be quantified by other techniques (spectrometry, immunological...) more easily implantable in general hospital laboratories. Several adaptations may be necessary to make the assay faster and more convenient without much impact on sensitivity or specificity. A major point in the final selection of biomarkers is the priority given to a high negative predictive value in this disease. These plasma biomarkers could be used alone or in a weighted combination (score) to improve their diagnostic performance. It is also possible to envisage integrating them into an algorithm taking into account other readily available known clinical or biological parameters.

While the number of emergency consultations is constantly increasing, acute abdominal pain is the first cause of emergency consultation in the US and Europe. (219, 220) In 2014 in the US, 138 million patients consulted the emergency room, 12% of which for acute abdominal pain. (220, 221) This primary reason for consultation still poses a real diagnostic challenge for

emergency physicians. Indeed, abdominal pain can reveal a mild illness as well as a life-threatening emergency such as AMI. In this high-risk setting, it was reported that 70 to 90% of patients consulting for acute abdominal pain had a blood tests in the emergency room.(219, 222) The availability of new diagnostic biomarkers for AMI might change the clinical picture of AMI and the prognosis of this condition in all its clinical applications, which are by nature translational and multidisciplinary (emergency physicians, intensivists, gastroenterologists, vascular and digestive surgeons, internists, pharmacists, researchers). Eventually, the application of these biomarkers could be generalized to the assessment and management of any acute abdominal pain in the emergency room, and their place could be of major importance in tomorrow's emergency medicine.

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APPENDIX

RÉSUMÉ SUBSTANTIEL EN FRANÇAIS

1. RATIONNEL

1.1. L'ischémie mésentérique aiguë, un pronostic inchangé depuis des décennies

L'ischémie mésentérique aiguë (IMA) est une urgence vitale, médico-chirurgicale, vasculaire et digestive. Elle peut être secondaire à une occlusion splanchnique artérielle ou veineuse, aux situations de bas débit splanchnique, à un clampage vasculaire en chirurgie aortique ou à une ischémie froide dans le cadre de la greffe intestinale (1, 4). L'évolution vers l'infarctus digestif et ses complications vitales est imprévisible et grevée d'une mortalité de 30 à 90% ou de lourdes séquelles intestinales en cas de survie (1). Cette évolution est principalement liée à un retard thérapeutique en l'absence de critères diagnostiques cliniques ou biologiques assez sensibles et spécifiques.(4)

Le syndrome d'IR mésentérique associe une composante vasculaire d'activation endothéliale et neutrophilaire et une composante digestive d'ischémie épithéliale. L'hypoperfusion de la muqueuse intestinale est responsable d'une desquamation précoce hypoxique des villosités intestinales. La rupture de la barrière épithéliale conduit à l'interaction du microbiote intestinal avec le système immunitaire sous-muqueux, stimulant les voies locales puis systémiques de l'immunité (223). Les polynucléaires neutrophiles, acteurs lésionnels majeurs, adhèrent et migrent dès l'ischémie pour assurer la détersion du tissu en cours de nécrose ischémique. L'absence d'un rétablissement rapide de la perfusion tissulaire conduit à une nécrose intestinale

irréversible et transmurale à l'origine des complications létales de l'IMA.(4) Cependant, la réoxygénation brutale de la muqueuse digestive peut également et paradoxalement aggraver les lésions épithéliales et vasculaires et leur retentissement systémique par un mécanisme d'explosion oxydative provoquant l'afflux et la mort des polynucléaires neutrophiles avec expulsion de NETs (neutrophil extracellular traps) et sécrétion du contenu, notamment granulaire (28, 224).

Notre équipe à l'Hôpital Beaujon a pu établir que, reconnue et traitée à un stade précoce, l'IMA est pourtant une maladie potentiellement totalement réversible.(2, 53) A ce stade, la présentation clinique est dominée par une douleur abdominale aiguë aspécifique sans autre caractéristique clinique ou biologique discriminante.(1) En outre, il n'existe à ce jour aucun biomarqueur assez sensible et spécifique du diagnostic,(1, 94) qui requiert un haut degré de suspicion clinique et sa confirmation par un angioscanner abdominal objectivant des signes d'insuffisance vasculaire splanchnique et de souffrance intestinale.(41) En pratique, les performances diagnostiques du scanner sont dépendantes de la probabilité clinique pré-test.(42) Il est donc crucial pour le clinicien de disposer de marqueurs permettant devant toute douleur abdominale, d'affirmer ou d'éliminer le diagnostic. Ainsi, et en dépit des nombreux progrès thérapeutiques réalisés dans les domaines de la chirurgie vasculaire et de la radiologie interventionnelle, la mortalité de l'IMA demeure inchangée depuis des décennies.(225)

1.2. Les biomarqueurs : un enjeu pronostique essentiel

L'amélioration du pronostic et du traitement de l'IMA nécessite l'identification de biomarqueurs diagnostiques sensibles et spécifiques du stade précoce et réversible de l'affection.(226) A ce jour, les approches analytiques conventionnelles n'ont pas permis de répondre à cet objectif.(1, 65, 91) En effet, la structure histologique complexe de la paroi intestinale, la modulation métabolique apportée par les facteurs environnementaux

alimentaires et bactériens, l'expression partagée de protéines par le foie et l'intestin et leur métabolisme hépatique à travers le sang portal sont autant de raisons expliquant les difficultés d'identification d'un biomarqueur moléculaire d'intérêt diagnostique.(4, 94, 145)

Certaines anomalies biologiques sanguines (hyperleucocytose, acidose métabolique, élévation de la CRP, LDH, ASAT, CPK, phosphatase alcaline, phosphate, amylase) sont inconstamment retrouvées au cours de l'IMA, avec une faible performance diagnostique.(93, 94) Les protéines pancréatiques, la neurotensine, la calcitonine, les D-Dimères et certains médiateurs inflammatoires (IL-2, IL-6, TNF) ne sont pas suffisamment spécifiques de la souffrance épithéliale intestinale. Les polypeptides gastrointestinaux (somatostatine, VIP, substance P), relativement spécifiques, sont rapidement éliminés par le métabolisme hépatique et non accessibles à un dosage sanguin périphérique.(102) L'augmentation de l'hexodesaminidase et du L-lactate est trop retardée.(37, 102, 227) Aucun des marqueurs ayant aujourd'hui la meilleure spécificité intestinale (Intestinal fatty acid binding protein, D-lactate, alpha-glutathion S transferase, ischemia modified albumin et citrulline) n'a d'application clinique dans le diagnostic d'IMA précoce.(65, 91) (1, 94, 102, 175)

Au-delà des difficultés fondamentales rencontrées, l'identification de biomarqueurs diagnostiques a été jusqu'à ce jour limitée par l'absence de centre expert colligeant des cas d'IMA au sein de cohortes bien caractérisées.

1.3. Perspectives ouvertes par la Structure d'URgences Vasculaires Intestinales (SURVI) et les approches globales non-ciblées

La nécessité d'une prise en charge multidisciplinaire d'urgence de l'IMA a justifié l'ouverture d'une première Structure d'URgences Vasculaires Intestinales (SURVI) en janvier 2016 à l'Hôpital Beaujon – Assistance Publique Hôpitaux de Paris. Cette première nationale est également une première mondiale.(134) Cette innovation est basée sur notre expérience

clinique prospective pilote prometteuse ayant démontré qu'une prise en charge multimodale, multidisciplinaire incluant une revascularisation précoce et ciblant la viabilité intestinale permet une survie à 2 ans de 89% sans nécessiter de résection intestinale dans 61% des cas (53). SURVI a ainsi vocation à améliorer le pronostic de l'IMA en offrant à tout patient une prise en charge d'urgence experte, multidisciplinaire et coordonnée 24h/24h et 7j/7. (135) En outre, elle permet un recrutement des patients, une standardisation des soins et le prélèvement d'échantillons biologiques dans des conditions standardisées. A ce titre, une biobanque associée à une cohorte prospective de cas bien caractérisés d'IMA a été constituée et a été utilisée dans ce projet pour identifier des biomarqueurs diagnostiques de l'IMA par une approche globale innovante non-ciblée d'analyse des métabolites libérés à partir du tissu ischémique par analyse du métabolome.

Le métabolome plasmatique représente, à un instant donné, l'ensemble des métabolites de faible poids moléculaire (<1kDa) contenus dans le plasma.(148, 149) L'ensemble des réponses cellulaires, tissulaires et organiques à un stimulus pathologique va contribuer à la complexité du métabolome plasmatique. Le plasma constitue ainsi l'échantillon idéal pour la recherche sans a priori de nouveaux biomarqueurs circulants, d'autant plus adapté à l'étude des pathologies vasculaires et d'urgence par sa facilité de recueil clinique répété et nécessitant de faibles volumes sanguins (<1ml). Le principe de cette approche repose sur l'analyse comparative de signatures métaboliques entre des échantillons de patients parfaitement phénotypés pour la maladie étudiée (cas) et de patients ne présentant pas la maladie (témoins douleurs abdominales non ischémiques) pour mettre en évidence des différences significatives de nature et/ou de concentrations de métabolites utilisables comme biomarqueurs. Le profilage du métabolome permet l'analyse qualitative et quantitative de structures biochimiques (nucléosides, acides aminés, sucres, lipides) endogènes et exogènes, qui représentent les métabolites les plus étroitement liés au phénotype pathologique.(141, 142) Les méthodes

utilisent essentiellement la spectrométrie par résonance magnétique nucléaire (RMN) et la spectrométrie de masse (MS) couplée ou non à une séparation par chromatographie en phase liquide (LC-MS) ou gazeuse (GC-MS). L'étude des métabolites a permis la caractérisation sous forme de signatures moléculaires de nombreux processus pathologiques.(141, 143-146) L'équipe d'Imperial College London est leader mondial en analyse métabolomique.(145)

2. PATIENTS ET METHODES

2.1 Population d'étude : la cohorte SURVIBIO

Nous avons les données et échantillons humains de la cohorte prospective SURVIBIO, qui inclut depuis le 4 janvier 2016 tous les patients de plus de 18 ans ayant un diagnostic d'IMA confirmé par un angioscanner abdominal, et admis dans l'unité SURVI de l'Hôpital Beaujon et dont le consentement écrit a été obtenu. Pour chaque cas, un témoin est également inclus, admis aux urgences de l'Hôpital Beaujon pour douleur abdominale aiguë ayant nécessité un angioscanner abdominal confirmant sa nature non-ischémique. Les données épidémiologiques, diagnostiques et thérapeutiques sont recueillies prospectivement. Cette cohorte a été approuvée par le comité d'éthique CEERB Paris Nord et la CNIL (déclaration n°1889983v0) et a été enregistrée à ClinicalTrials.org (NCT03518099). Les patients avec un diagnostic de colite ischémique gauche, ischémie mésentérique chronique (angor), dissection artérielle mésentérique sans IMA ou une occlusion intestinale par strangulation n'étaient pas inclus. Pour la recherche de biomarqueurs diagnostiques de l'IMA, nous avons déterminé que 50 cas et 50 témoins devaient être inclus pour permettre l'identification de biomarqueurs avec une AUROC ≥ 0.70 avec une puissance ≥ 0.95 .

2.2 Recueil des échantillons

Le sang veineux humain était prélevé à un seul temps à l'inclusion des patients cas et témoins, sur tube hépariné. Les tubes étaient alors immédiatement centrifugés à 4°C, et le plasma aliquoté est conservé à -80°C dans la biothèque du laboratoire de biochimie de l'Hôpital Beaujon (Dr Peoc'h / Pr Puy).

2.3 Acquisition et analyse des biomarqueurs candidats (Citrulline, D-lactate, I-FABP)

Les concentrations plasmatiques d'I-FABP étaient mesurées par 2 kits ELISA [Hycult Bioteck, Uden, The Netherlands, HK40602, range: 0 – 3000 ng/L;(99) (176) and R&D Systems, Liey, DY3078, range: 0 – 1000 ng/L(128)]. Les concentrations plasmatiques de Citrulline étaient mesurées par UPLC-MS (ultra-performance liquid chromatography-mass spectromete; Xevo TQS, Waters®). Les concentrations plasmatiques de D-lactate étaient déterminées par une méthode spectrophotométrique

2.4 Acquisition et analyse des données métabolomiques

Chaque plasma a été analysé par 2 méthodes complémentaires, la spectroscopie de masse haute-résolution (MS) couplée à une chromatographie en phase gazeuse (GC-MS) et la spectroscopie par résonance magnétique nucléaire (1H-RMN). Le profilage en GC-MS et en RMN (Bruker 700 MHz) a été réalisé sous la supervision du Pr Marc-Emmanuel Dumas au département de Chirurgie & Cancer dirigé par le Pr JK Nicholson à l'Imperial College de Londres (ICL). Cette approche combinée améliore la couverture des métabolites dans l'échantillon biologique et les performances d'identification de biomarqueurs métaboliques d'intérêt.(228, 229) De manière générale, la RMN est non destructrice et applicable à des échantillons intacts, alors que la SM est analytiquement plus sensible.

Une étape de prétraitement des données MS, comprenant la sélection et le regroupement des pics, la correction du temps de rétention et l'annotation des isotopes et des adduits, a été réalisée pour permettre 1) une correction de l'erreur analytique introduite pendant l'acquisition, 2) la détection des entités présentes dans l'échantillon, 3) leur quantification relative, 4) la comparaison des différents échantillons par des méthodes statistiques dédiées. Les matrices résultantes ont été utilisées pour des analyses univariées, multivariées et intégrées (analyse en composante principale, régression linéaire, algorithmes de machine learning). La haute résolution des techniques d'analyse du métabolome génère des données de très grande dimensionnalité, qui nécessitent une réduction des dimensions, qui a été effectuée par la méthode de recouplement statistique des variables (SRV). Afin d'optimiser leurs performances diagnostiques, nous avons sélectionné un panel de biomarqueurs par l'algorithme de machine learning LAMDA (Learning Algorithm for Multivariate Data Analysis)(164, 165) et une méthode de sélection MEMBAS (Membership Margin Based Attribute Selection). Un effectif aléatoire de 85% des patients a été utilisé pour développer le modèle et les 15% restant pour le valider (validation croisée). Pour chacun des tests statistiques, une valeur de $p < 0.05$ est considérée statistiquement significative. Les résultats d'association sont tous ajustés pour des tests multiples avec une correction False Discovery Rate (FDR).

3. RESULTATS

3.1 Population d'étude

Entre le 4 Janvier 2016 et le 5 Mars 2018, nous avons évalué 185 patients ayant une douleur abdominale aiguë pour lesquels un angioscanner abdominal était prescrit en urgence. Parmi eux, 48 patients n'ont pas été inclus en raison du diagnostic final portant le total des patients

inclus à 137 (52 cas confirmés d'IMA et 85 témoins). Les caractéristiques des patients et témoins sont résumées dans le tableau 1. Le diagnostic final des patients témoins est présenté dans le Tableau 2. Les patients avec IMA [age médian: 65 ans (55-74), 19 femmes] incluaient des causes artérielles et veineuses dans respectivement 65% et 35% des cas. La population des témoins incluait des douleurs abdominales d'origine infectieuse (n=20), inflammatoire (n=15), obstructive mécanique (n=13), fonctionnelles (n=13), pancréato-biliaires (n=12), uro-génitales (n=11) et une méningococcémie. Le traitement de l'IMA associait une antiagrégation plaquettaire (n=34, 100% des IMA artérielles), une anticoagulation (n=51, 98%), une antibiothérapie orale (n=51, 98%) et une antibiothérapie intraveineuse (n=21, 40%). Une revascularisation était réalisée chez 30 patients (88% des IMA artérielles) dans un délai médian de 14 heures (3-24). Enfin, une laparotomie était nécessaire chez 18 patients (35%), aboutissant à une résection intestinale dans 12 cas (23%).

3.2 Analyse des métadonnées cliniques et biologiques

Les patients avec IMA étaient significativement plus âgés, et avaient un BMI et un nombre de facteurs de risque cardiovasculaires plus élevé. A l'admission, les patients avec IMA se présentaient également plus souvent avec une douleur brutale et morphinoréquerante, une hémorragie digestive, des signes péritonéaux, une défaillance d'organe (définie par un SOFA score ≥ 2) et une C-reactive protéine élevée (Tableau 1). Il n'y avait pas d'autre différence significative entre IMA et témoins pour les autres variables cliniques et biologiques, incluant le L-lactate. En analyse multivariée (régression logistique), 3 facteurs indépendamment associés au diagnostic d'IMA ont été identifiés : le caractère brutal de la douleur (OR=13, IC95% : 3.3 – 52, $p < 0.001$), son intensité requérant des morphiniques (OR=7.6, IC95% : 2 – 29, $p = 0.003$) et une C-reactive protéine > 20 mg/L (OR=12.2, IC95% : 2.6 – 58, $p = 0.002$). Le risque d'IMA était de 0%, 22%, 68% et 90% en présence de 0, 1, 2 et 3 facteurs. Ces résultats suggèrent

qu'en l'absence ou en présence de ces facteurs, le diagnostic d'IMA pourrait être éliminé ou suspecté, indiquant alors sa confirmation par un angioscanner adapté.

3.3 Analyse de biomarqueurs candidats : Citrulline, D-lactate et I-FABP

Six patients témoins ont refusé le prélèvement sanguin, et en raison d'échantillons biologiques en volume insuffisant, 2 patients IMA ont été exclus portant le total des patients inclus pour cette analyse à 129 (50 cas confirmés d'IMA et 79 témoins).

Les concentrations plasmatiques de citrulline étaient significativement abaissées chez les patients avec IMA en comparaison aux témoins [15.3 $\mu\text{mol/L}$ (12.0-26.0) vs. 23.3 $\mu\text{mol/L}$ (18.3-29.8), $p=0.001$]. Néanmoins, l'aire sous la courbe ROC (AUROC) pour le diagnostic d'IMA par la Citrulline était insuffisant: 0.68 (IC95% = 0.58-0.78). Nous n'avons pas retrouvé de différence significative de concentrations plasmatiques en I-FABP [278 ng/L (209-544) vs. 348 ng/L (268-587), $p=0.06$] et en D-lactates [0.042 mmol/L (0.025-0.095) vs. 0.073 mmol/L (0.031-0.115), $p=0.28$] entre les IMA et les témoins. Les AUROC étaient de 0.44 et 0.40 pour IFABP et D-lactate respectivement. Nous avons donc conclu que Citrulline, IFABP et D-lactate ne permettaient pas le diagnostic d'IMA avec des performances acceptables et qu'il était essentiel de chercher de nouveaux biomarqueurs par les techniques omiques sans a priori.

Serum I-FABP concentrations tended to be lower in the AMI population compared to the controls using Hycult kit We then confirmed a similar trend using R&D kit (Supplementary data Fig1). Results obtained with Hycult kit were further used for calculation.

Plasma D-lactate concentrations did not significantly differ between AMI patients and controls Performances of the three biomarkers for the diagnosis of AMI are summarized in Table 3.

3.4 Analyse métabolomique

En raison d'échantillons biologiques en volume insuffisant, 2 autres patients ont été exclus. De plus, les analyses en composante principale ont permis d'identifier un patient extrême (outlier) correspondant à un profil métabolique et un phénotype extrême (décédé en 48h après l'inclusion). Ce patient a été exclu des analyses finales portant le total des patients inclus pour cette analyse à 126 (47 cas confirmés d'IMA et 79 témoins). La comparaison des échantillons sanguins des patients ayant une IMA aux patients témoins ayant une douleur abdominale aiguë d'origine non ischémique a permis l'identification d'un panel de métabolites plasmatiques (n=13, correspondant à des acides aminés, sucres et fragments lipidiques : glutamine, phenylalanine, isomaltose, threose, 1 monoolein, TPA2, L3TG, glycerol, L1PN, L1TG, H4A2, L1AB, and L1PL). Les concentrations plasmatiques de ces métabolites étaient significativement différentes entre les 2 groupes de patients en analyse univariée et multivariée ajustée sur l'âge et le sexe. Le développement de cette combinaison de biomarqueurs par l'algorithme de machine learning sur 85% de la cohorte (n=108) permettait l'identification des patients avec IMA avec une AUROC = 0.89. Après validation interne effectuée sur les 15% restant de la cohorte (n=18), tous les cas d'IMA étaient identifiés avec un taux d'erreur de 0% (AUROC = 1.00). Un nombre de métabolites plus restreint peut être utilisé avec une bonne performance diagnostique. Par exemple, la monoolein seule permettait le diagnostic d'IMA avec une AUC = 0.8 dans la cohorte de développement (n=118). Dans la cohort totale (n=126), monoolein, isomaltose et glutamine seuls permettait le diagnostic d'IMA avec une AUROC de 0.86 (IC95% = 0.78 – 0.93), 0.85 (IC95% = 0.77 – 0.93) and 0.78 (IC95% = 0.70 – 0.86), respectivement.

4. DISCUSSION

Dans ce travail de thèse, nous avons mis en évidence le besoin crucial d'outils diagnostiques pour aide au diagnostic précoce de l'IMA. L'analyse de la cohorte SURVIBIO des patients prospectivement inclus entre 2016 et 2018 a permis d'identifier trois critères cliniques et biologiques associés au diagnostic d'IMA : le caractère brutal de la douleur, son intensité requérant des morphiniques, son association à une C-reactive protéine > 20 mg/L. Ces critères disponibles dès le diagnostic pourraient permettre d'améliorer la suspicion d'IMA amenant à la réalisation d'un angioscanner abdominal, en attendant que des nouveaux biomarqueurs soient disponibles à la pratique clinique. Dans un deuxième temps, nous avons testé les trois biomarqueurs candidats de la littérature scientifique les plus prometteurs (Citrulline, IFABP, D-lactate) bien que de nombreuses études contradictoires aient été publiées. Nous n'avons pas validé ces biomarqueurs dont les performances diagnostiques mesurées par l'AUROC étaient toutes insuffisantes et < 0.70. Ces résultats négatifs encourageant à la recherche de nouveaux biomarqueurs, nous avons enfin comparé le métabolome plasmatique des IMA et témoins et nous avons identifié un panel de 13 métabolites hautement discriminants du phénotype ischémique. Ces résultats prometteurs nécessitent une validation sur une cohorte externe.

Alors que le nombre de recours aux services d'urgences est en augmentation constante, la douleur abdominale aiguë en est le premier motif aux USA et en Europe.(219, 220) En 2014 aux USA, 138 millions de patients ont consulté aux urgences dont 12% pour une douleur abdominale aiguë.(220, 221) Ce motif de consultation pose encore aujourd'hui un vrai challenge diagnostique aux médecins de l'urgence, pouvant aussi bien révéler une maladie bénigne qu'une urgence vitale. Dans ce contexte à haut risque, 70 à 90% des patients consultant pour une douleur abdominale aiguë ont une prise de sang aux urgences.(219, 222)

L'identification de nouveaux biomarqueurs diagnostiques est à même de changer le diagnostic précoce de l'IMA et le pronostic de cette affection dans toutes ses applications cliniques dont

la prise en charge et l'impact, sont par essence translationnels et multidisciplinaires (urgentistes, réanimateurs, gastroentérologues, chirurgiens vasculaires et digestifs, internistes, pharmaciens, chercheurs). L'application de ces biomarqueurs pourraient être généralisée à l'évaluation et la prise en charge de toute douleur abdominale aiguë aux urgences et leur place pourrait être majeure dans la médecine d'urgence de demain.