Application and Development of Contrast-Enhanced Ultrasound for Perfusion Quantification in Kidney Disease

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Publications and Impact

Peer reviewed publications

Publication	Author contributions
• Almushayt, S. J., Hussain, S., Wilkinson, D. J. & Selby, N. M. 2020. A Systematic Review of the Acute Effects of Hemodialysis on Skeletal Muscle Perfusion, Metabolism, and Function. *Kidney Int Rep,* 5**,** 307-317.	• Shatha Almushayt: Protocol design, screening of title/abstract and full texts for inclusion, critical appraisal stage using CASP tool forms, data collection, data analysis, writing of the manuscript. • Sam Hussain: contributed as 2nd reviewer: Screened full texts for inclusion, critical appraisal stage using CASP tool forms, edited manuscript tables, reviewed manuscript • Dr. Daniel Wilkinson: Reviewed manuscript/provided feedback • Professor Nicholas M Selby: Conception of project, oversight of review process, acted as third reviewer (solved disagreement between the 1st and 2nd reviewer), reviewed manuscript/provided feedback
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COVID-19 Impact

Due to the COVID-19 pandemic and the associated difficulties in conducting clinical research, I have been unable to complete data collection in my third year because all clinical studies at the University of Nottingham were halted at the beginning of the COVID pandemic, and again during the fourth year. As a result, the original plan of this thesis was amended so that one already approved and started recruitment study was stopped and alternative three studies were conducted. This required an extension for the data collection period. The reduced timeframe also limited my ability to recruit more patients in Chapter 6. Finally, I found mental health was a huge issue that I did not handle very well which heavily impacted my productivity.

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List of abbreviations

Abbreviation	Definition
AI	Acoustic index
AKI	Acute Kidney Injury
ADP	adenosine diphosphate
ATPase	adenosine triphosphatase
ATP	adenosine triphosphate
AEs	adverse events
ANOVA	analysis of variance
a.u.	arbitrary unit
ASL	arterial spin labelling
AVF	arteriovenous fistula
BIA	bioelectrical impedance analysis
BP	blood pressure
BMI	body mass index
CVC	central venous catheter
CKD	Chronic Kidney Disease
COV	coefficient of variation
CT	computed tomography
CA	contrast agents
CEUS	Contrast Enhanced Ultrasound
CASP	Critical Appraisal Skills Programme
DBP	diastolic blood pressure
DICOM	Digital Imaging and Communication in Medicine
ESRD	end stage renal disease
eGFR	estimated glomerular filtration rate
EFSUMB	European Federation for the Society of Ultrasound in Medicine and Biology
FOV	field of view
HDF	haemodiafiltration

HD	haemodialysis
HGS	Hand-Grip Strength
HI	harmonic imaging
HV	healthy volunteers
HR	heart rate
Pi	inorganic phosphate
ICU	intensive care unit
IL-6	Interleukin 6
IQR	interquartile range
ICC	intra-class correlation
IDH	intradialytic hypotension
MRI	magnetic resonance imaging
mTT	mean transit time
MI	mechanical index
uACR	micro Urine albumin to creatinine ratio
MHC	myosin heavy chain
NIRS-VOT	Near Infra-Red Spectroscopy with a Vascular Occlusion Test
PI	perfusion index
PCr	phosphocreatine
31P	Phosphorus
PET	positron emission tomography
PEW	protein energy wasting
PIH	Pulse-inversion harmonic imaging
ROI	Regions of interest
RRT	renal replacement therapy
RDH	Royal Derby Hospital
SCr	serum creatinine
SD	standard deviation
SBP	systolic blood pressure
TIC	Time-intensity curves

UF	ultrafiltration

Thesis abstract

Changes in microvascular perfusion play a critical role in the pathophysiology of kidney disease and its sequelae. This thesis describes research work that aimed to optimise and use an ultrasound imaging method to quantify tissue perfusion in patients with kidney disease, specifically Contrast Enhanced Ultrasound (CEUS) applied in the kidneys and in skeletal muscle. This work systematically reviewed prior evidence about the acute changes that haemodialysis exerts on skeletal muscle perfusion, metabolism and function. In a clinical study, changes in skeletal muscle microvascular perfusion during haemodialysis are then explored, as a potential factor that could contribute to skeletal muscle wasting in this population. Findings are then corroborated by an analysis of pre-existing data looking at the associations between hand-grip strength, dialysis related parameters (e.g. blood pressure and ultrafiltration volume) and nutritional measures. In the second half of the thesis, CEUS is used to assess renal perfusion, optimising the protocol for CEUS in healthy volunteers. Then, intra-subject repeatability of the different CEUS perfusion parameters was determined in healthy volunteers studied twice, as well as inter-operator repeatability of image analysis. Finally, a pilot study was undertaken in patients with AKI applying CEUS in this setting and generating preliminary clinical data.

Chapter 1: Introduction

This thesis aims to optimise an ultrasound imaging method to quantify tissue perfusion in patients with kidney disease. Specifically, the application of Contrast Enhanced Ultrasound (CEUS) to measure perfusion in the kidneys and in skeletal muscle of renal patients is studied.

This chapter provides context and the formation of hypotheses for the work conducted. It starts with an introduction to renal anatomy and physiology, followed by a description of the kidney blood supply and vasculature. Chronic Kidney Disease (CKD) and Acute Kidney Injury (AKI) are introduced, followed by a brief description of haemodialysis (HD) treatment. The next part of this chapter describes skeletal muscle structure and function. Skeletal muscle metabolism, and arterial vascular anatomy of skeletal muscle are explained. Finally, the problem of skeletal muscle wasting is discussed, along with an acknowledgment of the pressing need for new approaches to gain deeper insight into the human tissue microcirculation in kidney patients.

In this chapter, I will be reviewing available approaches for measuring kidney blood flow and some of their inadequacies. A systematic review for assessment techniques for assessment of skeletal muscle perfusion is provided in a separate Chapter 2. As a conclusion, CEUS is briefly introduced as a new method for the measurement of skeletal muscle and renal perfusion.

1.1 Kidney anatomy and physiology

Kidneys are bean-shaped structures situated in the retroperitoneal space at either side of the spine. A cross-sectional slice through the kidney reveals three distinct sections: the renal cortex (outer section), the medulla (middle section), and the renal pelvis (innermost section). Renal medulla consists of the pyramids which are separated from each other by renal columns, extensions of the renal cortex. Minor and major renal calyces are chambers through which urine passes into the renal pelvis before it passes to the ureters for excretion. Each kidney consists of more than one million nephrons, the functional units of the kidney that filter out wastes and toxins from blood.

Blood passes through the kidneys several times per day for filtration. Kidneys act as ‘chemical factories’ which remove waste products and excess water from the body, regulate the body fluids, minerals and chemicals, control blood pressure (BP) and red blood cell production, and activate vitamin-D that is necessary for bone health (Reilly and Perazella, 2014).

1.2 Kidney vasculature

The kidneys are extremely vascular organs which at rest receive about 25% of cardiac output. Such high perfusion is consistent with their function in filtering the blood and is pivotal for the kidney oxygenation per se. Reduced amounts of blood flow into a kidney have been implicated as pathological process in several forms of acute and chronic kidney disease.

Each kidney is supplied by a renal artery or arteries which originate directly from the descending aorta. Renal arteries are divided into interlobar arteries that run through renal columns to the renal cortex where they branch again into arcuate arteries, and then into afferent arterioles that supply the nephrons (Figure 1.1). Renal veins, through which filtered blood leaves the kidneys, are parallel to renal arteries and carry the blood to the inferior vena cava.

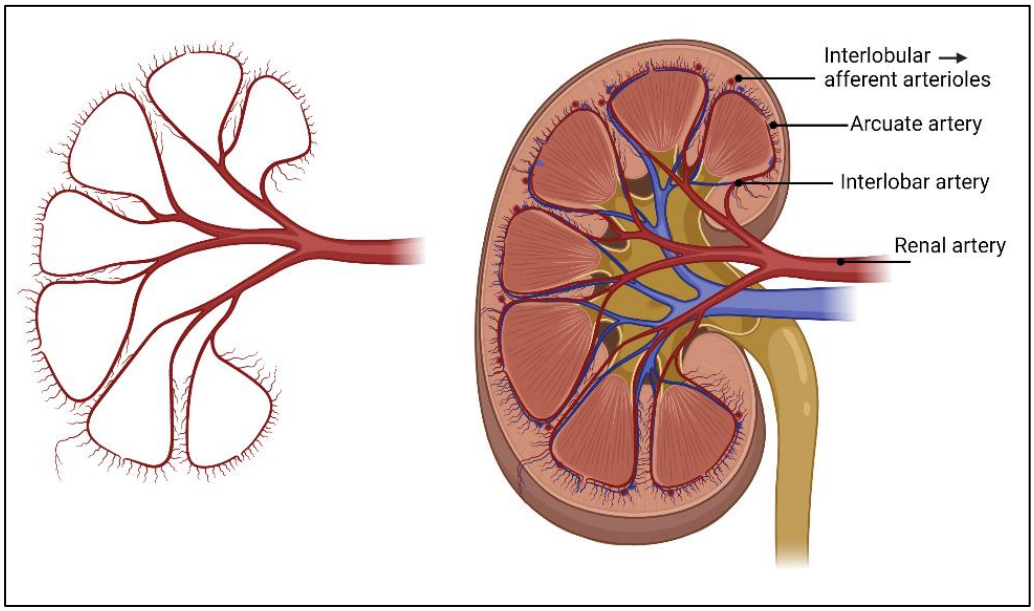


Figure 1.1: Kidney cross-section with vasculature highlighting the major blood vessels from renal artery extending to renal cortex. Illustration created using Biorender.com.

1.3 Kidney disease

The sections below introduce two common syndromes which are studied in this thesis.

1.3.1 Chronic kidney disease

Chronic kidney disease (CKD) is characterised with a gradual loss of kidney function over time. It is the most common form of kidney disease, with a global prevalence of 9·1%, as of 2017, which is an equivalent of roughly 700 million cases (Cockwell and Fisher, 2020). Unfortunately, CKD is irreversible and is usually asymptomatic until it is advanced in its course. However, its progression can be halted or slowed down in its early phases. The Kidney Disease: Improving Global Outcomes (KDIGO) defines CKD as abnormalities of kidney structure or function that last for more than 3 months, with implications categorised according to the aetiology, GFR, and albuminuria (2013) as demonstrated in Figure 1.2. When eGFR is below 15 mL/min/1.73m^2, CKD is classified as stage 5, the equivalent to end stage kidney disease (ESKD). A patient with ESKD must receive renal replacement therapy (RRT) in a form of dialysis or kidney transplantation.

Prognosis of CKD by GFR and albuminuria category

Prognosis of CKD by GFR and Albuminuria Categories: KDIGO 2012				Persistent albuminuria categories Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				<30 mg/g <3 mg/mmol	30-300 mg/g 3-30 mg/mmol	>300 mg/g >30 mg/mmol
GFR categories (ml/min/ 1.73 m^2) Description and range	G1	Normal or high	≥90			
	G2	Mildly decreased	60-89			
	G3a	Mildly to moderately decreased	45-59			
	G3b	Moderately to severely decreased	30-44			
	G4	Severely decreased	15-29			
	G5	Kidney failure	<15			

Green: low risk (if no other markers of kidney disease, no CKD); Yellow: moderately increased risk; Orange: high risk; Red, very high risk.

Figure 1.2: Implications of CKD by eGFR and albuminuria; Figure adapted from KDIGO guidelines (KDIGO, 2013).

1.3.2 1.3.2 Acute kidney injury

Acute kidney injury (AKI), previously known as acute kidney failure, occurs when the kidneys abruptly cease functioning within hours to a few days. Although it is a serious condition associated with high mortality, AKI is often reversible with early diagnosis and immediate treatment, especially if there is no accompanying underlying disease. Acute kidney injury is caused by multiple processes including reduced blood flow to the kidneys, direct damage to the kidneys, or blockage in the ureters that

keeps the urine from leaving the body. Most cases of AKI are due to impaired blood flow to the kidneys, which is induced by a variety of conditions including severe dehydration, sepsis, other causes of hypotension (e.g. bleeding, severe allergic reaction, severe burns), heart disease, and liver failure. Some drugs such as non-steroidal anti-inflammatory drugs also reduce blood flow via constriction of the glomerular afferent arteriole, mediated by reductions in prostaglandins; examples include Ibuprofen (Advil, Motrin IB, others), diclofenac and Naproxen. In terms of incidence of AKI, 5-10% of hospitalised patients experience AKI, with higher rates reported in intensive care unit (ICU). Renal replacement therapy can sometimes be required to replace some functions of the kidney when severe AKI causes kidney failure.

1.4 Haemodialysis treatment

Haemodialysis is a life-saving treatment for those with acute or chronic kidney failure. Prevalence data in 2019 from adults in the United Kingdom show that 24,365 adult patients receive in-centre haemodialysis (UK Renal Registry, 2021).The basic principle of HD is based on two processes, diffusion, and ultrafiltration (UF). During HD, the blood is pumped from the patient into blood lines to the dialyser (filter), which is surrounded by the dialysis fluid (dialysate). At this stage, an exchange of solutes between the blood and dialysate takes place by diffusion through the dialyser semi-permeable membrane. Excess waste products leave the blood, and deficient electrolytes such as Bicarbonate diffuse into the blood from the dialysate. Importantly, blood cells, proteins (such as

albumin), and other important particles do not pass through the membrane due to their large size.

Excess fluid is also removed during HD using UF, based on a hydrostatic pressure-gradient. The pressure in the dialysate side is lower than that in the blood. As a result of this, fluid is forced out of the blood into the dialysate. The amount of fluid to be removed is controlled by a volumetric pump over the course of dialysis (Wild and Stein, 2010) . The removal of excess fluid by UF can result in a rapid reduction in intravascular volume (hypovolaemia). Also, the relatively rapid removal of solutes (primarily urea) results in a reduction in plasma osmolality (Cernaro et al., 2012), which reduces compensatory plasma refilling where extravascular water is pulled into the blood as a result of colloid osmotic pressure. Therefore, sometimes plasma refilling falls short, prompting systematic circulatory stress and intradialytic hypotension (IDH) due to the reduced intravascular volume (Singh and Mc Causland, 2017). It has been long appreciated that HD-induced circulatory stress could exert cardiac ischaemic injury, but the growing evidence has now proved that this haemodynamic insult could cause end organ damage by tissue ischaemia on other organs including, brain, gut, liver, and the kidneys. Indeed, such presentations place a further health burden on dialysis patients and affects their quality of life (Canaud et al., 2020).

The relevance of describing the HD treatment above was twofold. First, it is a well-established RRT, and this thesis presents work in renal contexts. Secondly, this thesis discusses a clinical study in which CEUS was applied for skeletal muscles perfusion quantification in patients

receiving haemodialysis treatment. The next section, therefore, introduces basics of skeletal muscle anatomy and function and discusses skeletal muscle wasting in CKD.

1.5 Introduction to Skeletal Muscle

1.5.1 Skeletal muscle function and structure

Skeletal muscle has a pivotal role in the human body. It is the largest tissue by mass in the body, accounting for approximately 40% of human body weight. Skeletal muscle is attached to the skeletal bones, allowing for voluntary locomotion and posture, and it protects internal organs, generates heat, and acts as a reservoir for amino acids to maintain protein synthesis in tissue during starvation. Additionally, skeletal muscle is a primary site for glucose uptake and storage, playing a pivotal role in regulating whole-body glucose homeostasis (Wolfe, 2006, Mukund and Subramaniam, 2020).

Each skeletal muscle comprises multinucleated muscle fibres with threadlike striated and cylindrical muscle cells. Each fibre is covered with a thin layer of connective tissue, the *endomysium*. A group of fibres arranged in parallel forms the muscle fascicle, encapsulated by the *perimysium* layer. The muscle is formed by a group of fascicles encapsulated in a collagenous sheath, the *epimysium* (Boron and Boulpaep, 2009).

1.5.2 Skeletal muscle metabolism

Metabolism refers to the chemical reactions occurring in the living body, which involve catabolism (the breakdown of complex molecules to release energy) and anabolism (complex molecule synthesis from simpler molecules required by the cell). For skeletal muscle to be able to contract, it requires energy. This energy is supplied by an energy-storing molecule called adenosine triphosphate (ATP), which consists of adenine as a base, ribose as a sugar, and three phosphate molecules held to the sugar by energetic bonds. The hydrolysis of ATP results in the cleavage of the terminal phosphate. The results of this reaction are adenosine diphosphate (ADP) and inorganic phosphate (Pi), and the stored chemical energy in the bond is released in the form of mechanical energy (muscle contraction). Creatinine is a by-product of muscle metabolism in which phosphocreatine is converted nonenzymatically to creatinine. Creatinine is filtered by the glomerulus but is also secreted to a lesser degree by renal proximal tubule(Gomez et al., 2014)The daily synthesis of creatinine of about 20 mg/kg body weight reflects muscle mass and varies little from day to day (Perrone et al., 1992).

1.5.3 Arterial Vascular Anatomy of Skeletal Muscle

Different types of muscle fibres can be surrounded by varied numbers of capillaries. Slow fibres are surrounded by more capillaries than fast fibres, which has been confirmed in clinical (Andersen, 1975 , Sjøgaard, 1982) and preclinical (Acevedo and Rivero, 2006) studies using the capillary-to-fibre ratio and capillary density, respectively.

While it is acknowledged that muscle blood flow increases during exercise, it has been debatable whether all muscle capillaries are recruited (perfused) at rest or whether they are only recruited in response to training or insulin. Previous studies have presented consistent evidence that resting skeletal muscles are not fully perfused at the capillary level. A so-called capillary reserve is recruited during exercise or insulin uptake (Clark et al., 2008).

1.5.4 Skeletal-muscle blood circulation

The skeletal muscles have a unique blood flow characterised by a wide range from as low as 5 mL/min per 100 g of tissue at rest to at least 250 mL/min per 100 g of active muscle. Adequate muscle perfusion is essential for provision of nutrients and to maintain function, metabolism, and the rapid removal of waste products. The section below describes the arterial vascular anatomy supplying the thigh skeletal muscle, as this is the muscle examined in some of the clinical studies in this thesis.

1.5.5 Thigh-muscle arterial blood supply

The lower limb is supplied by the femoral artery, which is an extension of the external iliac artery. The quadriceps muscles are supplied by the descending branch of the lateral circumflex artery, which branches from the deep femoral artery (Standring S, 2016). An anatomical illustration of the femoral artery is depicted in Figure 1.3, presenting femoral artery branches in quadriceps muscles.

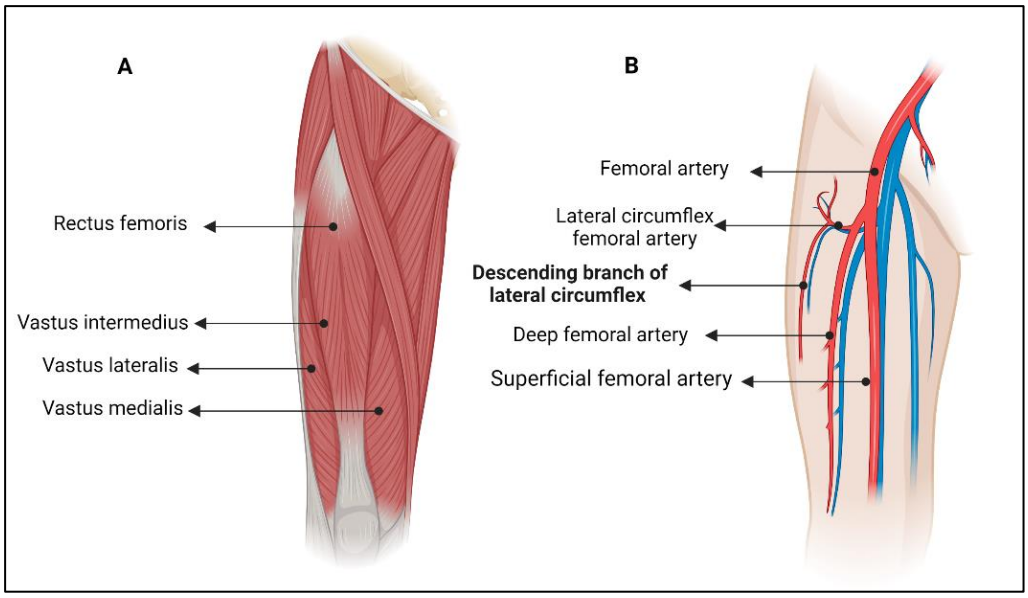


Figure 1.3: a) Quadriceps thigh muscles. b) Femoral artery branches in the thigh. Illustration created using Biorender.com.

Within the muscle, arterioles branch into the muscle until they become terminal arterioles that supply a group of capillaries. A microvascular unit comprises the capillaries supplied by a terminal arteriole and is the smallest functional unit of blood flow control in skeletal muscle. Each microvascular unit comprises 15 to 20 capillaries ending in a collecting venule. Figure 1.4 presents a diagram of the skeletal muscle arteriole network and microvascular unit (Bagher and Segal, 2011, Boron and Boulpaep, 2009).

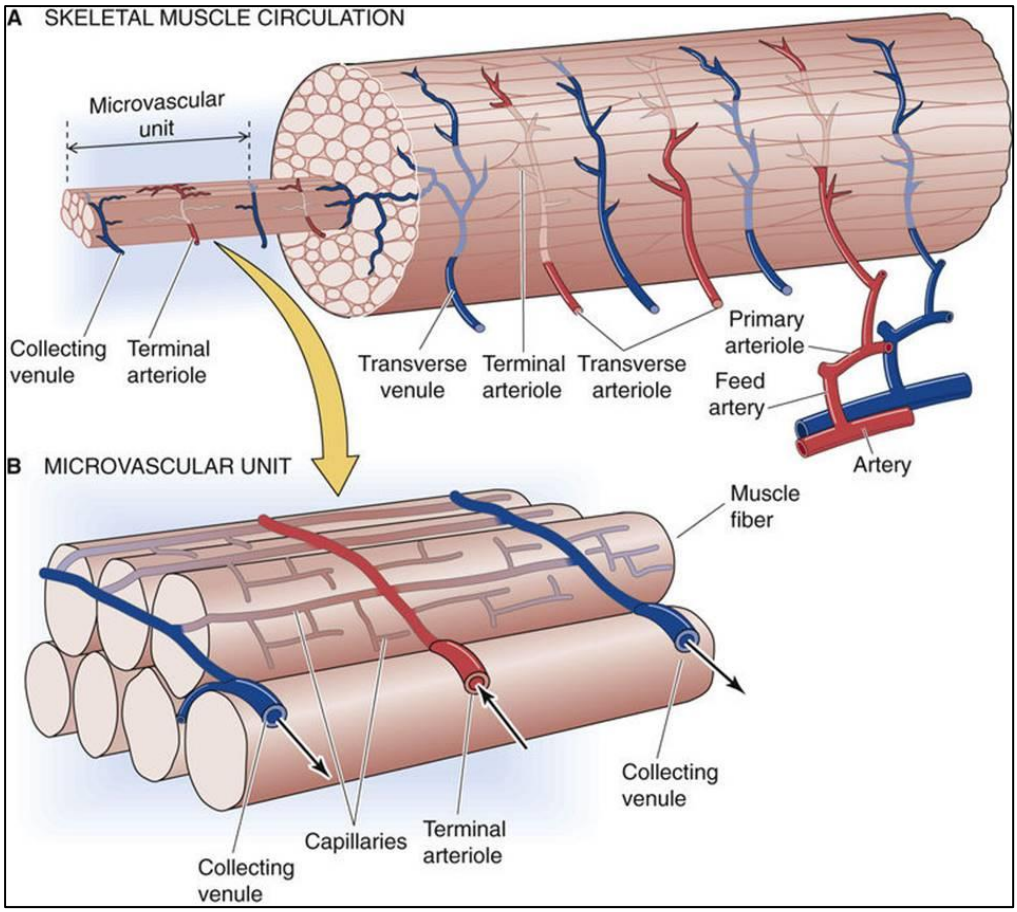


Figure 1.4: a) Skeletal-muscle circulation. b) Microvascular unit. Adapted from *The Skeletal Muscle - Special Circulations - The Cardiovascular System - Medical Physiology*, 3rd edition (Doctorlib.info).

1.5.6 Changes in muscle vasculature and muscle fibres in disease

A growing body of evidence has proved that skeletal muscle microvasculature is closely related to metabolic physiology and pathophysiology. Age-related endothelial dysfunction and impairment of vasoconstrictor responses leads to inadequate delivery of metabolites or distribution of blood flow (Muller-Delp, 2006). Similarly, endothelial dysfunction, impaired microvascular dilatation and capillary recruitment have been reported in obesity (Virdis et al., 2019). In type 2 diabetes,

reduced skeletal muscle capillary density and compromised endothelial wall function have been observed (Liang and Ye, 2019). Furthermore, patients with severe peripheral arterial disease experience vascular injury which extends beyond the atherosclerosis of main arteries and affects even the smallest microvessels. This is manifest as increased basement membrane thickness, amount of Collagen and diameter, and an increase of the pericyte coverage of terminal micro-vessels (Baum et al., 2005, Mietus et al., 2020).

Human skeletal muscle is composed of diverse fibre types distributed in various proportions according to the anatomical site, age, genetics, and gender. However, given muscle fibre plasticity, it can adapt to various functional or metabolic demands in a form of alterations in fibre distribution, fibre size, or number of capillaries around the fibre. The discussion of fibre distribution and adaptation may provide insights into muscle susceptibility to disease.

Fibre plasticity can be in response to muscle wasting states such as sarcopenia (Nilwik et al., 2013), cancer, cachexia and sepsis, which were associated with reduced size of Type II fibres as opposed to Type I fibres which showed more resistance to fatigue (Ciciliot et al., 2013). Type I oxidative fibres also appear more resistant to ischaemia-reperfusion than glycolytic fibres (Charles et al., 2017).

Muscle fibres also adapt to different types of exercises. For instance, endurance exercise has been reported to increase, albeit modestly, Type

I fibres, in contrary to resistance exercise which results in increased Type II fibres (Luden et al., 2012, Liu et al., 2003, Andersen et al., 1994).

Furthermore, several studies have reported an alteration in muscle fibre-type under pathological hypoxic conditions such as chronic obstructive pulmonary disease (Thériault et al., 2014, van de Bool et al., 2016, Ausin et al., 2017) and chronic heart failure (Kitzman et al., 2014, Lindsay et al., 1996). In these conditions, a transition from slow towards a fast fibre type has been frequently observed in the lower limb muscles of patients, yet the underlying mechanism of such fibre typing modulation remains unclear. Another hypoxic condition that affects as many as 60% of ESKD patients (Lin et al., 2019) is sleep apnoea where animal-based studies have revealed fibre-type disproportion in the upper airway muscles with no or limited change in hind limb muscles (Ferini-Strambi et al., 1998, Larsson et al., 2008)**.**

1.5.7 Skeletal Muscle Wasting in Chronic Kidney Disease

Muscle wasting and poor exercise capacity are highly prevalent among CKD patients (O'Sullivan et al., 2018, Roshanravan et al., 2017b, Carrero et al., 2008a). Prevalence is influenced by the different mechanisms of muscle wasting in CKD as well as the varied measurement tools used (O'Sullivan et al., 2018). Nevertheless, the clinical relevance of muscle mass wasting is evident through associations with mortality, morbidity, and quality of life (MacKinnon et al., 2018, Carrero et al., 2008a).

Protein energy wasting (PEW) is characterised by a reduction in body protein and energy fuels, which is not easily reversed simply through

nutritional intervention. PEW primarily results from increased catabolism, inflammation, acidosis or endocrine disorders (Fouque et al., 2008). In contrast, sarcopenia is the ageing-related progressive reduction in skeletal muscle mass and function (Cruz-Jentoft and Sayer, 2019). Cachexia is a complex metabolic syndrome linked to an underlying chronic disease and the loss of muscle (Evans et al., 2008). Finally, the state of muscle wasting is a common feature in PEW, sarcopenia, and cachexia. Therefore, muscle wasting has been proposed as a combining term to describe any condition that involves muscle wasting, regardless of its specific aetiologies (Anker et al., 2014).

Loss of muscle mass is associated with the deterioration in muscle strength and physical function (Zhou et al., 2018a, Segura-Ortí et al., 2018). Such muscle wasting in CKD is associated with a lower quality of life (Martinson et al., 2014) and is associated with increased mortality (Pereira et al., 2015). Many scholars over the years have conducted empirical studies on humans, animals, and cell cultures to examine the cause of the loss of muscle mass in CKD (Wang and Mitch, 2014). Fundamentally, there is a disturbed balance between protein synthesis and degradation caused by uraemic complications (e.g., insulin resistance, metabolic acidosis, and inflammation) in CKD (Bailey et al., 1996, Bailey et al., 2006, Wang et al., 2009, Wang, 2015). Nevertheless, knowledge gaps remain in the exploration of other potential causes of muscle wasting in CKD patients. For example, very little attention has been paid to the effect of HD on the vascular beds of skeletal muscle.

Demonstration of dialysis-induced reductions in blood flow could reveal a new process by which skeletal muscle wasting may occur in CKD.

1.5.7.1 Mechanisms of muscle wasting in CKD

CKD-associated muscle wasting results from interconnected mechanisms that interact and disturb the balance between catabolic and anabolic processes controlling muscle homeostasis. The underlying pathways inhibit protein synthesis, increase protein degradation and impair muscle regeneration. I highlight in this section several cellular signalling pathways that contribute to muscle wasting in CKD.

1.5.7.1.1 Ubiquitin-Proteasome System and Caspase-3 Activity

Ubiquitin-proteasome system, the major mechanism of proteolysis, is activated in skeletal muscle in CKD patients. Protein degradation is increased mainly through activation and upregulation of the UPS (Rajan, 2008). Caspase-3 is a proteolytic enzyme that participates in cell apoptosis. It cleaves actin-myosin protein complex in muscle fibres and generates the 14 kDa actin fragment (biomarker of protein breakdown). Hence, the activation of Caspase-3 accelerates protein degradation in muscles. Elevated levels of 14 kDa actin fragment were revealed in muscle biopsies obtained from patients diagnosed with ESRD on HD.

1.5.7.1.2 Impaired Insulin, Insulin-Like Growth Factor-1, and Insulin Resistance

Insulin hormone is a major regulator of protein metabolism in skeletal muscle by promoting protein synthesis and inhibition of protein degradation. It has been well established that insulin resistance is a common feature of CKD, which may contribute to muscle wasting (Siew et al., 2007). Insulin resistance leads to impaired insulin/Insulin-Like Growth Factor-1 signalling in skeletal muscle which leads to muscle protein degradation. Furthermore, a low testosterone level may induce muscle protein degradation by impaired Insulin-Like Growth Factor-1 signalling (Sun et al., 2006). Evidence shows that low testosterone concentrations are highly prevalent in CKD patients and closely correlated with muscle wasting and mortality in HD patients (Gungor et al., 2010, Carrero et al., 2009).

1.5.7.1.3 Inflammation

Inflammation is a major consequence of CKD (Schindler, 2004). It starts in CKD well prior the need for dialysis, suggesting that both CKD and dialysis are considered as low-grade inflammatory processes. Chronic inflammation triggers other complications besides muscle wasting, including vascular calcification, osteoporosis, and frailty (Kooman et al., 2017). As CKD progresses, increases are seen in the levels of inflammatory markers such as interleukin-6, tumour necrosis factor-alpha (TNF-α), and C-reactive protein, which are highly predictive of mortality (Meuwese et al., 2011) and inversely related to thigh muscle area in HD patients (an index of muscle wasting) (Kaizu et al., 2003). Also, low levels

of albumin and high levels of fibrinogen (acute-phase reactants) are observed in CKD (Panichi et al., 2002) (Muntner et al., 2004).

Tumour necrosis factor-alpha interferes with the insulin signalling through its receptor in skeletal muscle and induces insulin resistance (Gao et al., 2003). Additionally, it activates Caspase-3 which stimulates Ubiquitin-Proteasome System, leading to muscle wasting (Li et al., 2003, Du et al., 2004). Also, the release of Interleukin-6 expression from CKD patients skeletal muscle is linked with increased muscle net protein catabolism, which suggests that muscle interleukin-6 could contribute to a catabolism (Garibotto et al., 2006).

1.5.7.1.4 Metabolic acidosis

Metabolic acidosis is also common in patients diagnosed with CKD. Metabolic acidosis has been shown to cause negative nitrogen balance (nitrogen is a fundamental component of amino acids) and decrease albumin synthesis, leading to muscle wasting. Moreover, metabolic acidosis activates the Ubiquitin-Proteasome System and caspase-3, leading to muscle protein degradation (Franch et al., 2004, Du et al., 2004).

1.5.7.1.5 Other factors

Alterations in miRNA expression in CKD can lead to abnormal protein metabolism and muscle regeneration (Robinson et al., 2020). Also, disruption of gut microbiota homeostasis is increasingly recognized to play a critical role in inflammation, insulin resistance, and mitochondrial dysfunction, which are closely associated with muscle wasting in CKD

(Ramezani and Raj, 2014). Finally, the haemodialysis treatment also boosts the catabolic and atherogenic pathways which are already upregulated in the muscle of non-dialyzed CKD patients (Garibotto, 2012). This is not an exhaustive list of physiological factors leading to muscle wasting, and other factors such as oxidative stress, nutritional factors and inactivity can also contribute.

1.5.7.2 Significance of muscle mass in chronic kidney disease and dialysis

Maintenance of the protein of certain organs, such as the skin, brain, heart, and liver, is essential for survival. These tissues rely on a steady supply of amino acids via the blood for the synthesis of new proteins to balance the persistent rate of protein breakdown that occurs in all tissues. In the absence of nutrient intake, skeletal muscle plays a key role in whole-body protein metabolism by serving as the principal reservoir to replace blood amino acid taken up by other tissues and serve as precursors for protein synthesis (Felig et al., 1969) as well as precursors for hepatic gluconeogenesis (Felig, 1973). Therefore, if there is adequate muscle mass, protein mass of essential tissues and organs as well as plasma glucose concentration can remain relatively constant in the absence of nutritional intake.

Loss of muscle mass is a common feature in CKD and dialysis patients and is an important determinant of survival (Carrero et al., 2008a). It is linked with fatigue, poor physical activity, increased risk of falling and

eventually decreased quality of life (Kooman et al., 2013, Carrero et al., 2016, Zhou et al., 2018b, Carrero et al., 2008a, Cook and Jassal, 2005). Larger muscle mass may help in counter-acting some of the metabolic changes associated with CKD such as insulin resistance (Moon, 2014) as skeletal muscle mass is a key factor in glucose and energy homeostasis (Wohlgemuth et al., 2010) and is positively correlated with insulin sensitivity (Cleasby et al., 2014). Moreover, concentrations of inflammatory markers (C-reactive protein, interleukin-6) were also reduced in CKD patients following a resistance training program (that increases muscle mass) (Castaneda et al., 2004). It is, therefore, thought that training programmes are a potentially beneficial approach to increase muscle mass, muscle strength, fatigability, and physical performance in maintenance HD patients (Storer et al., 2005).

1.5.7.3 Muscle mass and creatinine

The discussion of muscle mass in the context of kidney disease has also relevance to the established estimates of renal function. Creatinine, which is a waste product from muscle metabolism is normally filtered by the kidneys, and hence used as a surrogate to predict renal function. However, generation of creatinine is dependent on muscle mass which varies according to age, gender, race, and weight. Therefore, estimation of renal function using the estimated GFR equation was developed which considers these factors along with the serum creatinine. One equation that has garnered widespread acceptance is the Modification of Diet in Renal Disease (MDRD) Study equation (Levey et al., 1999, Levey et al.,

2006) which was developed in people with CKD, but is limited by imprecision and systematic underestimation of measured GFR (bias) at higher levels (Stevens et al., 2007). Therefore, the CKD Epidemiology Collaboration (CKD-EPI) equation has been developed as a new equation using pooled data from multiple studies. Researchers randomly divided ten studies which included 8254 participants, into separate data sets for development and internal validation. Additional 16 studies included 3896 participants were used for external validation. Linear regression was used to estimate the logarithm of measured GFR from standardized creatinine, sex, race and age available in the dataset and comparison of new equations to the MDRD Study equation showed that the CKD-EPI equation performed better than the MDRD equation, particularly at higher GFR, with less bias and greater accuracy (Levey et al., 2009). Nonetheless, eGFR can still be imperfect estimate of true renal function in people with high muscle mass (e.g., body builders) who demonstrate high creatinine levels and consequently low eGFR even with normal renal function. Conversely, eGFR may remain within the normal range despite marked renal impairment in patients with low muscle mass (e.g., malnourished patients) and their kidney function would be overestimated. Therefore, cautious interpretation of eGFR in the context of body habitus (or muscle mass) and clinical condition is recommended.

1.6 Assessment of Tissue Perfusion in Kidney Disease

Measurements of blood flow and perfusion could provide valuable insights into tissue function and pathophysiology in kidney and muscle, and the unifying theme of this thesis is the minimally-invasive assessment of microvascular perfusion.

Blood flow is the continuous circulation of blood in the circulatory system. The amount of blood delivered to an organ is measured in mL/min. Perfusion is the volume of blood that travels through the capillary bed per unit of time to supply oxygen and nutrients to tissues and is usually expressed in mL/g/min.

Several techniques are used for the in vivo measurement of renal blood flow and perfusion. Para-aminohippurate clearance is a gold-standard method in renal physiology to estimate renal plasma flow as 100% of para-aminohippurate is extracted by the kidney, so the clearance is used to estimate renal plasma flow, a renal function measure. Nevertheless, this technique is not suitable for renal disease, as the disease may invalidate the 100% clearance assumption (Todo, 1995) and it is not practical outside of specific research settings.

In positron emission tomography (PET), radiopharmaceuticals are intravenously administered, and the PET scanner is used to map the radioactively labelled blood has travelled. Tracers used for renal perfusion imaging and quantification are cyclotron-produced ^{15}O-water and ^{13}N-ammonia, as well as using generator-produced ^{82}RbCl and ^{62}Cu-ETS. However, the problem with PET is the exposure to

ionising radiation and its low spatial and temporal resolution. A better spatial resolution can be obtained from computed tomography (CT) but exposure to ionising radiation persists in this technique. Moreover, CT typically requires contrast agents (CA), which are associated with a risk of contrast-induced AKI, and hence often avoided for patients with renal impairment, adding to the limitations of this technique for this patient group.

Dynamic contrast-enhanced magnetic resonance imaging (MRI) has been largely used in renal imaging, showing promise, and providing valuable information about renal perfusion and GFR (Tofts et al., 2012, Lee et al., 2007). Nevertheless, as with the CT, in patients with impaired kidney function, the linear chelate gadolinium-based CA used in MRI has been restricted since 2006 to mitigate against the risk of the rare but life-threatening condition of nephrogenic systemic fibrosis. Therefore, the use of lower doses of the newer macrocyclic chelate gadolinium-based CA is advised in patients with renal impairment, which resulted in no new incidences of nephrogenic systemic fibrosis since then in Europe. (The Royal College of Radiologist, 2019, Wang et al., 2011). However, the risk-benefit risk in research settings is different, with gadolinium rarely justifiable. Additionally, the clinical significance of the potential long-term effect of gadolinium retention, especially in the brain, remains unclear (Errante et al., 2014)

Another MRI technique known as arterial spin labelling (ASL) allows for a quantitative perfusion assessment using magnetically labelled subject's blood as an endogenous contrast agent, and a subtraction technique to

detect perfusion. This has the benefit of being free from ionising radiation and the need for contrast agents. However, restrictions related to the logistics and high cost remain challenges hindering the advancement of this technique for acutely unwell patients in hospital wards.

Doppler ultrasound is established in renal imaging and has long been used, particularly for visualising transplanted kidney vessel blood flow and tissue perfusion. However, tissue perfusion cannot be quantified, and only the flow within macro-vessels (e.g. the renal artery) can be measured using Doppler-derived parameters. Moreover, the Doppler ultrasound has limited accuracy in renal arterial blood flow changes in animals (Wan et al., 2008). Recently, CEUS has emerged as an alternative minimally invasive technique with potential for measuring tissue perfusion in kidney patients, with an excellent safety profile of the CA and the usual ultrasound advantages of portability, lack of ionising radiation, and cost-effectiveness.

CEUS is increasingly accepted clinically for diagnosis and interventional work-up (Sidhu et al., 2018). It has improved the detection and characterization of different abnormalties. For example, CEUS is widely applied in liver disease for detection and characterization of liver lesions and metastasis (Friedrich-Rust et al., 2013) as well as for detection of portal vein thrombosis (Tarantino et al., 2006). It has also been used to improve delineation of tumours in other organs such as pancreas and spleen (Kersting et al., 2009, Stang et al., 2009). Moreover, CEUS has been used to quantify tumour perfusion, which is essential to assess data objectively, and to further characterize focal lesions. It is also useful to

evaluate therapeutic response in cancer patients as treatments that target tumour vascularity have highlighted the need for accurate and reproducible quantitative techniques to evaluate alterations in tumour vascularity.

Perfusion studies using CEUS have been performed in the bowel to differentiate between normal and diseased bowel (Girlich et al., 2009) and to quantify bowel wall vascularity in Crohn's disease (Pascale et al., 2006). Another successful application is the improved detection of splenic infarction by demonstrating avascular wedge-shaped lesions (Catalano et al., 2003, Catalano et al., 2004, Li et al., 2014). In pancreatic transplant imaging, CEUS adds extra value to conventional ultrasound by assessing perfusion and vascular complications. Assessment of microcirculation using CEUS allows evaluation of pancreas viability and may offer prognostic information (Ito et al., 2014, Aboutaleb et al., 2011). Other applications of CEUS include contrast echocardiography in which the contrast improved visualization of endocardial borders in patients with suboptimal images by conventional ultrasound (Nanda et al., 2002, Wake et al., 2006). Such applications increased the overall knowledge about the technique's capability and encouraged the exploration of new applications, including the kidneys.

In nephrology, CEUS has been extensively applied for renal lesion characterisation (Li et al., 2013, Barr et al., 2014, King et al., 2015, Park et al., 2007). Also, CEUS has demonstrated excellent performance in the detection of renal parenchymal ischaemia that is comparable to computed tomography and superior to Doppler ultrasound. Infarctions

appear as wedge-shaped and non-enhancing areas within an otherwise enhanced kidney (Bertolotto et al., 2008), which is distinguished from cortical necrosis in that the latter has a preserved hilar vascularity (Bertolotto et al., 2008, Correas et al., 2006). It can also detect renal ischaemia which is manifested as hypoperfusion (Fernandez et al., 2013).

As with pancreatic transplant, CEUS can assess microcirculation in renal transplant. Perfusion quantification of renal transplant has been shown to be useful in evaluating vascular complications and in improving the detectability of perirenal hematomas (Alvarez Rodriguez et al., 2017, Pan et al., 2017, Korda et al., 2016, Grzelak et al., 2013). Moreover, measures related to reduced perfusion (e.g. longer time to peak, or longer mean-transit time) have been associated with a worse prognosis of graft function and survival (Kay et al., 2009, Wang et al., 2015b).

In a research setting, CEUS has also been applied to assess renal cortex perfusion in CKD patients, which revealed reduced CEUS perfusion measures compared to healthy volunteers, consistent with hypoxia and vascular rarefaction commonly associated with CKD (Dong et al., 2014, Tsuruoka et al., 2010a). CEUS was also able to detect how reduced renal perfusion in CKD can increase in response to a low salt intake diet (Garessus et al., 2021). Renal cortical perfusion has also been assessed in septic shock patients (Harrois et al., 2018) which was reduced compared to the control group, with variable perfusion at the individual level. Moreover, the reduction of perfusion in the sepsis group was associated with the development of AKI.

Other scenarios where CEUS was able to detect changes in renal cortical perfusion were in smaller studies in ICU patients receiving noradrenaline (Schneider et al., 2014), around cardiac surgery (Schneider et al., 2013), in response to blood pressure (Angiotensin II) drugs (Schneider et al., 2012), and in hepatorenal syndrome (Schneider et al., 2015). However, there remains a gap in our understanding about the technique's repeatability and validity against gold standard measures of renal perfusion. Additionally, more studies are needed to generate descriptive data about perfusion variation among clinical groups. More studies are needed to address these questions before translation to clinical practice.

1.6.1 Principles of Contrast-Enhanced Ultrasound

CEUS is a promising imaging technique that has garnered growing interest. With this technique, a micro-bubble CA enhances the vascular compartment, different to the CAs used in CT and MRI. Ultrasound CAs are tiny microbubbles measuring only 2 to 6 μm, which are smaller than red blood cells but with similar rheology. They are too large to pass into the interstitium, which classifies them as blood pool CAs suitable for tissue microcirculation imaging (Correas et al., 2001, Wescott, 2011). This enhancement results from CA oscillation under the ultrasound beam, creating a robust echo (reflected sound wave).

1.6.1.1 Development of Ultrasound Contrast Agents

Ultrasound CAs have gone through several developmental trials since 1968 (Gramiak and Shah, 1968, Ophir and Parker, 1989). The production of an ideal CA for clinical use has demanding specifications for both safety and effectiveness, including easy intravenous administration, intact passage through the cardiac circulation and pulmonary circulation, stability in the circulation for the duration of the exam, and safety for the kidneys (Wescott, 2011).

The initial structures used free gas microbubbles and presented limited stability and efficacy, which provoked more trials aiming to meet the ideal specifications. In 1984, the first encapsulated stable microbubbles were produced, followed by the first well-known CA Levovis (Schering AG, Berlin, Germany) in the 1990s. However, these CAs are no longer marketed since the introduction of the second-generation CAs in 1996, based on improved size and stability and, hence, improved effectiveness.

Second-generation CAs differ according to their structure. They consist of a poorly soluble gas (perfluorocarbon or sulphur hexafluoride) encapsulated by biocompatible shells made of protein, lipid or biopolymer (Quaia, 2005) to maintain the contrast in the blood for a longer time. Examples of the current commercially available CAs are Optison (GE Healthcare, Milwaukee, Wisconsin, United States), Definity (Lantheus Medical Imaging, Boston, Massachusetts, United States), and SonoVue (Bracco, Milan, Italy). However, some restrictions in terms of availability exist across the world regarding different CA applications. In Europe,

SonoVue (sulphur hexafluoride gas encapsulated in a phospholipid shell) is the only CA approved for cardiology and radiology indications.

1.6.1.2 Interaction of Microbubbles with the Ultrasound

Microbubbles oscillate when exposed to an ultrasound, and at sufficiently higher acoustic powers they respond to the wave nonlinearly. Their contraction and expansion are unequal due to stronger bubble resistance to compression than expansion, resulting in reflected harmonic signals (multiples of transmitted frequency) from the bubbles back to the transducer. In conventional harmonic imaging (HI), the fundamental frequency is filtered out so that the harmonic signals form bubbles are distinguished from fundamental signals reflected by the background tissue. Figure 1.5 presents a schematic diagram for microbubble behaviour at different ultrasound amplitudes, including the nonlinear response. While this filtration technique is effective, it limits the available imaging bandwidth, decreasing the resolution and leading to the development of another technique that overcomes such limitations (Phillips and Gardner, 2004).

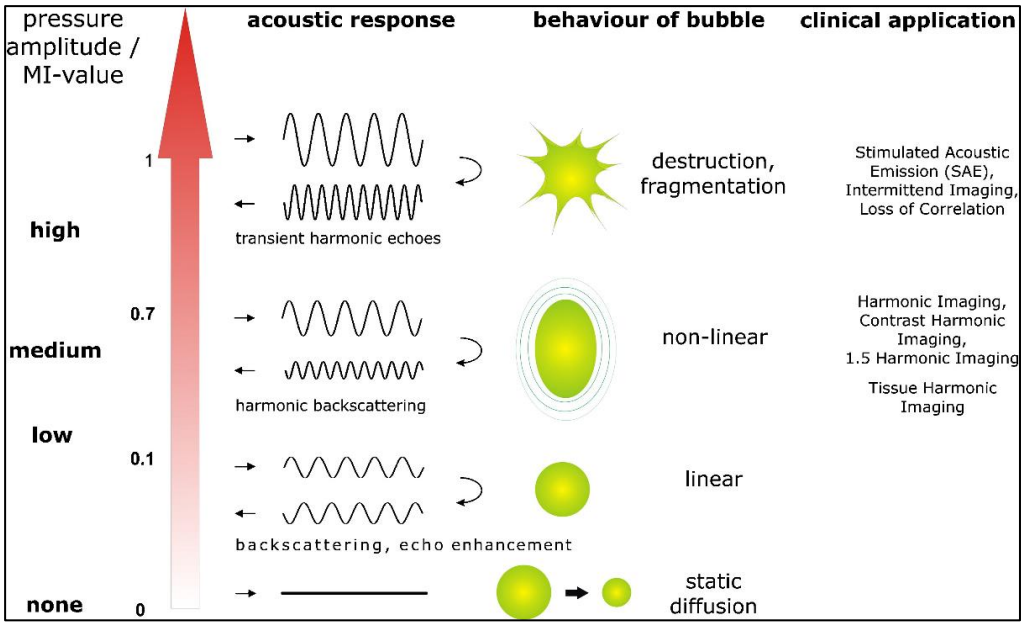


Figure 1.5: Diagram of microbubble behaviour at different ultrasound amplitudes of the propagating pulse with mechanical index (MI) values and the corresponding clinical imaging applications. Adapted from (Kollmann, 2007).

1.6.1.3 Pulse-inversion harmonic imaging

Pulse-inversion harmonic imaging (PIH) was developed to avoid harmonic imaging bandwidth limitations by subtracting rather than filtering out the fundamental signals (Burns et al., 2000). In pulse-inversion harmonic imaging, two pulses are transmitted rapidly into the tissue, where the second pulse is a mirror of the first. These inverted pulses are reflected by normal tissue, which normally responds linearly as identical but opposite echoes, yielding a sum of zero. However, as the bubble response is nonlinear, the reflected echoes from the two transmitted pulses are not identical inverted pulses. Therefore, the sum of the echoes is a detectable bubble signal rather than zero (Figure 1.6),

which forms the basis for most bubble-specific imaging methods using modern scanners (Simpson et al., 1999).

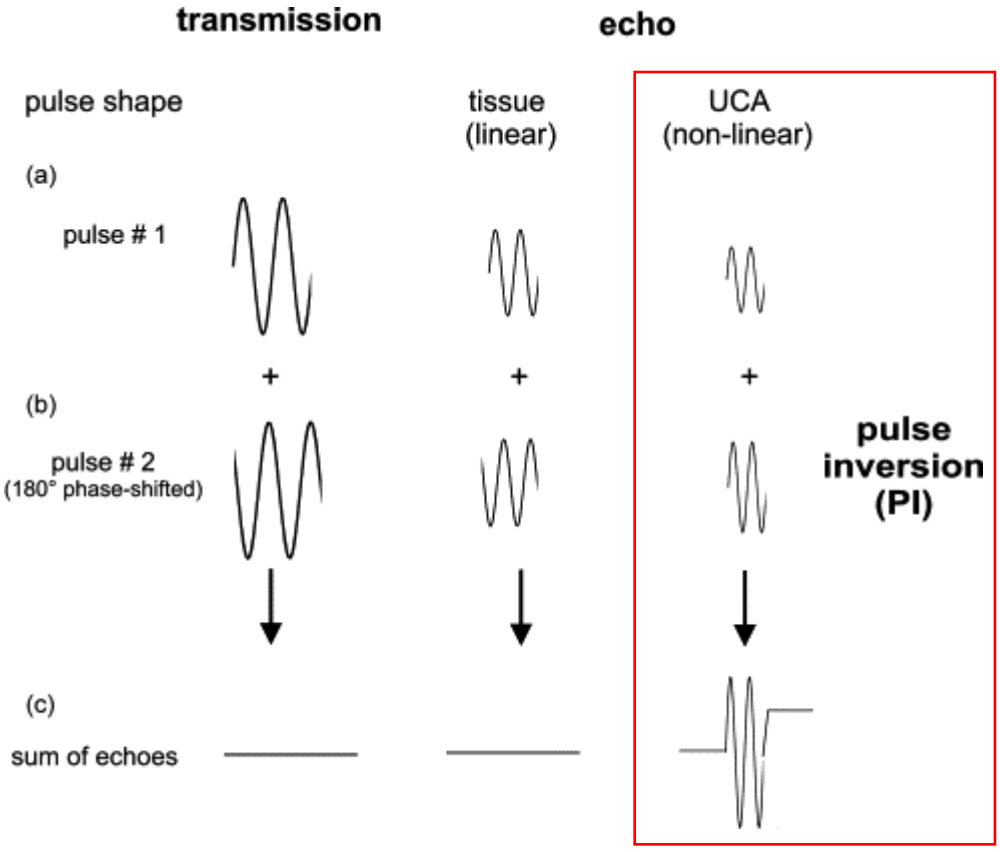


Figure 1.6: Pulse-inversion method. (a and b) Short pulses are transmitted, and the second pulse is inverse. (c) The echoes from tissue are identical forms but inverted with different amplitudes, which are cancelled when summing the two echo sequences. The echoes of microbubbles or the ultrasound contrast agent (UCA) do not cancel and yield a signal at summation. Adapted from (Kollmann, 2007).

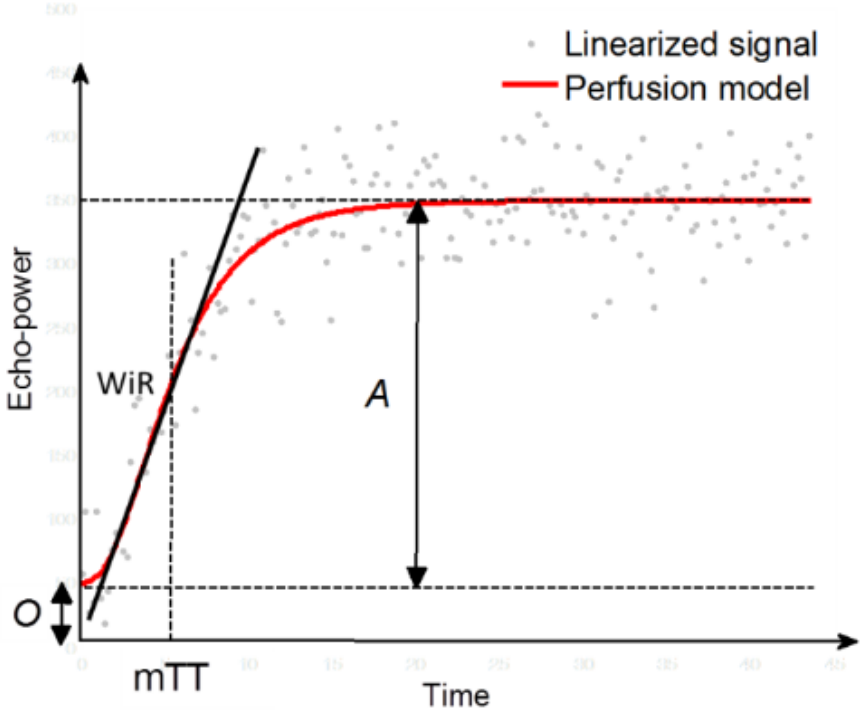
1.6.1.4 Contrast Enhanced Ultrasound Perfusion Imaging

Assessment of microvascular perfusion may provide crucial insight into the pathophysiology of different diseases. Recently, CEUS has emerged as a novel method for perfusion imaging and has shown encouraging

potential as a portable, cost-effective, real-time substitute for MRI and CT imaging.

The continuous infusion of contrast provides a steady state of microbubbles in plasma after 1-2minutes. Once the steady state is achieved, brief (flash) frames of high acoustic power US waves, represented by the mechanical index (MI), are applied to burst all of the infused microbubbles in the imaging plane. Lower acoustic powers are then used to record the refilling of microvessels (replenishment) immediately afterwards (Wei et al., 1998). Time-intensity curves (TICs) are generated by plotting the acoustic index (intensity) against mean transit time as the CA replenishes the tissue for offline quantitative analyses, from which several parameters that express tissue haemodynamics can be drawn. For replenishment kinetics, the following perfusion parameters are used to describe perfusion: Acoustic index (AI or A) in arbitrary units (a.u.), sometimes referred to as the relative blood volume, is the plateau or maximal intensity after reperfusion; mean transit time (mTT) in seconds, which is the time needed after bubble destruction to reach 50% of the maximal intensity; and perfusion index (PI) in a.u., which is the ratio of AI to mTT. The Wash-in rate (WiR) in a.u., reflects the maximum slope of the TIC. (Figure 1.7 A). For Bolus kinetics, other parameters are reported such as the peak enhancement, time to peak, rise time, and wash-in rate, where blood flow is the ratio of peak enhancement to rise time (Figure 1.7 B).

A


Linearized signal
Perfusion model
Echo-power
WiR
A
O
mTT
Time

B

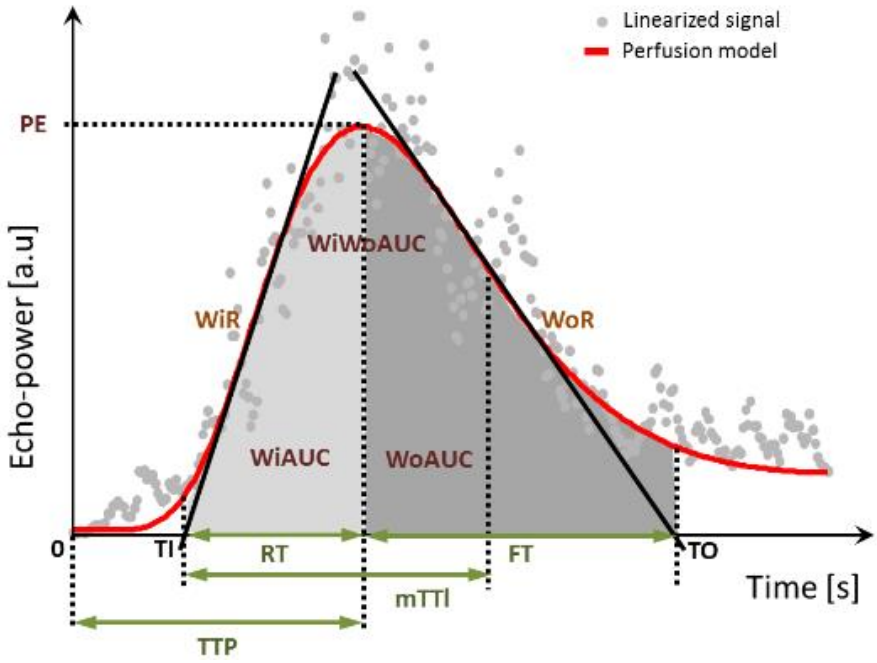

Linearized signal
Perfusion model
PE
Echo-power [a.u]
WiWoAUC
WiR
WoR
WiAUC
WoAUC
0
TI
RT
FT
TO
mTTl
TTP
Time [s]

Figure 1.7: adapted from Bracco manual.(Bracco, 2019). A: TIC for the replenishment kinetics. B: TIC generated from the bolus technique.

1.6.1.5 Infusion Versus Bolus Contrast Administration

Ultrasound contrast agents can be administered either via continuous infusion or bolus injections. The choice between the two approaches is contingent upon study indication and the required outcome. While both approaches provide similar tissue enhancement, there may be differences when CEUS is used for perfusion assessment. First, bolus injections reach the peak intensity quickly, but the enhancement lasts only for a brief time before it gradually drops. In contrast, infusion has longer-lasting contrast enhancement, as a contrast steady state is achieved (Figure 1.8) and is thereby often preferred for perfusion imaging. In addition, bolus requires an independent operator to perform the injection manually and continuously agitate the contrast in the syringe before injection, which is subject to variation in injection speed that may affect enhancement. On the other hand, infusion can be performed independently using an agitating pump. Moreover, the quick, concentrated enhancement in the bolus approach increases the likelihood of a glare artefact (bright signal enhancement beyond the true boundaries of a vessel), which is less likely with an infusion (European Society of Cardiology, 2020).

The bolus approach is well established for nonperfusion CEUS studies (e.g. liver lesion characterisation) and supported by the European

Federation of Societies for Ultrasound in Medicine and Biology guidelines. Nevertheless, no guidelines exist regarding the use of CEUS for perfusion studies.

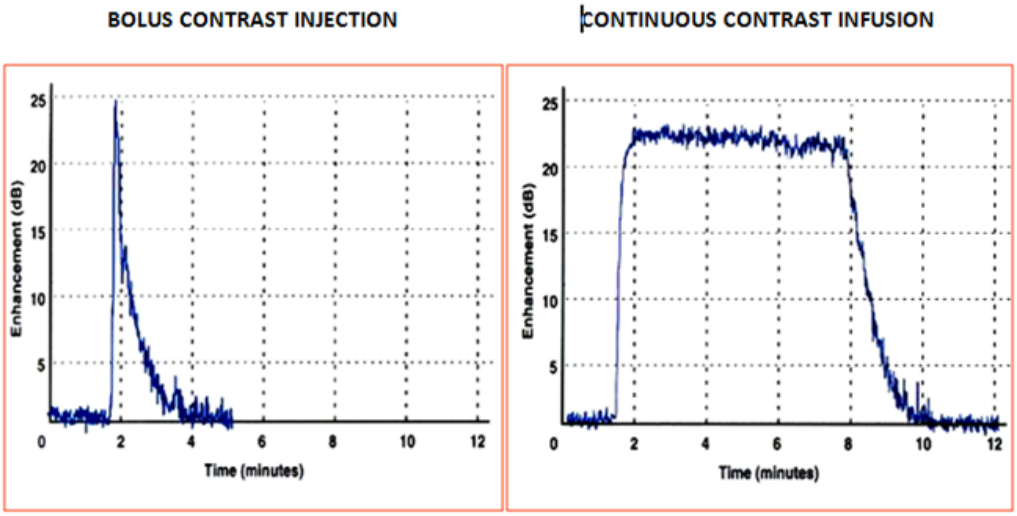


Figure 1.8: Concentration curves for a contrast agent after bolus injection or continuous infusion administration. Adapted from the (European Society of Cardiology, 2020).

1.6.1.6 Safety of the Ultrasound Contrast Agent

Ultrasound CAs exhibit an excellent safety profile. Unlike CT and MRI CAs, ultrasound microbubbles are eliminated via the lungs (exhaled) within minutes of administration and are not filtered by the kidneys. Therefore, they are not nephrotoxic and do not require renal or hepatic function tests prior to their use. Patients with renal failure and those on dialysis can undergo CEUS without concerns (Granata et al., 2012).

The International Contrast Ultrasound Society recently reported that the rate of serious reactions from iodinated contrast used (potential or

immediate) in CT was 1 in 5000 for low osmolar agents. In contrast, the rates of anaphylactoid and serious allergic reactions with ultrasound CAs were 1 in 15,000 and 1 in 500,000, respectively (International Contrast Ultrasound Society).

The incidence of adverse events (AEs) in humans is extremely rare, and those that occur are minor and self-resolving, including taste alteration, a warm sensation, general flush (Cohen et al., 1998, Myreng et al., 1999 , Kaps et al., 2016), headache and nausea, which have also been observed in control groups who received a placebo (saline) (QUAY and EISENFELD, 1997). The more serious anaphylactic or anaphylactoid reactions that have been previously reported with early ultrasound CAs are extremely rare (Torzilli, 2005). The rate of such serious AEs was 1 in 10,000 patients, as reported in two large studies with more than 100,000 patients(Wei et al., 2008, Piscaglia and Bolondi, 2006). SonoVue is contraindicated for patients with certain cardiac conditions, including recent acute coronary syndrome, acute myocardial infarction, coronary artery intervention, unstable angina, acute or Class III/IV chronic heart failure, or severe rhythm disorders (Torzilli, 2005).

The Food and Drug Administration approved SonoVue only for cardiac imaging, liver ultrasound, and paediatric voiding cystourethrograms; however, it has been used off-label for decades for kidney indications. The use of SonoVue for kidneys is accepted by the European Federation for the Society of Ultrasound in Medicine and Biology and the General Medical Council of Great Britain because sufficient evidence and experience demonstrate its safety and effectiveness.

1.7 Clinical significance of proposed work

Renal disease is a worldwide growing health problem that merits more attempts at understanding the underlying mechanisms and associated complications. There is accumulating evidence that changes in microvascular perfusion play a crucial role in the pathophysiology of kidney disease and its consequences. This is backed up by the basic physiological fact that an intact microcirculation is fundamental for cell survival and the function of every tissue in the human body as it provides oxygen, nutrients, hormones as well as the removal of toxins and carbon dioxide. The microcirculation is tightly regulated to meet the metabolic demands of organs and hence, impaired microcirculation can contribute to the progression or possibly the initiation of disease including kidney and skeletal muscle diseases (Basile, 2004, Bobik, 2005).

Proposed work in this thesis provides improvement in the understanding of the microvascular perfusion measurement methods using CEUS. In particular, the potential of CEUS in the assessment of the perfusion of the skeletal muscle of CKD patients as well as the kidneys of healthy volunteers and AKI patients are explored.

Skeletal muscle wasting (i.e., atrophy) is common in CKD patients including patients on HD. It increases the risk of falling and affect the quality of life and the ability to perform daily activities. Fatigue and longer recovery time is one of the most frequent complaints of dialysis patients. These are devastating complications that have significant impact on

morbidity and mortality. The aetiology of muscle wasting is multifactorial, and our understanding of the underlying pathophysiology is incomplete, hindering preventative measures. Therefore, exploring how microvascular perfusion alteration may play a role as a potential underlying mechanism is of great significance to develop effective interventions. In particular, interventions may include modifying haemodialysis to lessen the impact of haemodynamic changes, or in the more distant future new drug therapies that target angiogenesis.

Each muscle fibre (cell) is surrounded by a network of capillaries allowing for the efficient exchange of oxygen and nutrients required for contraction and the rapid removal of waste products. In dialysis patients, oxygen transport is impaired in skeletal muscle (Sala et al., 2001). Since not all capillaries are perfused in the resting muscle, studies have been performed on muscle during exercise when precapillary sphincters relax and allow perfusion of more capillaries, and oxygen uptake during exercise was also reduced (Marrades, 1996, Stray-Gundersen et al., 2016). Muscle biopsies showed altered capillary structure, with an increased thickness of the basement membrane and the capillary endothelium (Stray-Gundersen et al., 2016), suggesting that microvascular changes contribute to diminished exercise tolerance in CKD. However, measurement tools that can visualise and quantify skeletal muscle microvascular perfusion at bedside are not readily available.

The second half of this thesis includes a clinical application of CEUS in AKI. AKI is associated with increased risks of adverse outcomes such as

progression to chronic kidney disease (CKD), end-stage kidney disease (ESKD), and mortality. Therefore, early diagnosis, treatment, and proper follow-up are essential. The pathophysiological mechanisms of AKI are still not fully understood. However, it is well known that alterations to renal microcirculation and subsequent impaired oxygen delivery to the respiring renal cells play a major role in renal dysfunction (Zafrani and Ince, 2015, Guerci et al., 2017).

The kidney receives blood from the renal artery which gives rise to a complex microcirculatory network that provides sufficient intraglomerular pressure, peritubular capillary pressure, perfusion, and oxygenation to sustain glomerular filtration (GFR) and function. This microcirculation is uniquely orchestrated via renal autoregulation mechanisms mainly via the myogenic response and the macula densa tubuloglomerular feedback (MD-TGF) response. Autoregulation keeps renal blood flow and GFR constant over a defined range of blood pressure (80–180 mmHg), resulting in complete recovery of renal function. However, a marked reduction in renal perfusion may overwhelm the autoregulation and precipitate an acute fall in GFR. In patients with impaired autoregulation, the GFR will fall even if the mean arterial pressure remains within the normal range and hence, the delivery of oxygen and metabolic substrates becomes inadequate. In fact, the kidney is vulnerable to ischemic injury due to the complexity of the renal microvasculature and tubular system, and the high energy demand in the kidney from its functional workload.

Previous experimental studies have shown that microvascular flow can be reduced even with preserved macrovascular flow (Maiden et al., 2016, Seely et al., 2011, Calzavacca et al., 2015). Hence, whether alterations of microvascular renal blood flow are due to insufficient haemodynamic optimization or to specific microvascular injuries needs further clarification. In addition to that, the few studies on animal microvascular change during sepsis-induced AKI show that microvascular perfusion within the kidney is variable (normal (Auguste et al., 1980), increased (Ravikant and Lucas, 1977), or decreased (Calzavacca et al., 2015). Therefore, measurement of renal microvascular perfusion is necessary to guide therapy, and CEUS has great potential as it is a bedside technique that is eminently appropriate for the acute clinical setting. For instance, fluid resuscitation is a cornerstone in the treatment of sepsis and it aims to promote tissue perfusion and oxygenation by the preservation of adequate intravascular volume and the maintenance of blood pressure (Vincent and Gerlach, 2004). However, there is little evidence to guide clinicians in its administration. Fluid resuscitation can have severe deleterious effects on microcirculation and haemodilution may contribute to AKI (Johannes et al., 2006). Both excessive and restrictive fluid administration have been found to be associated with renal failure in different circumstances (Payen et al., 2008). Therefore, determining the optimal fluid volume to administer lacks guidance.

Current available measures such as mean arterial pressure do not provide direct measures of renal microcirculation and since renal hypoperfusion may persist (Prowle et al., 2012) even with optimised

macrovascular haemodynamics, assessment of intrarenal blood flow is paramount in understanding the pathophysiology of AKI. Therefore, there is a crucial need for a measurement tool to assess renal microvascular perfusion in daily practice.

CEUS is a direct, quick and repeatable measure that can be performed at the bedside that has the potential to offer a fresh insight into the skeletal muscle of dialysis patients and into renal microcirculation and the development of AKI. The findings of this work may inform future researchers, and may ultimately help establish guidelines, that are currently lacking, for tissue perfusion quantification using CEUS in preparation for its incorporation in the clinical practice. Patients will also ultimately benefit from the findings as better understanding of perfusion changes can guide therapeutic interventions.

Another area in which CEUS can be applied, though beyond the scope of this thesis, is in CKD. CKD is a progressive disease with increased rates of cardiovascular disease. It is caused by a number of clinical conditions including diabetes mellitus, hypertension, and glomerulonephritis. However, once renal fibrosis (a major consequence of CKD) reaches a certain threshold, CKD progression becomes irreversible and independent of the initial cause (Fine et al., 2000). Thus, renal hypoxia acts as a marker and a mechanism in a final common pathway of CKD progression (Nangaku, 2006, Mimura and Nangaku, 2010).

Hypoxia occurs as a result of inadequate oxygen supply or due to excessive energy demands in the context of a continuous blood supply. Even though the kidney is highly perfused organ, it paradoxically uses no more than 10% of the oxygen delivered by the renal artery with most of the blood transported to the renal cortex and only 10% – 15% of blood delivered to the renal medulla. The kidneys are susceptible to hypoxia, probably due to their unique microvascular structure in which arterial and venous vessels run strictly parallel. The oxygen shunt between arterial and venous vessels can bypass the capillary bed and blood circulation and make the oxygen tension in renal tissue relatively low (Schurek et al., 1990). Therefore, impaired renal perfusion may lead to CKD progression. CEUS could potentially be utilised to further confirm this hypothesis in clinical cohorts by providing a more sufficient understanding of perfusion. It then could help researchers develop new drugs that target the vasculature (e.g. angiogenic molecules) and the reduction in perfusion, which ultimately may be of key importance to improve quality of life and survival of CKD patients.

1.8 Summary

Kidney diseases (such as AKI or CKD) and muscle wasting in ESKD are common disorders that may be driven by changes in perfusion. This chapter has provided a brief introduction to the kidney and skeletal muscle anatomy along with short backgrounds on the pathophysiology of AKI, CKD and muscle wasting in CKD. The principles of CEUS imaging with a focus on infusion-based CEUS-perfusion imaging are also presented, because this is the technique that I have used in this thesis to study kidney and muscle perfusion in relation to patients with kidney disease. These clinical studies will be presented in the subsequent chapters.

1.9 Thesis aims

The aims of this thesis are as follows:

1. To summarise the evidence around the short-term effects of HD on skeletal muscle perfusion, metabolism, and function. Hence, establishing prior knowledge on HD-induced circulatory stress on the microvascular perfusion of human skeletal muscle.
2. To investigate the acute effects of HD on skeletal muscle microvascular perfusion and macrovascular blood flow. I hypothesise that HD results in a reduction in skeletal muscle perfusion, which could be a factor in skeletal muscle wasting in this patient group.

3. To seek supporting evidence for this hypothesis by performing a secondary analysis of a study in which hand-grip strength, an indicator of muscle function, was collected prospectively and at serial timepoints in dialysis patients and investigate the associations with dialysis related parameters such as blood pressure and ultrafiltration volume that are known to contribute to dialysis-induced vascular bed injury (hypoperfusion).
4. To optimise the protocol and methods for performing renal CEUS to measure renal perfusion in healthy volunteers and to establish the repeatability of CEUS-derived perfusion parameters.
5. To describe the variation in renal microvascular perfusion in AKI patients and to explore the association between CEUS measures of renal cortical perfusion and clinical measures of AKI. I also aim to determine the feasibility of the application of CEUS in the acute care setting. I hypothesise that CEUS can provide a bedside measure of renal cortical perfusion changes during early AKI onset and that it associates with AKI clinical measures.

Chapter 2: A Systematic Review of the Acute Effects of Haemodialysis on Skeletal Muscle Perfusion, Metabolism and Function

This chapter presents a systematic review of published studies that assessed intra-dialytic changes in skeletal muscle perfusion, metabolism, and function that I performed prior to planning my first clinical study to use CEUS in haemodialysis patients to address whether dialysis resulted in reductions in skeletal muscle perfusion. Fourteen studies were finally included and assessed for methodological quality and adequacy of reporting. The primary outcome measures of muscle perfusion, metabolism and function were summarised and discussed.

2.1 Abstract

Introduction: The underlying mechanisms of skeletal muscle wasting in haemodialysis patients are complex. I performed a systematic review to summarize evidence on whether haemodialysis has acute effects on skeletal muscle perfusion, metabolism, and function.

Methods: The protocol was registered on PROSPERO (Registration number CRD42018103682). A systematic search was performed in MEDLINE, PubMed, Cochrane, Embase, Scopus, and Web of Science. Citation, and reference list were also performed. Studies were selected in 2 stages: title and abstract review, then full-text review.

Results: A total of 65 full-text articles were reviewed, and 14 studies were eligible for inclusion. No studies were identified that assessed

muscle perfusion during dialysis. Two studies used near-infrared spectroscopy to indirectly measure skeletal muscle oxygen consumption, which increased during dialysis in 1 study but only in patients with diabetes in the second. Metabolism was examined in 9 studies. A number of acute metabolic changes were reported (e.g., caspase-3 activity, polyubiquitin, and interleukin-6 protein increased in response to haemodialysis) as was a net negative protein balance over the dialysis session. Three studies examining muscle function did not produce consistent findings.

Conclusion: Gaps remain in understanding the acute effects of haemodialysis on skeletal muscle, particularly for changes in perfusion and function, although there does appear to be an acute effect on muscle metabolism.

2.2 Introduction

Skeletal muscle wasting is a common complication of HD. It is seen in 18-80% of patients, and is associated with mortality, lower quality of life, reduced activity and immunity (Roubenoff et al., 1997). The underlying mechanisms of muscle wasting are complex and unclear, with several factors identified to which muscle wasting could be attributed. These factors include nutritional deficiency, hormonal abnormalities, chronic inflammation, metabolic acidosis, and regular hospitalizations. There is evidence that endurance and resistance exercises may ameliorate reductions in muscle strength (Gianola et al., 2013). However,

comorbidity, psychological status or logistical barriers often interfere with easy and sustainable exercise (Bennett et al., 2010, Roshanravan et al., 2017a). There have also been suggestions that the dialysis treatment per se is implicated in muscle wasting. Some studies examining the metabolic effects of HD have reported that it exerts an acute catabolic effect on whole body and muscle protein (Ikizler, 2012, Rhee and Kalantar-Zadeh, 2014). In parallel, evidence has grown to show that circulatory stress induced by HD causes hypo-perfusion in certain vascular beds. Specifically, myocardial stunning and cerebral ischemia have been reported in the heart and the brain respectively (Polinder-Bos et al., 2018, McIntyre, 2010a). Our aim was therefore to perform a systematic review to provide a summary of the best available evidence on the acute effects of haemodialysis treatments on skeletal muscle perfusion, metabolism, and function in ESKD patients.

2.3 Methods

A systematic review of the published literature was conducted on the acute effect of haemodialysis on muscle perfusion, metabolism, and function according to the PRISMA checklist statement. The methods were registered at PROSPERO (CRD42018103682) prior to commencement. The research question was formulated according to PICO (population, intervention, comparison, outcomes) strategy (Table 2.1).

P	Population	end-stage renal disease patients receiving in-centre haemodialysis
I	Intervention	Haemodialysis
C	Comparison	pre- versus post-haemodialysis, or pre- versus intra-haemodialysis
O	Outcomes	skeletal muscle perfusion, metabolism, or function

Table 2.1: PICO terms

Inclusion criteria for studies were set according to PICO. Articles were included if they: 1) involved human adult ESKD patients receiving any form of HD or haemodiafiltration (HDF); 2) compared an outcome of interest before and after or before and during dialysis; 3) reported the effect of HD or HDF on at least one of skeletal muscle perfusion, metabolism, or function. Articles were excluded if they: 1) only involved animals; 2) only focused on any other renal replacement therapy (e.g., peritoneal dialysis or transplantation) as an intervention; 3) were review articles; 4) were studies of children. Studies that included more than one group, one of which met the inclusion criteria, were included if data could be extracted separately.

The search strategy was restricted to adult human studies presented in English language, but there were no restrictions in study design, year of publication, or geographic area. However, the nature of the intervention required a hospital or a medical/haemodialysis centre setting.

The systematic search was carried out from 13 July 2018 to 27 July 2018. The following databases were searched from their inception: MEDLINE,

PubMed, The Cochrane Central Register of Controlled Trials (CENTRAL), EMBASE, Scopus, and Web of Science (core collection). All citations were imported to Endnote for deduplication, screening, and management. Full text articles were retrieved by Endnote. If not retrieved, articles were found through online database searches and imported to Endnote as an attachment. The applied search limits in each database along with the date of search can be found in Table.2.2

Database	*Limits*	*Date*
Cochrane	None	15 July 2018
PubMed central	English –Human	13 July 2018
Medline ovid	English-human and adult	July week 4, 2018
EMBASE	English-human	July week 4, 2018
WOS *Core collection*	No limit	19 July 2018
SCOPUS	English only	27 July 2018

Table 2.2: Databases' search limits

In addition, using WOS, a citation author search was performed to identify earlier and more recent studies from key papers that were identified from the initial database search. Reference lists for the identified studies were systematically searched for potential studies that may have been missed by electronic database searches. Free text and Subject Heading key terms were used to ensure a thorough search. Also, word synonyms, relevant abbreviations, alternative spellings, and

potential spelling mistakes were considered in the search strategy. Boolean line-by-line searches for each database can be found in Appendix A. Selection of studies was performed according to the eligibility criteria. It involved two stages: Title and Abstract Review, and Full-Text Review. The Title and Abstract Review was performed by a single author (SA), whereas the Full-Text Review was performed on all retained articles from stage 1 by two authors with disagreements resolved by a third reviewer. Checklist and questions for these stages can be found in Appendix B.

The methodological quality of included studies was assessed using the Critical Appraisal Skills Programme (CASP) tool for cohort studies (CASP, 2018). The appraisal was carried out by two individual reviewers (SA & SH). Disagreements were resolved by a third reviewer (NS).

A data extraction form tailored to the review questions was designed by (SA) and used to extract data from selected studies Appendix C. Extraction was carried out by two authors (SA, SH) and cross-checked by (NS).

2.4 Results

A systematic review flow diagram is shown in Figure 2.1. A total of 1118 articles were screened, and 14 studies were eligible for inclusion. All studies were prospective. Characteristics of included studies are summarised in Table 2.3 and characteristics of patients included in studies in Table 2.4.

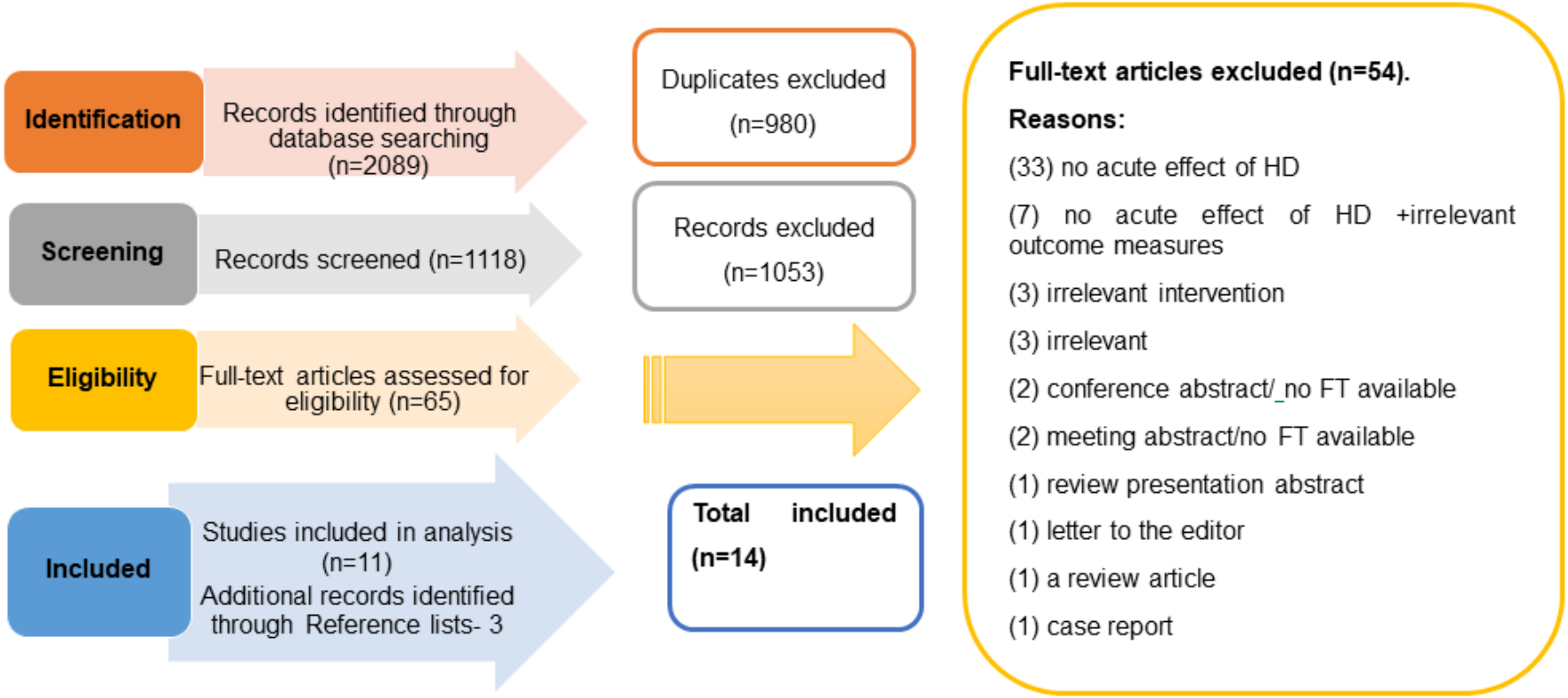


Figure 2.1: The systematic review flow diagram. HD: haemodialysis; FT: full text

AUTHOR	SAMPLE	OUTCOME MEASUREMENT TOOL
(Pipili, 2015)	20	• Near Infra Red Spectroscopy with vascular occlusion test.
(De Blasi, 2009)	20	• Near Infra Red Spectroscopy with vascular occlusion test.
(Cardoso et al., 1988)	3	• 31P- Magnetic Resonance Spectroscopy, using1.5 Tesla magnet and 6 cm surface coil
(LOFBERG, 1991)	8	• Muscle biopsies
(Taborsky et al., 1993)	7	• 31P Magnetic Resonance Spectroscopy, using 1.5 Tesla magnet and 8cm surface coil.
(IKIZLER ET AL., 2002)	11	• Primed constant infusion of stable isotopes tracers: L-[1-^{13}C] leucine and L-[ring-$^{2}H_5$] phenylalanine with a-v blood sampling.
(Raj, 2003)	12	• Muscle biopsy: mRNA levels of caspase-3, and ubiquitin. • Plasma levels of cytokines, interleukin-1 (IL-1), IL-6, and TNF
(Raj et al., 2004a)	9	• Primed constant infusion of stable isotopes tracer: L-[ring-$^{13}C_6$] phenylalanine with a-v blood sampling. • Blood samples to estimate fractional synthesis rates of albumin (FSR-A), fibrinogen (FSR-F) • Muscle biopsies to measure isotopic carbon enrichment, • Cytokines (IL-1, IL-6, IL-10, C-reactive protein and TNF-α)
(Raj et al., 2004b)	6	• Primed constant infusion of stable isotopes tracers: phenylalanine, leucine, lysine, alanine, and glutamine before and during HD with a-v blood sampling and muscle biopsies to measure isotopic carbon enrichment • Cytokines (IL-1, IL-6, IL-10, and TNF-α) in plasma samples
(Raj, 2005)	17	• Muscle biopsy, Femoral a-v balance of IL-1, IL-6, IL-10, and TNF-α cytokines were measured using ELISA kit. • Levels of cytokines quantification in the skeletal muscle
(Boivin, 2010)	8	• Primed constant infusion of stable isotope of L-(ring 13C6) phenylalanine and a-v blood sampling. • Muscle biopsy: Caspase-3 enzyme activity, TUNEL: To detect apoptotic DNA damage. Percentage of apoptotic cells was calculated by a pathologist, and IL-6 levels in skeletal muscle extracts were quantified/
(Saiki et al., 1980)	10	• Handgrip and quadriceps muscle strength.
(Harrison et al., 2006)	25	• Surface Electromyography • Set-To-Stand test
(Soangra et al., 2013)	6	• Sit-to-walk test

Table 2.3: Characteristics of included studies

Study ID	Sample	Age M/F	Gender M/F %	BMI	ESKD cause	Co-morbidity
(Pipili, 2015)	HD: 11 HDF: 9	69.5±12.0	Both gps: 75/25% HD: 82/18%;HDF: 67/33%	26.0±3.4 kg/m2	NR	Diabetes Mellitus: 5 (25%) HTN: 14 (70%)
(DE BLASI, 2009)	20 : 10 DM, 10 non-DM	DM gp (60.1 ± 10.1); non-DM gp (57.8 ± 11.5)	DM gp: 60/40% non-DM gp: 70/30%	NR	10: DN (DM gp); of non-DM gp: Lupus nephritis 1, PKD 2, nephrosclerosis 7	NR
(Cardoso et al., 1988)	3	NR	NR	NR	NR	NR
(Lofberg, 1991)	8	52.1± 24.89	50/50%	wt (kg): 58.2	6 chronic GN , 1 IgA nephritis , 1 nephrosclerosis and GN.	NR
(Taborsky et al., 1993)	7	48± 9	NR	NR	NR	NR
(IKIZLER ET AL., 2002)	11	43.8± 3.7	55/45%	28.3±1.9 kg/m2	2 (18%) DM 4 (36%) HTN, 2 (18%) GN, 1 (9%) APCKD , 2 (19%) Unknown	NR
(Raj, 2003)	12	46.1 ± 3.6	92/8%	wt(kg): 76.2 ± 14.4	NR	6 (50) diabetes %
(Raj et al., 2004a)	9	43± 5.9	83.3/16.7%	wt(kg)= 74.8±3.4	2GN , 2 HTN, 1 TIN , 2 DM, 2 unknown	diabetes: 2 (22.2) %
(Raj et al., 2004b)	6	43± 5.10	83.3/16.7%	23.6±1.2	1GN , 2 HTN, 1 TIN , 2 unknown	NR
(Raj, 2005)	17	44 ± 5.4	NR	wt (kg): 75.2 ± 5.5	2 HTN, 6 DN, 3GN, 2 TIN, 4 unknown	35.3% diabetic
(Boivin, 2010)	8	43±5.9	NR	wt (kg): 75.2±3.5	2 GN, 2 HTN, 1 TIN, 3 unknown	NR
(Saiki et al., 1980)	10	20-71 range	60/40%	NR	1. GN, HTN, heart failure, 2. nephroangiosclerosis, 3. TIN, 4. DN, 5. PKD, 6. GN, 7. IN, 8. GN, 9. SLE Nephropathy, 10. Hypertensive nephroangiosclerosis.	NR but myopathies were excluded.
(Harrison et al., 2006)	25	54.5± 2.6	64/36%	(M): 25.8 ± 1.3 kg/m2 ; (F): 22.4 ± 0.8 kg/m2	GN (5); NAS (3); PKD (6); renal failure (6); other or unknown (5)	NR, but excluded patients with malignancy, severe heart, lung or liver disease, and DM.
(Soangra et al., 2013)	6	54 ± 4	33/67%	NR	NR	NR, free of orthopaedic injury

TIN: tubulointerstitial nephropathy, PKD: polycystic kidney disease, HTN: Hypertension, GN: Glomerulonephritis, DN: diabetic nephropathy, DM: Diabetes mellitus, APCKD: autosomal polycystic kidney disease, CHRONIC IN: chronic interstitial nephritis, COPD: chronic obstructive pulmonary disease. NR: not reported.

Table 2.4: Patients' characteristics

2.4.1 Methodologic Assessment

Table 2.5 provides a summary of the methodological quality of the included studies. All the included studies had methodological weaknesses including risk of selection bias, measurement bias and confounding (Table 2.6). Adequacy of study reporting was also variable (Table 2.7).

	Clearly focused issue?	Acceptable recruitment? (Selection bias)	Exposure & outcome accurately measured? (Measurement bias), Objective, validated measures? Reliable system for	Conf. factors in the design/ analysis (Conf. bias) Restriction in design, or sensitivity analysis	Believable results? Big effect, consistency	Results can be applied locally?	Results fit with other evidence?	Implications for practice, robust evidence to change practice.	Score (yes:1;no:0,max possible 8)	Percentage of ' yes' scores
(Saiki et al., 1980)	Y	N	N	N	Y	Y	Y	N	4	50%
(Cardoso et al., 1988)	Y	N	N	N	Y	N	Y	N	3	38%
(Lofberg et al, 1991)	Y	N	N	N	Y	Y	Y	N	4	50%
(Taborsky et al., 1993)	Y	N	N	N	Y	Y	N	N	3	38%
(Ikizler et al., 2002)	Y	N	N	N	Y	Y	Y	N	4	50%
(Raj et al, 2003)	Y	N	N	N	Y	Y	Y	N	4	50%
(Raj et al., 2004a)	Y	N	N	N	Y	Y	Y	N	4	50%
(Raj et al., 2004b)	Y	N	N	N	Y	Y	Y	N	4	50%
(Raj et al, 2005)	Y	N	N	N	Y	Y	N	N	3	38%
(Harrison et al., 2006)	Y	N	N	N	Y	Y	Y	N	4	50%
(De Blasi et al, 2009)	Y	N	N	N	Y	Y	N	N	3	38%
(Boivin et al, 2010)	Y	N	N	N	Y	Y	Y	N	4	50%
(Soangra et al., 2013)	Y	N	N	N	Y	N	Y	N	3	38%
(Pipili et al, 2015)	Y	N	N	N	Y	Y	Y	N	4	50%
CASP contains 12 questions. No scoring system is provided by CASP, but for the purpose of this review scores were allocated as follows: "1" was awarded for a "yes", "0" for a '"no"; overlapping questions were merged into one point (Q3 and 4, Q 5a and 5b). This resulted in a maximum score of 8 points, with higher scores representing better methodology.										

Table 2.5: Summary of CASP quality assessment tool.

	Recruitment & selection bias:	**Confounding factors** **Potential confounding factors present or not reported**	**Measure bias**: **details of measure method/operator? Other measures?**
Pipilli et al., 2015	Small sample (HD: 11, HDF: 9), age range not reported	Patients' food intake and exercise history	No
De blasi et al., 2009	NA	Patients' food intake and exercise history, medication, dialysis access.	No
Cardoso et al., 1998	Small sample (only 3), patients' gender and age not reported.	Patients'gender, food intake & exercise history, medication, dialysis membrane & access, co-morbidity, baseline data were not compared with controls.	No Acetate HD was used.
Lofberg et al., 1991	Small sample (8), mean age (52 yr)	Exercise history, dialysis access, co-morbidity.	No
Tabarosky et al., 1993	Small sample (only 7 out of 21 chronic renal failure patients had pre-& post HD measurements), mean age was 48±9yr	Food intake & exercise history. medication, dialysis membrane & access, co-morbidity.	No
Ikizler et al., 2002	Small sample (11), mean age was 43.8yr	Food intake & exercise history, medication, co-morbidity	No, no muscle intracellular pool sample to measure protein turnover.
Raj et al., 2003	Small sample (12), 1 F:11 male. Mean age was 46yrs	Exercise history, dialysis access and vintage	No
Raj et al., 2004 a	Small sample (9). 1F:8 M. Mean age was 43 yrs	Exercise history, dialysis access and vintage	No
Raj et al., 2004 b	Small sample (6),1 F:5 M, mean age 43yrs	Exercise history, dialysis access, baseline data were not compared with controls.	No
Raj et al., 2005	Small sample (17), mean age was 44 yrs	Food intake & exercise history, dialysis access, vintage, gender.	Yes
Boivin et al., 2010	Small sample (8), mean age was 43 yrs	Exercise history, dialysis access, vintage, co-morbidity, gender.	No
Saiki et al., 1980	Small sample (10)	Exercise history, diabetes, co-morbidity, was not identified. some patients baseline data were not compared with controls	Yes
Harrison et al., 2006	NA	Patients' food intake & exercise history, dialysis membrane & access, baseline data were not compared with controls	No. Intra-subject variability was used
Soangra et al., 2013	Small sample (6), age range not reported, Females > males	Patients' food intake & exercise history, dialysis membrane, access & vintage, baseline data were not compared with controls	No. Intra-subject variability was used

Table 2.6: Included studies' recruitment, measurement, and confounding biases; NA: not applicable.

Author	**Judgment**	**Description**
Pipilli et al., 2015(Pipili, 2015)	Y	NIRS variables were fully reported in text/tables with P-values.
De blasi et al., 2009	Y	NIRS variables were fully reported in text/tables with P-values.
Cardoso et al., 1998	N	ADP values were not reported. No P-values for ATP and pyrophosphate accumulation but mean standard error SEM was reported.
Lofberg et al., 1991	Y	Concentration of ribosome content and amino acid is fully reported with p-values
Tabarosky et al., 1993	Y	p-value for Pcr/ATP ratio of for the pre-post dialysis values for the seven patients group.
Ikizler et al., 2002	Y	Fully reported with P –values
Raj et al., 2003	Y	Fully reported with P –values
Raj et al., 2004 a	Y	Fully reported with P –values
Raj et al., 2004 b	Y	Fully reported with P –values
Raj et al., 2005	Y	Fully reported with P –values
Boivin et al., 2010	Y	Fully reported with P –values
Saiki et al., 1980	Y	P-values for the pre and post dialysis mean (as collective) values of muscles strengths were reported.
Harrison et al., 2006	N	Electromyography signal Peak-to-peak amplitude and signal root mean square (RMS) data were not reported. Electromyography frequency pre and post dialysis are only presented in figure.
Soangra et al., 2013	Y	Fully reported with P –values

Table 2.7: Adequacy of study reportin

2.4.2 Primary Outcome measures

Measurement techniques varied among studies (detailed in Table 2.3 above). A meta-analysis was deemed inappropriate due to the differences in the studies' methodologies. Figure 2.2 illustrates the potential mechanisms contributing to the acute effects of HD on skeletal muscle metabolism and the lack of evidence regarding the acute effect of HD on skeletal muscle perfusion.

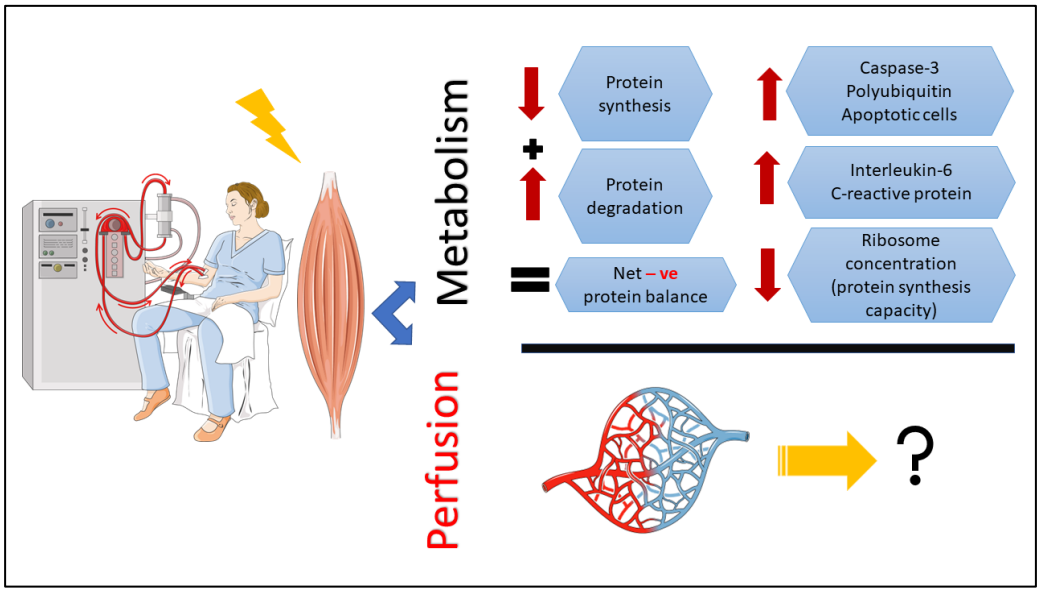


Figure 2.2: A diagram summarising the potential mechanisms contributing to the acute effects of HD on skeletal muscle metabolism and lack of evidence regarding the acute effect of HD on skeletal muscle perfusion.

2.4.2.1 Perfusion studies:

No studies were identified that measured changes in muscle perfusion in response to HD. Two prospective studies examined the acute effects of HD on skeletal muscle oxygenation and microcirculation using Near

Infra-Red Spectroscopy with a Vascular Occlusion Test (NIRS-VOT), This method involves placing the NIRS probe on the tissue of interest and placing a blood pressure cuff connected to a rapid inflation system above the joint. The purpose of the cuff is to cut off the blood flow to the distal extremity. The occlusion lasts for a few minutes before releasing the cuff. The NIRS device collects signal during the ischaemic occlusion and recovery period. NIRS-VOT measures a dynamic assessment of the tissue oxygenation response to ischaemia, the percentage of oxyhaemoglobin in total haemoglobin for a certain tissue volume (tissue oxygen saturation, StO2) and allows other measures to be derived that indirectly provide information on oxygen consumption (mVO2) and vascular reactivity.

In the study conducted by Pipili et al (Pipili, 2015), NIRS-VOT was used to assess thenar muscle microcirculation in patients undergoing HD and HDF. The only measure to change significantly after dialysis was mVO2 (24.5±7.5%/min versus 40±17.7%/min post dialysis, p = 0.03) but this was only observed in the HD subgroup. There was a non-significant trend towards an increase in vascular reactivity demonstrated by endothelial function percent per minutes post-dialysis in HD group (from 9.9± 5.5 pre-HD to 11.4± 6.5 post HD; p = 0.062), with no such trend apparent following HDF. Vascular reactivity was significantly lower in dialysis patients (9.1±5.6), as compared to healthy controls (15.7±6.3), p = 0.003.

De Blasi et al (De Blasi, 2009) used a different NIRS-VOT device applied to the gastrocnemius muscle. Two equal groups of participants (10 diabetic and 10 non-diabetic) were enrolled. The authors did not find any

change in StO2 in either group in response to dialysis. The calculated values for mVO2 results did differ between the diabetic and non-diabetic group. In the non-diabetic group, there was no change in mVO2 values during dialysis, whereas in the diabetic group, values increased during dialysis from 0.29±0.15ml/min/100ml to 0.72±0.21ml/min/100ml in the third hour and to 0.58±0.20 ml/min/100ml in the fourth hour of treatment. In both groups, total haemoglobin increased significantly from baseline during dialysis, reflecting haemoconcentration in response to ultrafiltration. There was also a rapid and significant decrease in microvascular compliance within the first hour of dialysis for both groups. This decrease was more pronounced in the diabetic group and microvascular compliance diminished further throughout the whole dialysis session in both groups. Microvascular reactivity (ml/mmHg/100ml) was 0.161± 0.110 and dropped to 0.061± 0.041 within the first hour in the non-diabetic group; $p< 0.05$ whereas in the diabetic group, it dropped from 0.221± 0.140 to 0.045± 0.051; $p< 0.05$ in the diabetic group withing the first hour. Microvascular compliance was calculated as haemoglobin concentration changes at different venous occlusion pressures and expressed as millilitres of blood increase in 100 ml tissue per change in pressure (mmHg),

2.4.2.2 Functional studies:

Three studies examined the acute effect of HD on skeletal muscle function. The study by Saiki et al (Saiki et al., 1980) produced diverse results at individual level, with the mean value for quadriceps muscle strength foot-pounds increased after dialysis from 53.2±30.4 to 58.8±

25.9; p=0.1 for a collective ten patients. Results for quadriceps muscle strength test at individual level showed that muscle strength increased after HD in six patients, decreased in three patients, and was unchanged in one patient. Similarly, results for handgrip strength for the whole group was increased from 54.4± 15.7 pounds before dialysis to 56.1± 15.8 pounds after dialysis but did not reach statistical significance (p-value was not reported). At individual level, the handgrip strength test showed that muscle strength had increased after HD in five patients, decreased in three patients, and was unchanged in two patients. Crucially, intra-individual repeatability of the test was not reported. In the study by Harrison et al (Harrison et al., 2006), surface electromyography was used on the hand (2nd dorsal interosseous) and on the leg (vastus lateralis). For the hand muscle, a comparison between pre-HD and post-HD showed a significant overall increase of 18Hz in signal frequency (a change towards normality after dialysis) pre-dialysis value was not reported but data were presented on a graph ($p<0.01$). In the leg, there was no significant change. Two studies, Harrison et al (Harrison et al., 2006) and Soangra et al (Soangra et al., 2013), examined Sit-To-Stand and Sit-To-Walk tests respectively before and after HD. Harrison et al (Harrison et al., 2006) reported a small number of complete stands from a sitting position within 30 seconds (6% from 12.0± 0.08 to 12.7±0.8; $p<0.05$) yet significant increase in the number of stands immediately following HD, compared to the pre-HD test. Soangra et al (Soangra et al., 2013) used a Sit-To-Walk test and observed a significantly slower rise in patients following the dialysis session. The average post-HD initial flexion

angular acceleration was 2.48°/s^2 compared to pre-HD value (3.23°/s^2) (p-value=0.02) and the time needed to produce peak flexion angular velocity increased (mean time extending from 0.47±0.04 to 0.79±0.07s post HD, p-value=0.03). It should be noted that in both studies, patients had not been trained prior to the Sit-To-Stand or Sit-To-Walk tests.

2.4.2.3 Metabolism studies

Nine prospective studies were included in this section of the review of which four studied protein turnover, while the remaining five studied static markers of metabolic changes.

2.4.2.3.1 Protein turnover

Protein turnover was measured in four studies using 2H, 13C, and15 N stable isotope labelling of amino acids. Phenylalanine was used in all of the four studies. Other utilized amino acids were leucine, lysine, alanine, and glutamine (Ikizler et al., 2002, Raj et al., 2004b, Boivin, 2010, Raj et al., 2004a). Ikizler et al (Ikizler et al., 2002) studied protein turnover using L-[1-13C] leucine and L-[ring-2H5] phenylalanine. The Infusion of the stable isotopes and blood sampling were done using the arteriovenous shunt created for haemodialysis in the forearm. Given the fistula was already in place, and providing the fistula blood flow was not <750 ml/min and/or without any recirculation, it was easier and tolerable to patients to use it for measurement of forearm synthesis. Muscle protein breakdown and synthesis were measured in three phases: before, during and after single dialysis session. Results showed that muscle protein breakdown was significantly increased during dialysis from baseline. Although

forearm protein synthesis also increased, the magnitude of increase was less than the increase in protein breakdown. This resulted in an increase in net forearm protein loss by around threefold during dialysis. In the post-dialysis period, forearm protein breakdown was significantly decreased from the dialysis period but remained significantly higher (84% greater) than the basal period. Similarly, forearm protein synthesis also fell from during dialysis to the post-dialysis period, but not back to baseline levels. However, net forearm protein loss was similar between basal and post-dialysis periods.

Raj et al.(Raj et al., 2004a) estimated the fractional synthesis rates and supporting the results of Ikizler et al, both muscle protein synthesis and breakdown increased significantly during HD. Again, the increase in muscle breakdown was higher than synthesis during HD, resulting in net muscle protein loss. Arteriovenous balance of amino acids was also measured. Results showed that phenylalanine concentration in the artery decreased from 86.1±7.7µmol/l to 67.6±6.4µmol/l ($p<0.01$) during dialysis whereas the venous concentration did not show significant change (86.6±7.4µmol/l versus 76.2±6.8µmol/l, suggesting intradialytic muscle breakdown.

Another study conducted by Raj et al (Raj et al., 2004b) studied intracellular amino acid transport kinetics and protein turnover using before and during HD. Arteriovenous balance was also measured. In addition, muscle biopsies were obtained to calculate intracellular amino acid transport and muscle protein synthesis and breakdown. The fractional synthesis rate was increased during HD (0.0521 ± 0.0043 vs.

0.0772 ± 0.0055%/h, $p<0.01$). Compartmental modelling showed that both protein synthesis and breakdown increased during HD ($p<0.01$), with intradialytic protein breakdown being greater than synthesis ($p<0.05$). These results suggest that HD alters amino acid transport kinetics and increases protein turnover with net increase in protein catabolism.

In the study conducted by Boivin et al (Boivin, 2010), skeletal muscle metabolism was measured with tracer labelling. Leg muscle protein synthesis and breakdown increased significantly during HD. However, the increase in muscle breakdown was significantly higher than synthesis during HD, resulting in a net negative protein balance. Table 2.8 shows protein breakdown and synthesis values.

Study	**Baseline muscle protein breakdown**	**Intra-dialytic muscle protein breakdown**	**Baseline muscle protein synthesis**	**Intra-dialytic muscle protein synthesis**	**Net muscle protein loss**	**Post dialysis Muscle protein breakdown**	**Post dialysis muscle protein synthesis**	**Post dialysis Net muscle protein loss**
Ikizler µg.100 $ml^{-1}.min^{-1}$	77±13	180± 17	56±8	123±19	from -22±7 to -58±17	127±19	98±16	-28±12
Raj et al (2004 a) $nmol.min^{-1}.100\ ml^{-1}$	40.7±2.4	83.1±3.6	39.1±7.3	54.7±4.0	from 1.5±1.9 to 29.1±5.3			
Raj et al (2004 b) $nmol.min^{-1}.100\ ml\ leg^{-1)}$	57.8 ± 13.8		28.0 ± 8.5		Protein breakdown being greater than synthesis ($p<0.05$).			
Boivin et al $nmol.min^{-1}.100\ ml$	41.63± 2.47	84.61± 3.65	41.19 ± 3.03	55.15± 4.48	net negative protein balance.			

Table 2.8: Protein breakdown and synthesis between studies

2.4.2.3.2 Protein breakdown markers

Five studies assessed skeletal muscle metabolism by measuring static markers of metabolic change. These markers were protein breakdown markers, inflammatory markers, a marker of protein synthesis capacity and metabolite levels (Cardoso et al., 1988, Lofberg, 1991, Taborsky et al., 1993, Raj, 2003, Raj, 2005). Skeletal muscle biopsy samples showed increased Caspase-3 enzyme at the end of dialysis in two studies from 0.50±0.01units to 0.81±0.04units (Raj, 2003), and from 25±40 to 38±42units (Boivin, 2010). In addition, polyubiquitin was reported to increase during dialysis (Raj, 2003). One study also reported a significant increase in the percentage of apoptotic cells in muscle samples obtained following HD (6.9%), as compared with pre-HD samples (4.3%) (Boivin, 2010).

2.4.2.3.3 Inflammatory markers

Interleukin 6 (IL-6) was measured in four studies: in blood plasma, a-v balance, and in muscle extract, all of which showed consistent results in terms of increased levels of IL-6 during dialysis (Raj, 2005, Raj et al., 2004a, Raj, 2003) and at the end of dialysis (Boivin, 2010). Raj et al (Raj, 2003) reported that plasma IL-6 significantly increased from 7.54±2.24pg/dL pre dialysis to 27.86±4.94pg/dL during dialysis; $p<0.001$. In a different study, the same authors also reported similar results (IL-6 increased from 11.53±6.73pg/dL to 27.86±14.83pg/dL during dialysis; p<001) (Raj et al., 2004a). In a third study (Raj, 2005), Raj et al demonstrated higher concentrations of IL-6 in the femoral vein than in the femoral artery (16.27±2.42 vs. 11.29±2.17 pg/dL; $p<0.01$) during dialysis.

femoral vein IL-6 (pg/dL) was 5.23±0.59 pre-dialysis; p<0.001). In the latter study, two patients underwent muscle biopsies for IL-6 prior to and at the end of dialysis, which showed an intradialytic increase of IL-6 in muscle from 0.028 ± 0.02 to. 6.69 ± 0.21; p < 0.01). IL-6 levels were also measured in the muscle extract in a study conducted by Boivin et al (Boivin, 2010) and again results showed increased IL-6 from 7.74± 8.1 pg/ml to 347.0± 209.2 pg/m post dialysis; p<0.001. Additionally, one study reported an increase in plasma IL-10 during dialysis from 0.63±0.73pg/dL to 2.22± 1.07pg/dL; p<0.01. Also C-reactive protein (mg/dL) was increased from 3.72± 0.89 to 4.12± 0.99; p<0.01 during dialysis (Raj et al., 2004a). Levels of IL-1 and TNF- α did not change significantly (Raj, 2005, Raj et al., 2004a, Raj, 2003).

2.4.2.3.4 Muscle energy metabolism

Distinct from studies that examined protein turnover, studies have also attempted to assess the acute effects of haemodialysis on skeletal muscle energy metabolism. Magnetic spectra from (31P) MRS shows resonances from Pi, PCr and ATP allowing their quantification. Additionally, other function-related measures can be retrieved indirectly from the 31P spectra: ADP, and intracellular pH (Prompers et al., 2006). 31P MRS was used in two studies to assess the effect of haemodialysis on skeletal muscle energy metabolism (Cardoso et al., 1988, Taborsky et al., 1993). In both studies, gastrocnemius muscle was assessed. The aim of the study conducted by Cardoso et al. was to examine the effect of dialysis with an acetate buffer on the concentration of phosphate-containing metabolites in the muscle. The MRS spectra were obtained

before and during dialysis and it was reported that Muscle ATP and ADP concentration did not change significantly during dialysis, and no significant pyrophosphate Ppi accumulation was noted (P-values were not reported in the original study). Although the authors concluded that dialysis did not affect the energy status of the gastrocnemius muscle, the study included only 3 patients. In the study conducted by Taborsky et al, MRS was performed on seven patients before and after dialysis. PCr/Pi ratio before dialysis was 1.18± 1.17 and showed a slight but significant increase after dialysis to 1.22±0.11; p= 0.010).

2.4.2.3.5 Ribosome concentration

Ribosome concentration is a marker of protein synthesis capacity. In a study conducted by Lofberg et al (Lofberg, 1991), muscle biopsies were performed to assess ribosome concentration before and after dialysis. Total ribosome concentration was expressed as optical density units per mg of DNA. The results showed that total ribosome concentration declined by 22.8±6.7 optical density units per mg of DNA from a basal pre-dialysis value of 71.3±7.4 OD units/mg of DNA (p=0.02). The relative proportion of polyribosomes also declined by 3.2±1.35% of total ribosomes as compared with pre-dialysis ($p<0.05$), which indicates lower capacity for protein synthesis in dialyzed patients.

2.4.3 Secondary outcomes:

The following secondary outcomes of the systematic review were not assessed due to insufficient data: correlation between change in muscle

perfusion, metabolism or function and clinical variables or dialysis details; effect of patient age on the primary outcomes; and effect of dialysis vintage on the primary outcome

2.5 Discussion

In this systematic review of 14 prospective studies, I aimed to assess the acute effects of HD on skeletal muscle perfusion, metabolism, and function. This is a relatively under-studied area, and all of the included studies were of low to medium methodological quality. Despite this, there were consistent results regarding the effects HD on skeletal muscle metabolism, generally suggesting an acute increase in protein breakdown during dialysis, associated with an inflammatory response. However, studies investigating the effect of dialysis on muscle perfusion and function have shown diverse findings from which it is not possible to draw definite conclusions.

Skeletal muscle wasting is a common complication of HD, occurring in 18-80% of patients (Roubenoff et al., 1997) and is associated with significant morbidity and mortality rates (Huang et al., 2010, Morishita, 2014). Mechanisms leading to muscle wasting are complex. Putative causative factors include nutritional deficiency (Ikizler and Hakim, 1996), hormonal abnormalities (Fouque et al., 2000), chronic inflammation (Avesani et al., 2006), metabolic acidosis (REAICH et al., 1992) and gastroparesis (Bammens et al., 2003). Over recent years, there has been a recognition that the acute effects of dialysis are implicated in a variety

of pathophysiological processes. For example, HD can result in acute reductions in blood flow to the heart and brain that over time results in ischaemic damage and organ dysfunction (McIntyre, 2010a, Polinder-Bos et al., 2018). Our aim was therefore to review the current literature and assess the current evidence as to whether the dialysis process may contribute to pathological changes in skeletal muscle. In addition, the observation that dialysis patients often have long recovery times following HD treatments raises the question as to how muscle function may be affected by haemodialysis (PL et al., 2011, Rayner et al., 2014, W.F. et al., 2017)

My review suggests that there is limited evidence as to whether HD results in altered perfusion of skeletal muscle. The studies by Pipili et al.(Pipili, 2015) and De Blasi et al. (De Blasi, 2009) did not demonstrate changes in tissue oxygen saturation. Increases in muscle oxygen consumption were reported, suggesting an increase in muscle oxygen utilisation during dialysis, although these were not universally observed, and a number of other measures did not change. Some discrepancy between the two studies could be due to the different muscle groups studied (thenar muscles versus gastrocnemius) and differences in the NIRS models. Additionally, both studies categorized participants into two subgroups, (in one study HD and HDF groups and in the other diabetic and non-diabetic), which made the already modest sample sizes yet smaller. I found no studies that directly studied muscle perfusion, and from this review it is not possible to draw any conclusions as to whether HD alters muscle perfusion.

Similarly, I found very limited data to inform the effect of HD on short-term muscle function. The three studies used different methods of assessment, produced conflicting results and were of small sample size. In contrast, much more is known about the change in muscle mass and function over time. It has been shown in several studies that dialysis patients have reduced muscle strength than healthy subjects (Pupim et al., 2005a, Pupim et al., 2005b, Workeneh, 2010, Fouque et al., 2011, McIntyre et al., 2006b). When compared with controls, patients on dialysis are weaker, walk slower, and show slower phosphocreatine recovery following exercise which results in slower recovery from muscle contraction. The latter implies a functional defect in energy metabolism (Johansen et al., 2003, Kemp, 2004). Muscle mass and function also deteriorates over time, as reported in a study of peritoneal and haemodialysis patients, in which muscle mass and sit-to-stand test were assessed (John et al., 2013). This is particularly true with elderly patients. The incidence of sarcopenia In 131 patients receiving dialysis tested with BIA and grip strength was 13.7%, but was much higher at 33.3% in patients over 60 years (Ren et al., 2016). Also, in a cross-sectional study performed on 95 elderly ESKD patients, sarcopenia was highly prevalent (37.0% in males and 29.3% in females) (Kim et al., 2014).

A relationship to short-term changes during dialysis and longitudinal deterioration in muscle physiology was suggested by the results from the studies examining short-term changes in muscle metabolism during HD. This was an area that was examined in nine of the fourteen included studies, and in general their results were broadly consistent. In addition,

some of these studies used gold standard techniques such as muscle biopsy and tracer techniques with arteriovenous sampling. The invasive nature and technical complexity of this type of study goes some way to explaining the small sample size of these studies. A number of acute metabolic changes were reported. The gold standard for measuring protein turnover is the fractional synthesis rate and fractional breakdown rate with muscle biopsies to look at incorporation of tracer into muscle protein. This approach was used by Raj et al. (Raj et al., 2004a) to measure isotopic carbon enrichment of bound and free phenylalanine in the muscle. Results from this study showed an increase in muscle protein breakdown and net protein loss during dialysis. Whilst other studies also reported similar results, it should be noted that different methods were employed across the studies, meaning that direct comparisons are more difficult. For example, for a-v balance studies used either two-pool (Ikizler et al., 2002) or three pool models (Raj et al., 2004b, Boivin, 2010, Raj et al., 2004a). The two-pool model samples only from the artery and vein, making the assumption that skin, tendon, ligament and other tissues within the sampling bed do not contribute to amino acid levels, which may introduce a degree of inaccuracy. The three-pool model additionally takes samples from the muscle intracellular pool in an attempt to improve accuracy but there remains debate as to which approach is optimal.

The assessment of whole-body protein kinetics together with the muscle protein synthesis provides deeper insight into body protein status. Such combination of measurements allows for comparisons between metabolism in muscle and in the whole body. Studies on arm and leg for

regional protein turnover measurement have been conducted over the years using stable isotope methodology, where the rate of tracer uptake into skeletal muscle proteins can be evaluated from the arterio-venous difference. Additionally, in the cases where muscle biopsies have been taken, this enabled the direct estimate of fractional synthesis rate of muscle from the biopsy with the indirect estimate from amino acid uptake by the limb. The fractional synthesis rate is considered the gold-standard measure of skeletal muscle protein synthesis since measurement of arterio-venous difference can be affected by amino acid metabolism in other tissues and altered blood flow (Biolo et al., 1994).

To further support the findings of increased catabolism during dialysis, a number of studies reported acute increases of static muscle protein breakdown markers (Caspase-3 activity and Polyubiquitin), as well as increases of cytokines, especially IL-6, which has a major role in the balance between protein breakdown and synthesis in inflammatory conditions (Raj, 2003). It induces expression of genes regulating protein catabolism (Morishita, 2014), increases muscle protein breakdown (Johansen et al., 2003) and facilitates acute phase protein synthesis (Ikizler and Hakim, 1996). Whilst the mechanisms that may cause increased protein breakdown during dialysis are not fully described, the included studies also reported that these changes occurred in association with increased expression of inflammatory cytokines (Li et al., 2011, Niewczas et al., 2012) that may influence the metabolism of muscle protein.

Previous studies have demonstrated that systemic inflammation is associated with reduced rates of protein synthesis along with increased protein breakdown, and consequently loss of muscle mass (BIOLO et al., 2002). In HD patients, there is also a widely reported link between malnutrition, inflammation, and adverse outcomes, including decreased quality of life, increased hospitalization and greater mortality (Harrington et al., 1997, Castets and Ruegg, 2013, Sandri et al., 2004).

To the best of our knowledge, this is the first systematic review to examine the acute effect of a single HD session on skeletal muscle perfusion, metabolism, and function. There are some limitations, including that this review did not include studies published in other language than English and hand searches of journals were not performed. All included studies were of small sample size and of low to medium quality, which limits definitive conclusions from being drawn.

2.6 Conclusion

In conclusion, based on studies included in this systematic review, gaps remain in our understanding of the acute effects of HD on skeletal muscle and further research in this field is warranted. This is particularly true for changes in perfusion and physical functioning, whilst there does appear to be an acute effect of dialysis on skeletal muscle metabolism, with increased inflammatory signalling and catabolism.

Chapter 3: Muscle Stunning in Haemodialysis

Based on the conclusions from the previous chapter, there is a lack of evidence as to whether changes in skeletal muscle perfusion occur during dialysis. This phenomenon has been reported in other vascular beds (e.g. heart, brain), and could potentially have relevance to the acute intra-dialysis changes in muscle metabolism. Here, I present a clinical study where CEUS was applied for the measurement of skeletal muscle microvascular perfusion in patients receiving chronic haemodialysis. Femoral artery Doppler ultrasound was also performed to measure macro-vascular blood flow.

3.1 Abstract

Introduction: Skeletal muscle wasting is a common complication of haemodialysis (HD). It has adverse effects on physical function, frailty, and quality of life, but mechanisms leading to muscle wasting are complex. The aim of this study was to determine whether HD results in hypoperfusion of skeletal muscle as a potential mechanism of muscle wasting.

Methods: An observational study was conducted over the course of single dialysis sessions. Dialysis patients underwent intradialytic Contrast Enhanced UltraSound (CEUS) to measure skeletal muscle microvascular perfusion in the quadriceps muscle, and also had ultrasound Doppler of the mid-femoral artery to measure large artery blood flow. The CEUS studies were undertaken with a microbubble

contrast agent (Sonovue) that was infused through the dialysis venous line (post-dialyser), and three studies were performed: at time of dialysis initiation; after 30 minutes; and within 30 minutes of the end of dialysis. High frequency ultrasound pulses were used to destroy the microbubbles following which the time and intensity of microvascular reperfusion was recorded. One-way repeated measures ANOVA was performed to compare CEUS and Doppler measures between time points.

Results: Twelve patients were recruited, of whom 11 had satisfactory images included in the analysis. There were six males and five females. Median age was 52 (48 to 69) years. Mean ultrafiltration volume was 2.15 (0.86) L. The mean acoustic index (AI) plateau, mean transit time (mTT), and perfusion index (PI) were compared between the three time points of 9 patients who completed three CEUS exams and showed no significant difference between them. There was no difference in PI values between the three time points ($p = 0.52$) or in its components, AI ($p = 0.23$) and mTT ($p = 0.17$). However, there were some individuals who had lower perfusion values during dialysis. No difference in Doppler peak velocity values was seen between the time points ($p = 0.58$).

Conclusion: A well-established technique was applied in a new population to assess skeletal muscle blood flow during dialysis. HD did not result in acute changes to skeletal muscle perfusion, suggesting that intra-dialytic changes in muscle perfusion are unlikely to be a significant contributor to skeletal muscle wasting.

3.2 Introduction

HD is a lifesaving renal replacement therapy, but it is not without complications. A large body of evidence has accumulated to support the concept of HD-induced physiological and haemodynamic stress. This stress is responsible for acute complications, such as IDH and skeletal muscle cramps (Hassan et al., 2016, Wizemann et al., 2009) or accumulated complications over time, such as the irreversible myocardial fibrotic changes, chronic heart failure, arrhythmias, sudden cardiac death (Burton et al., 2009b) and ischaemic white matter brain injury (Eldehni et al., 2015a). Such evidence raises the question as to whether similar processes occur in skeletal muscle, which may then contribute to the acute catabolic changes during dialysis and subsequent skeletal muscle wasting. The systematic review in Chapter 2 found that HD results in catabolic effects on muscle metabolism, whereas literature on its effects on muscle perfusion remains scarce (Almushayt et al., 2020). Addressing this question has a pivotal role in expanding our knowledge about the complex processes behind muscle wasting experienced by HD patients. If HD results in hypo-perfusion to the skeletal muscle, it can be implied that recurrent ischaemic injury (with repeated HD) may contribute to skeletal muscle wasting in the long run.

A variety of studies have established that CEUS is a valid technique for quantifying microvascular muscle perfusion. As muscle perfusion changes markedly to meet metabolic demands, CEUS has been used to assess real-time muscle perfusion in response to various physiologic stimuli, such as exercise (Krix et al., 2010, Dawson et al., 2002), insulin

(Vincent et al., 2002, Clerk et al., 2004), and disease (Womack et al., 2009 , Amarteifio et al., 2011a). Its application using SonoVue® CA was established for the first time by Mitchell et al. (2013), who extended the evidence about muscle microvascular flow changes in response to nutrition.

At rest, skeletal muscle has a low blood flow of 5 to 10 $mL \cdot min^{-1} \cdot 100\ g^{-1}$), which is attributed to the relatively high vascular resistance caused by the inherent smooth vascular muscle myogenic tone and the activity of the supplying sympathetic nerves (Korthuis, 2011). It is also widely known that exercise increases blood flow in the skeletal muscle by up to 100 fold (Radegran, 1999). This increase may have motivated researchers to develop the rest-stress imaging method for skeletal muscle. At rest, the CEUS perfusion measurement of skeletal muscle fails to distinguish healthy subjects from those with peripheral artery disease with clinically evident skeletal muscle hypoperfusion. In contrast, stress imaging could detect that difference (Amarteifio et al., 2011b, Davidson et al., 2017).

We hypothesised in the present work that HD results in acute changes in skeletal muscle perfusion. Therefore, I aimed to apply CEUS for muscle perfusion measurement during HD for the first time and assess the acute change in muscle microvascular perfusion in response to a single dialysis session. In addition, I compared the Doppler peak velocity of the femoral artery before and after dialysis as a proxy for macrovascular changes (flow in large vessels).

3.2.1 Objectives

The primary outcome measures were to assess the changes, as follows:

- In skeletal muscle CEUS perfusion variables throughout a single dialysis session and
- In femoral artery Doppler variables throughout a single dialysis session.

The secondary outcome measures were to assess the following:

- The relationship between changes in muscle perfusion with clinical and dialysis treatment variables and
- The effects of age and dialysis vintage on the outcome measures.

3.3 Methods

3.3.1 Application of Contrast-enhanced ultrasound during haemodialysis

Prior preclinical studies (unpublished) demonstrated that the CA microbubbles were not destroyed or dialysed during the dialysis procedure, which was expected as they have rheological characteristics similar to red blood cells. Dummy dialysis circuits were set up with a saline reservoir, the contrast was infused, and the saline bag was scanned using an ultrasound transducer and CEUS settings for signal visibility. This visibility was demonstrated on the ultrasound screen with the clear and persistent presence of microbubbles that did not diminish over time in the saline.

3.3.2 Overview: Muscle stunning in haemodialysis

The current investigation is a prospective observational study involving assessments of skeletal muscle perfusion changes over a single dialysis session. All subjects were recruited from the dialysis unit at Royal Derby Hospital (RDH). Each subject underwent three CEUS scans: immediately with dialysis commencement, 30 min into dialysis, and 30 min before the end of dialysis. Femoral artery blood flow was also measured four times: predialysis, 30 min into dialysis, 30 min before the end of dialysis, and immediately after dialysis. Figure 3.1 illustrates the study design.

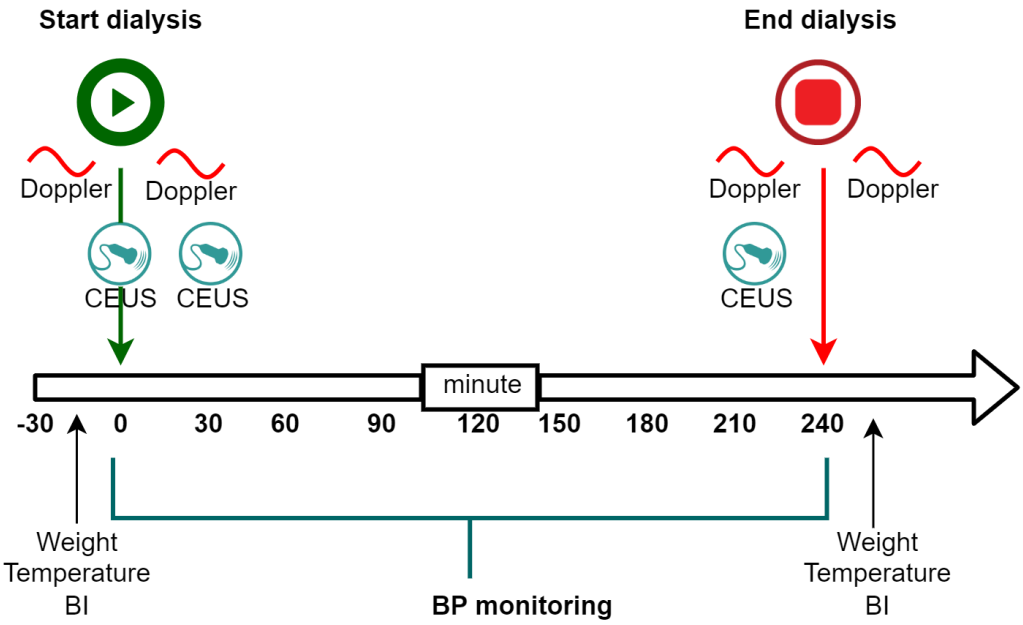


Figure 3.1: Flow diagram presenting measurements during a study day. BI: bioelectrical impedance measurement; BP: blood pressure.

3.3.3 Ethics approval

The study was approved by the East of Scotland Research Ethics Service (EoSRES reference number: 18/ES/0106). Approved protocol,

participant information sheet and consent form are attached in Appendix D, E, and F respectively). The study was conducted using the Good Clinical Practice standards.

3.3.4 Recruitment

A list of existing dialysis patients at RDH was screened for potential candidates via VitalData software. Candidates were approached during their usual dialysis sessions at RDH. A brief introduction to the study and a written participant information sheet (attached in Appendix E) were provided to read at home and consider. These candidates were revisited during their next dialysis session and offered an opportunity to ask questions. If they were interested in participating, they were asked to read, sign and date a consent form (attached in Appendix F). A screening log containing the patient's name, date approached, and decision was logged and kept confidential. One of the consenting patients was excluded from the study due to a subsequent illness.

Eligibility criteria were adults (18 to 80 years old) on HD for at least 3 months. They were excluded if they presented pre-existing myopathy, active malignancy, severe active chronic obstructive pulmonary disease, inflammatory bowel disease, known sensitivity to the CA, pregnancy, or lack of mental capacity to consent.

3.3.5 Study procedure

Patients were informed of the date of the study prior to the study day. The patients required no preparations prior to the study day except for optional clothing preparation (skirt or wide-leg trousers) to allow easy access to the thigh muscle.

Each patient was assigned a unique trial code for use on case report forms (the primary data collection tool) and other electronic files. Patients' demographics, medical history and current medications were collected from their electronic medical files. The details about the patients' smoking status and alcohol consumption were collected on the study day by asking the subjects directly while they were on dialysis. Dialysis details were collected from the dialysis monitor before the end of treatment.

BP was measured before and after dialysis and every 15 min during dialysis. Body weight, body temperature, and bioelectrical impedance analysis (BIA) measures were obtained before and after dialysis as described in Section 3.3.12.

Figure 3.2: Picture of a haemodialysis bay displaying the dialysis apparatus and dialysis bed.

3.3.6 Dialysis set up

The unit dialysis nurses/technicians set up the dialysis machine as usual. The dialysis treatment was delivered to each patient per their usual prescription, using their usual vascular access via arteriovenous fistula (AVF) or tunnelled central venous catheter (CVC).

3.3.7 Contrast preparation

SonoVue ® (Bracco, Italy) was used as the CA. Three contrast kits (one for each time point) were used for each patient. Prior to each CEUS exam, the contrast was prepared per the manufacturer's instructions (Bracco). The 25 mg of dry lyophilised powder was reconstituted by adding a 5-mL prefilled syringe of a sodium chloride 9 mg/mL (0.9%) solution provided in the kit. An additional 1 mL of sodium chloride solution was added to the infusion syringe, which was inserted into a dedicated oscillating pump (VueJect®, Bracco, Italy) for contrast infusion and agitation.

The infusion rate was 2 mL/min for the first minute and 1 mL/min for the rest of the exam. A plastic tube provided with the administration set from the same provider as the contrast, Bracco, was used as an infusion line connecting the infusion syringe to the dialysis machine's venous chamber port. The venous chamber is part of the out-flow bloodline that links back to the venous access and is routinely used for drug or fluid administration. The infusion pump was placed on a tabletop at the same level as the venous chamber. Trained and delegated clinicians performed preparation and administration of contrast. The dialysis blood-flow rate was reduced to 200 mL/min during the contrast infusion. This reduction in the blood-flow rate reduced the venous pressure to avoid the pump error/occlusion alarm. The contrast could not be infused through the dialysis line without running the dialysis. Therefore, only intradialytic CEUS scans were technically possible in this study.

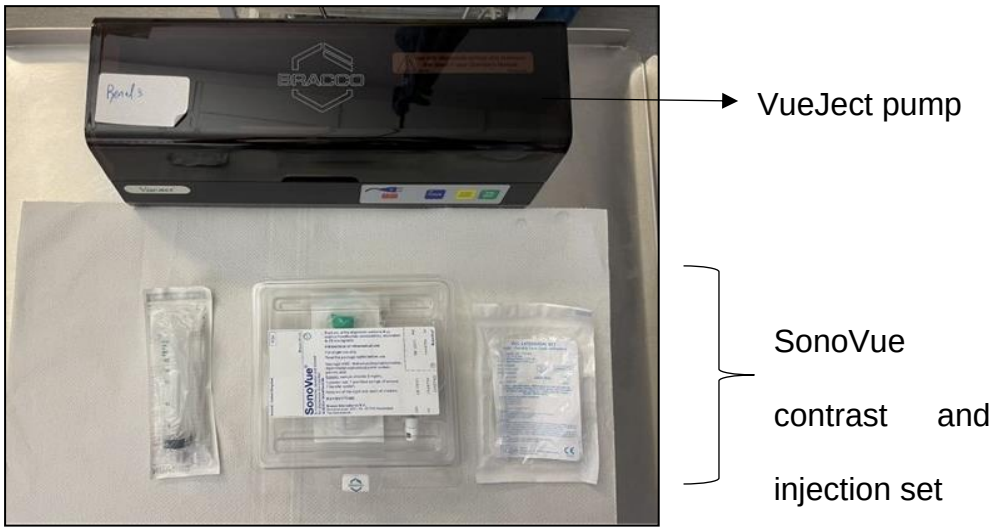


Figure 3.3: Picture of the VueJect oscillating injector, SonoVue contrast agent kit and administration set.

3.3.8 Ultrasound machine

Ultrasound imaging was performed using an iU22 Phillips (Bothell, WA, USA) ultrasound machine equipped with contrast-specific software and a preset dedicated to the Philips L9-3 MHz linear transducer. The ultrasound machine was placed opposite the dialysis machine to allow space around the machines. The participant trial number was entered into the machine to start a new exam.

3.3.9 CEUS measurement

A plastic transducer housing unit manufactured by the medical engineering department at the University of Nottingham was used to hold the transducer on the patient's thigh muscle (quadriceps). The housing was secured using Velcro straps (Amsterdam, NL) wrapped around the

thigh. The housing design was adapted from prior muscle studies on healthy volunteers (Mitchell et al., 2013). An acoustic gel was applied inside the housing before inserting the transducer, and patients were instructed to remain still during the exam.

The ultrasound contrast imaging mode was used, and the settings were optimised as follows: low mechanical index of 0.08 and high mechanical index range [>0.8 to 1.32], time-gain-compensator gain at 62, 64, 66, 68, 70, 71, 71, 71 from the top, overall gain set at 95%, a frame rate of 21 Hz and a contrast resolution of C30. As CEUS quantification is based on measuring real-time perfusion, clips were captured rather than frozen images. For the first CEUS, the contrast infusion and recordings were started at the commencement of dialysis.

Prior studies have shown that 90s is the time necessary for microbubbles to reach a steady state in the circulation (Mitchell et al., 2013). Therefore, as soon as the 90s elapsed, two cycles of flash/reperfusion were captured. The infusion was stopped immediately at the end of the last clip. The transducer was removed from its housing while keeping the housing in place to ensure position consistency for the next (mid-dialysis) CEUS scan at 30min, which was performed using the same steps after resuming the contrast infusion. Upon completing the mid-dialysis scan, the transducer housing was removed for patients' comfort during the remaining dialysis time. Finally, towards the end of the dialysis and within the last 30min, the third (late-dialysis) CEUS was performed using a freshly prepared contrast.

Each CEUS session was completed in 2.5min. At the end of the three CEUS sessions, clips were exported as Digital Imaging and Communication in Medicine (DICOM) files to a 500 GB hard drive (Seagate, USA, CA) for off-line analysis using VueBox (version 7.2.0.58362, Bracco, Geneva, Switzerland) and Gastrointestinal perfusion package quantification software.

3.3.10 Doppler ultrasound

The Doppler ultrasound is used extensively for vascular haemodynamic assessment. In particular, the spectral Doppler generates a spectrum (signature waveform) for each vessel, whose shape and velocity can be used for assessment. In addition, the blood-flow volume can be measured using the spectra and a special formula.

A pre-HD Doppler ultrasound on the femoral artery was performed with the arterial imaging preset. Using the B-mode and with a transverse transducer orientation, the superficial femoral artery was traced from its bifurcation at the groin to mid-thigh, which was rotated clockwise to view the mid-artery longitudinally. The imaging mode was switched to colour Doppler and pulse-wave modes to sample the mid-SFA flow. A Doppler spectrum was displayed, from which measures of blood vessel velocity (peak systolic and end-diastolic velocities) and resistive index were obtained. The Doppler scan was repeated for the mid and late CEUS and post-dialysis.

3.3.11 Contrast-enhanced ultrasound analysis

The CEUS for muscle perfusion is a validated technique described previously (Herrod et al., 2021, Deane et al., 2022, Mitchell et al., 2013). All ultrasound recordings were exported as DICOM files to a 500 GB hard drive (Seagate, USA, CA) for off-line analysis using VueBox (Bracco, Italy) quantification software. Regions of interest (ROI) were drawn to include as much muscle tissue as possible, clear from large vessels and adipose fascia. The ROIs were copied into all subsequent 30 s clips to ensure that the same ROI was measured. Minor ROI adjustments were made to compensate for motion, as necessary. VueBox measures the average signal intensity within the drawn ROI and displays TICs. Raw data from the TICs were then exported into GraphPad Prism® (version 9, San Diego, California) for one-phase association curve fitting.

A nonlinear one-phase exponential decay model $y = (y_{0--} Plateau) * \exp(-K * X) + plateau$ was used, with y_0 constrained to $y_0 = 0$. The following perfusion parameters were derived for each loop individually: AI or the plateau, which is the maximal intensity after reperfusion; mTT, which is the time needed after bubble destruction to reach 50% of the maximal intensity; and perfusion index, which is the ratio of AI to mTT. Each destruction-replenishment loop was analysed, and the mean value was calculated for each parameter.

3.3.12 Bio-impedance analysis

The BIA measures whole-body impedance (resistance) to an applied low-intensity alternating current through the body (Piccoli and Italian, 2004). Electrical conductivity is proportional to the amount of water in the body. The other impedance measures are the reactance (*Xc*), which is the opposition of cells to the flow of an alternating electrical current in ohms, and the phase angle (the ratio between the resistance and reactance) in degrees.

For each participant enrolled in this study, the BIA was assessed using a portable device before and after a single dialysis session (*Akern*, Florence, Italy). Two electrodes were attached to the participant's right foot and two to the hand, as demonstrated in Figure 3.4. To prevent any electric current sensation, participants had to be free from any metal (e.g., jewellery). The dialysis chair was flattened as much as conveniently possible. Afterwards, a current of 200 μA was sent at a frequency of 50 kHz. Two parameters were obtained: Body cell mass in kilograms, and Extracellular water (as percent of total body water).

The time required for the BIA measurements was about 2 to 3 min for each participant, and the body composition data were instantly analysed. The acute change in BIA measures was used to describe the nature of the dialysis sessions, allowing for further interpretation of the CEUS results.

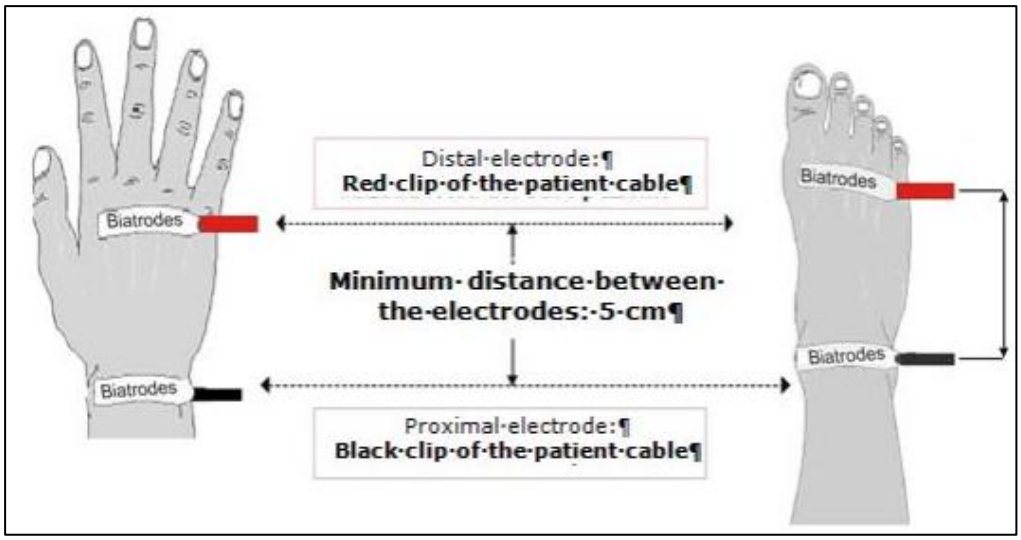


Figure 3.4: Electrode positioning; adapted from (Akern, 2015), all rights reserved.

3.3.13 Blood samples

Blood samples were collected from each patient via the dialysis port needle immediately before and after dialysis. Four tubes were collected: two ethylenediaminetetraacetic acid (EDTA) and two serum tubes. One of each type was sent to the local NHS laboratory at the RDH with a blood test request that included urea, creatinine, sodium, potassium, calcium, magnesium, phosphate, albumin, bicarbonate, troponin T, NT pro-BNP, C-reactive protein parathyroid Hormone, a full blood count and liver function tests. Remaining blood sample tubes were centrifuged at 3,500 revolutions per minute for 15 minutes and stored as plasma and serum in aliquots at -80 °C in a secure freezer at the University of Nottingham for possible future studies.

3.3.14 Statistical analysis

Statistical analyses were performed using SPSS (version 26, IBM, Chicago, Illinois, USA) and GraphPad Prism (version 9, San Diego, CA). All continuous data were graphically tested for normality. Data are presented as the mean and standard deviation (SD) or, when not normally distributed, the median and interquartile range (IQR) Positively skewed data were transformed to log (10) for normality. A comparison between time points was performed using the repeated-measure analysis of variance (ANOVA) for the three time points whereas a paired two-tailed *t*-test was used to compare two time points for the extended analysis to include data from a couple of patients who only had two scans. Nonnormal pairs were tested using the Wilcoxon signed-rank test. An alpha error of ≤0.05 was considered significant. Fisher's exact test, a variation of the chi-square test, was used to compare the proportions between categorical variables.

Five out of 176 readings were randomly missing values in the 15 min BP, which was replaced by the last observation carried forward strategy. Blood tests and BIA missing data were replaced using the series mean.

3.3.14.1 Sample size justification

This is the first time the acute effects of dialysis on muscle perfusion have been assessed. So, there was no prior information to base the sample size on. This study was a pilot work to explore new methodologies and to inform future studies design. A formal sample size calculations was not

performed, but was rather determined based on a pragmatic basis and is comparable with published studies where CEUS was used for muscle perfusion assessment in other clinical settings(Krix et al., 2005, Phillips et al., 2014, Phillips et al., 2015, Hildebrandt et al., 2017).

3.4 Results

3.4.1 Study population and haemodialysis details

Twelve patients were recruited; however, one was excluded due to unusable CEUS images, leaving 11 participants in the analysis. Two patients could not complete the third (late) CEUS scan for the pump error/occlusion alarm, possibly due to the raised pressure in the venous chamber. For analysis, patients were divided into two groups: those who completed all three CEUS exams (n = 9) and those who completed only early and mid-CEUS exams (n = 11) to avoid combining complete and incomplete datasets.

All patients tolerated the exams well, with no reactions noted or reported. Participant demographics and medical history data are presented in Table 3.1. One patient had IDH within the preceding month. The details of the HD treatment delivered to patients are also summarised in Table 3.1.

Demographics	N = 11
Gender (male/female)	(6/5)
Ethnicity (White/Asian)	(8/3)
Age (years)	52 (48 to 69)
Diabetes (Yes/No)	(4/7)
HTN drug (Yes/No)	(8/3)
β-blocker	4
CCB	2
β-blocker and CCB	1
CAD	1
Dialysis details	
IDH in preceding month (Yes/No)	(1/10)
Dialysis access (AVF/CVC)	(10/1)
Haemodialysis duration (4 h/3.5 h)	(10/1)
Haemodialysis vintage (months)	26 (14 to 63)
Dialyzer type: Revaclear/Theranova/Nephral (AN-69 ST)	(3/6/2)
Dialysate temperature (36ºC /37ºC)	(10/1)
Dialysate flow rate (mL/min)	750
Last ultrafiltration volume(L)	2.15 (0.86)
Last prescribed blood flow (mL/min)	380 (320 to 400)
Relative blood volume (%)	-6.80 (-12.30 to -4.30)
Last dialysis adequacy (Kt V^{-1})	1.37 (1.07 to 1.42)
IDWG (kg) on CEUS day	1.6 (1.2 to 2.1)

HTN: hypertension; β: beta; CCB: calcium channel blocker; CAD: centrally acting drug; IDH: intra dialytic hypotension and defined by a decline to <100 mmHg; AVF: arteriovenous fistula; CVC: central venous catheter; IDWG: intradialytic weight gain. CEUS: contrast-enhanced ultrasound. Data are expressed as mean (SD) or median (IQR), as appropriate.

Table 3.1: Demographics, medical history, and dialysis detail summary

3.4.2 Intradialytic skeletal muscle perfusion

Perfusion measurements obtained at three time points are presented in Box and Whiskers plots (Figure 3.5). There was no difference in PI values between the three time points ($F = 0.64$, $p = 0.52$, $n = 9$) or in its components, AI ($F = 1.61$, $p = 0.23$, $n = 9$) and mTT ($F = 2.19$, $p = 0.17$, $n = 9$).

I performed a further analysis to include the data from the other two patients who only had early and mid-CEUS exams (Figure 3.6). Similarly, the PI, AI, and mTT values $t(10) = 1.18$, $p = 0.27$; $t(10) = 1.54$, $p = 0.15$; and $t(10) = 0.40$, $p = 0.70$, respectively) exhibited no difference between early and mid-dialysis. Figure 3.7 displays the mean TICs for the two groups.

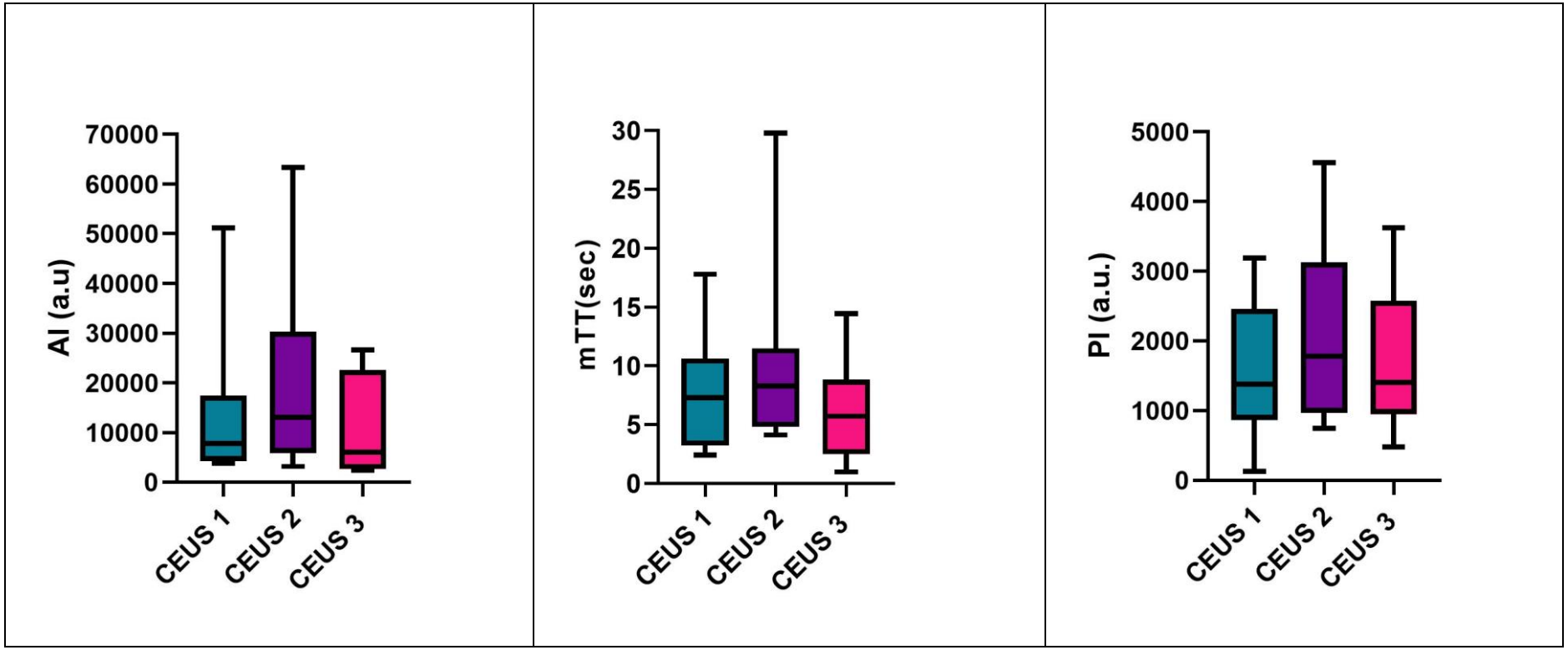


Figure 3.5: Intra-dialytic skeletal muscle perfusion using contrast-enhanced ultrasound (CEUS) in nine included patients; AI: acoustic Index; mTT: mean transit time; PI: perfusion index; a.u: arbitrary unit.

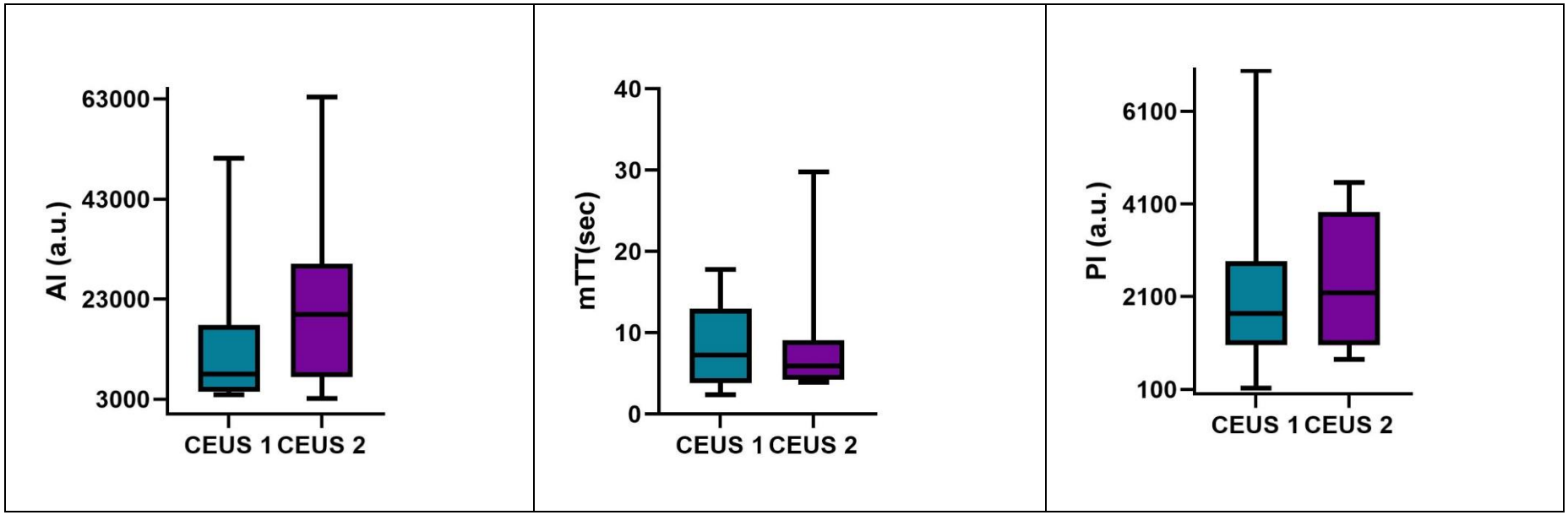


Figure 3.6: Intra-dialytic skeletal muscle perfusion using contrast-enhanced ultrasound (CEUS) in 11 included patients; AI: acoustic Index; mTT: mean transit time; PI: perfusion index; a.u: arbitrary unit.

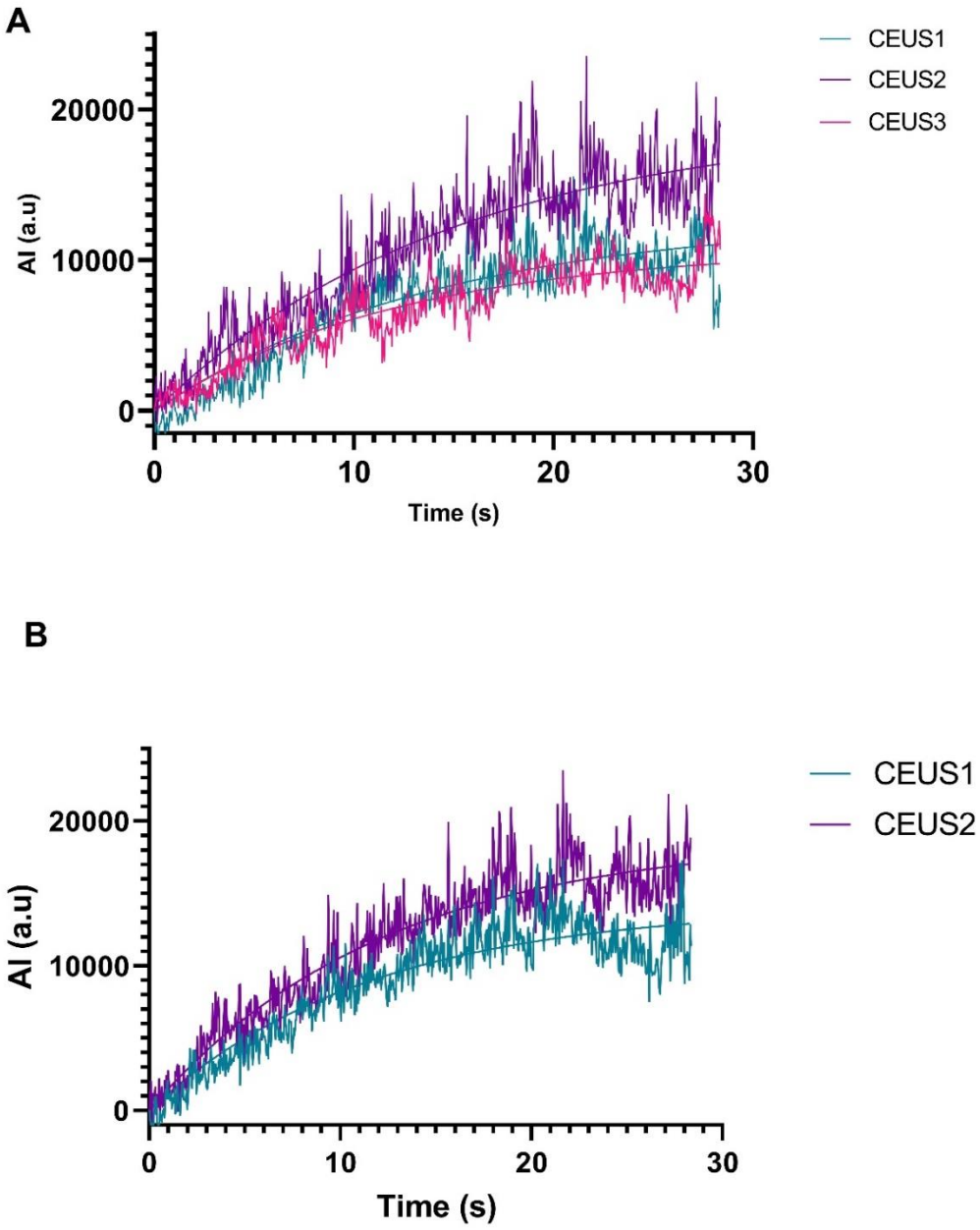


Figure 3.7: Means of the acoustic index plateau for **A:** 11 patients who completed early (at time of dialysis initiation), mid (30 minutes into dialysis), and late (within 30 minutes towards the end of dialysis) contrast-enhanced ultrasound (CEUS) exams. **B:** nine patients completed early

(at time of dialysis initiation) and mid (30 minutes into dialysis) CEUS scans.

3.4.3 Individual TIC analysis

On inspection of the individual cases, it was possible to see a fall in CEUS perfusion measures in in either mid or late CEUS perfusion in 8 out of the 11 patients. Individual TICs are shown in Figure 3.8, and lower perfusion was shown with either a lower degree of curve steepness (higher mTT) or lower AI values. The demographics and dialysis details between the two groups (those who did or did not have a decline in perfusion) are demonstrated in Table 3.2. Due to the small number of patients, a statistical assessment of the relationship between the demographics and dialysis variables between the two groups was not performed. This prevents definitive conclusions, but it is possible that dialysis vintage, ultrafiltration volume, and intradialytic weight gain were higher in those with a fall in CEUS measures of perfusion. However, these observations should not be over-interpreted.

It should also be noted that three participants (7, 9, 10) had higher CEUS perfusion values at later timepoints during dialysis.

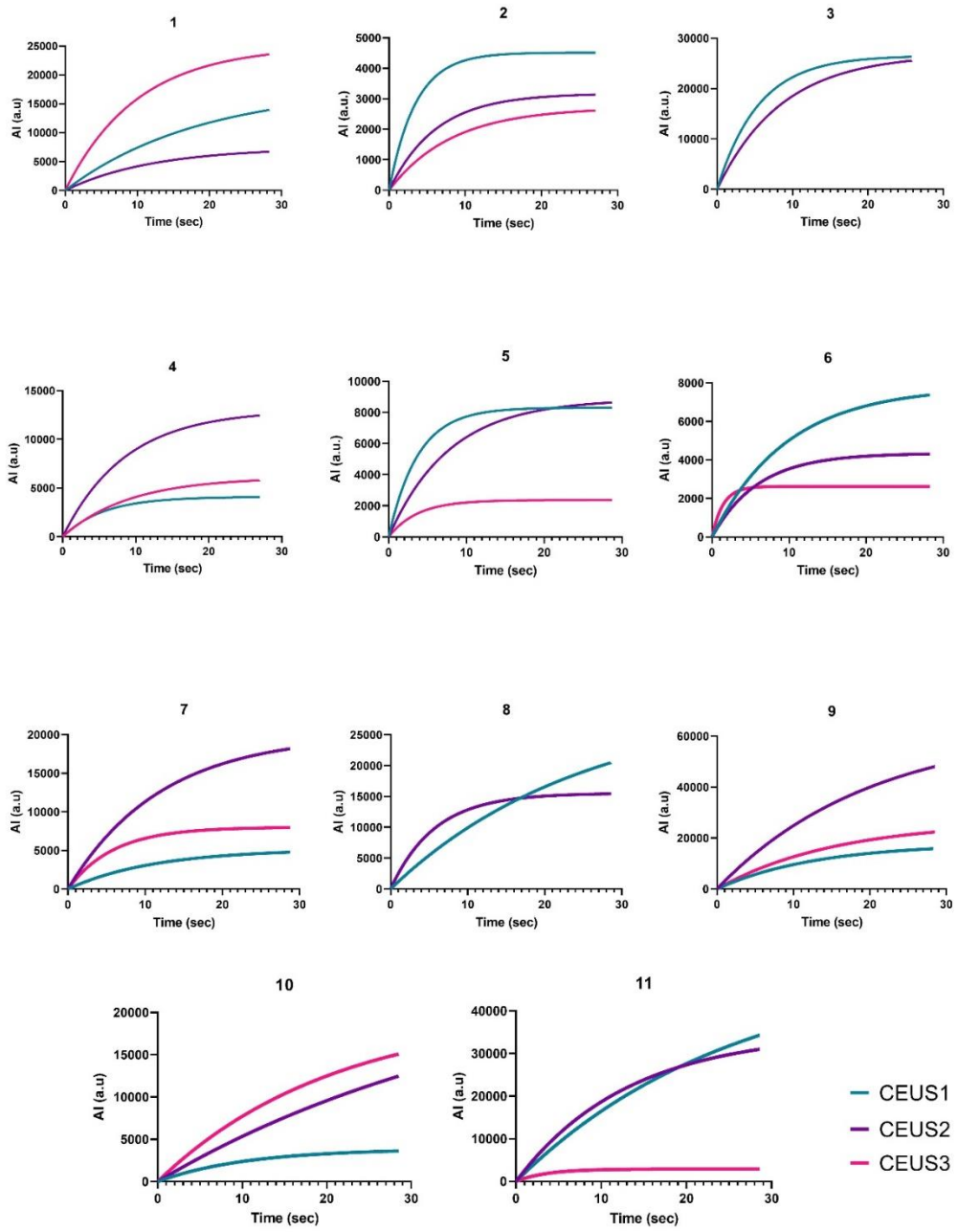


Figure 3.8: Individual time-intensity curves. Patients 4, 9 and 10 did not exhibit a drop after the first contrast-enhanced ultrasound (CEUS) at time of dialysis initiation but had increased acoustic index (AI) values or steeper curves. CEUS 2 is 30 minutes into dialysis; CEUS 3 is within 30 minutes towards the end of dialysis.

Demographics	**Drop** ***n*** **= 8**	**No drop** ***n*** **= 3**
Gender (Male/Female)	(4/4)	(2/1)
Ethnicity (White/Asian)	(7/1)	(1/2)
Age (years)	55 (39 to 69)	52 (51 to 61)
Diabetes (Yes/No)	(3/5)	(1/2)
HTN drug (Yes/No) β-blocker CCB B-blocker and CCB CAD	(6/2) (4/4) (1/7) (1/7) (1/7)	(2/1) (1/2) (1/2) (0/3) (0/3)
Dialysis details		
IDH in preceding month (Y/N)	(0/8)	(1/3)
Dialysis access (AVF/CVC)	(7/1)	(3/0)
Haemodialysis duration (4 hrs/3.50 hrs)	(7/1)	(3/0)
Haemodialysis vintage (months)	51 (17 to 66)	16 (5 to 19)
Dialyzer type: Revaclear/Theranova/Nephral (AN-69 ST)	(3/4/1)	(0/2/1)
Dialysate temperature (36ºC/37ºC)	(7/1)	(3/0)
Dialysate flow rate (mL/min)	750	750
Last ultrafiltration volume (L)	2.24 (0.78)	1.89 (1.2)
Last prescribed blood flow (mL/min)	375 (313 to 400)	380 (320 to 400)
Relative blood volume (%)	-8.80 (-12.17 to -3.25)	-6 (-17 to -5)
Last dialysis adequacy (Kt V^{-1})	1.32 (1.02 to 1.50)	1.37 (1.10 to 1.42)
IDWG (kg) on CEUS day	1.8 (1.2 to 2.1)	1.2 (0.4 to 3.0)

HTN: hypertension; β: beta; CCB: calcium channel blocker; CAD: centrally acting drug; IDH: intra dialytic hypotension and defined by a decline to <100 mmHg; AVF: arteriovenous fistula; CVC: central venous catheter; IDWG: intradialytic weight gain. CEUS: contrast-enhanced ultrasound. Data are expressed as mean (SD) or median (IQR), as appropriate.

Table 3.2: Patient demographics with and without a drop in perfusion variables.

3.4.4 Macrovascular blood flow in skeletal muscle

All recruited patients (n = 11) completed four ultrasound Doppler exams. Box and whisker plots for the different time points are presented in Figure 3.9. No difference in peak velocity values was seen between the time points ($F = 0.51$, $p = 0.58$).

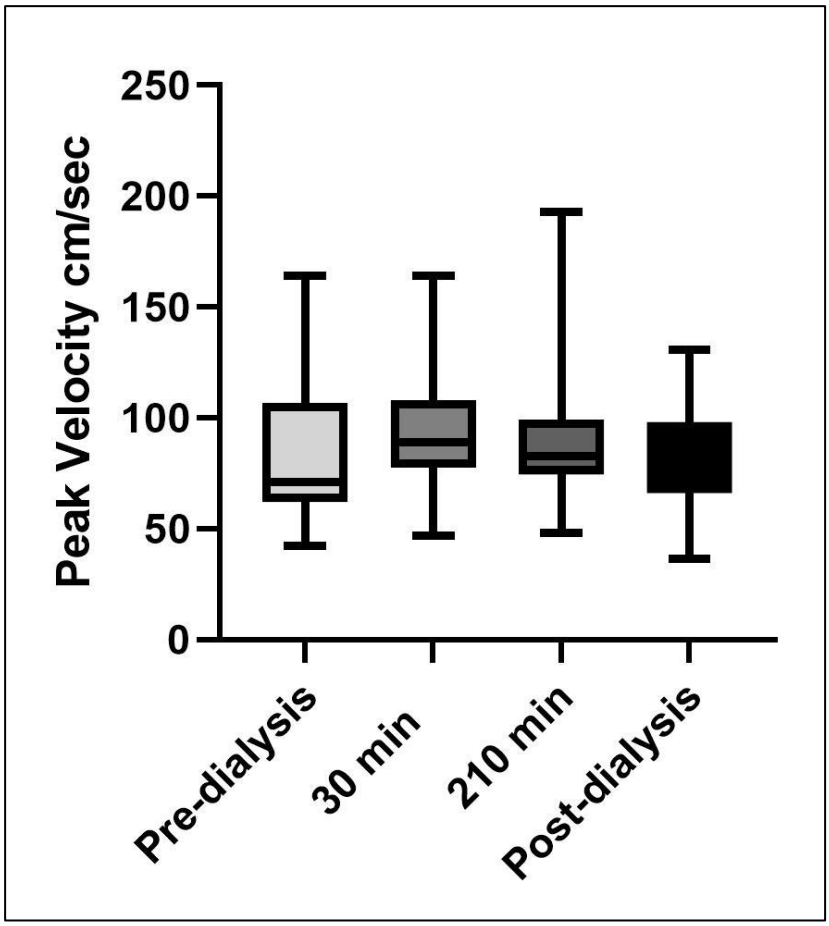


Figure 3.9: Box and whisker plot showing the median, maximum and minimum values and the lower to upper quartiles for the femoral artery peak blood velocity before, during (30 minutes and 210 minutes into dialysis) and after dialysis (n = 11). HD: haemodialysis

3.4.5 Analysis of blood pressure

Mean systolic blood pressure (SBP) and diastolic blood pressure (DBP) were calculated for all recruited patients (n = 11) to assess the BP stability during dialysis and are illustrated in Figure 3.10. Intradialytic hypotension was defined as a decline to <100 mmHg. Four (36%) HD patients had IDH episodes during study sessions.

There was no statistically significant difference between the SBP or DBP time points (every 15 min for 4 h); t (F = 0.88, p = 0.46) for SBP and (F = 0.61, p-value = 0.66) for DBP. The mean (SD) predialysis SBP was 134.64 (17.30) mmHg, and the mean (SD) post-dialysis SBP was 135.09 (17.22); mmHg the paired t-test was t(10) = 0.06, p = 0.96.

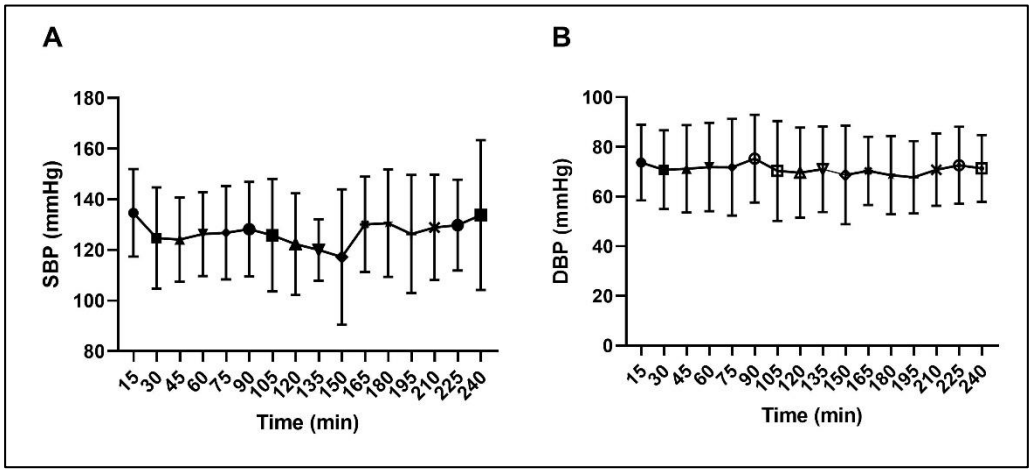


Figure 3.10: Change in the mean (a) systolic blood pressure (SBP) and (b) diastolic blood pressure (DBP) with standard deviation bars over the 4-h dialysis session for the 11 participants combined.

The blood test results before and after dialysis are presented in Table 3.3, which describes the patients and shows the effects of the dialysis. BIA before and after dialysis to assess hydration status are in Table 3.4.

Variable	**Predialysis**	**Post-dialysis**	**P-value**
Serum creatinine (µmol/L)	753.82 (238.76)	291.01 (132.13)	<0.001
Serum albumin (g/L)	32 (3)	33 (5)	0.59
Serum potassium (mmol/L)	5.0 (0.8)	3.5 (0.4)	<0.001
Serum sodium (mmol/L)	138 (3.61)	138.36 (1.91)	0.48
Serum phosphate (mmol/L)	1.5 (0.46)	0.8 (0.32)	0.02
Urea (mmol/L)	20 (7.1)	6 (4.2)	0.004
Serum calcium (mmol/L)	2.14 (0.21)	2.17 (0.15)	0.52
Bicarbonate (mmol/L)	21 (1.87)	26 (2.18)	0.007
NtproBNP (picograms/mL)	5761 (6935.39)	2244 (3329.83)	0.005
Troponin T (ng/mL)	37.88(10.12)	25.80(9.14)	<0.001
C-reactive Protein (mg/L)	15.14(15.32)	16.37(15.40)	0.03
Parathyroid Hormone (pmol/L)	506 (441.04)	293 (181.15)	0.24
Total Bilirubin (µmol/L)	16 (32.83)	17 (32.75)	0.74
ALT (U/L)	16 (4.81)	17 (4.78)	0.15
GGT (U/L)	31 (16.66)	36 (14.22)	0.07
Total protein (µmol/L)	67 (7.6)	69 (10.30)	0.52
Globulin (g/L)	35 (6.35)	36 (7.72)	0.54
Alkaline phosphatase (mmol/L)	117 (36.91)	122 (35.4)	0.10
WBC 10^9/L	7.89 (2.34)	7.25 (2.27)	0.04
RBC 10^{12}/L	3.70 (0.43)	3.88 (0.45)	0.04
Haemoglobin (g/L)	112.82 (7.05)	118.91 (13.07)	0.05
Haematocrit	0.34 (0.03)	0.65 (1.00)	0.33

Data are expressed in the mean (SD); ALT: alanine aminotransferase; GGT: gamma-glutamyl transferase; WBC: white blood cells; RBC: red blood cells.

Table 3.3: Blood test results before and after dialysis, *n* = 11

	Predialysis	Post-dialysis	*P*-value
Body cell mass (kg)	33.10 (22.90 – 35.13)	28.1 (19.30 – 38.70)	0.93
Extra cellular water (% total body water)	46.45 (10.47)	46.56 (10.14)	0.98

Table 3.4: Bioimpedance analysis measurements before and after dialysis, *n* = 11.

3.5 Discussion

This study used CEUS during dialysis to assess the acute effect of HD treatment on skeletal muscle microvascular perfusion. The data from this study revealed no change between early, mid, and late CEUS sessions. Similarly, no change occurred between macrovascular blood-flow time points.

Patients receiving HD exhibit more functional skeletal muscle wasting than nondialysis CKD patients (McIntyre et al., 2006a). Muscle atrophy is common in dialysis patients (Carrero et al., 2008b) with rapid and persistent deterioration in physical function (Marcus et al., 2015, Tamura et al., 2009), which is even worse in young dialysis patients (Marcus et al., 2015). This outcome is linked to high mortality rates (Noori et al., 2010) and poor physical function and quality of life, imposing an additional concern for the already burdened patients (DeOreo, 1997, Martinson et al., 2014).

The current study results yielded no signs of changes in perfusion measures during dialysis, in contrast to our original hypothesis. These findings suggest that changes in perfusion during dialysis are not likely to be a major contributor to muscle wasting in patients undergoing HD, or the reduced physical function and increased levels of muscle wasting in patients receiving HD (Marcus et al., 2015, Tamura et al., 2009, Carrero et al., 2008b, McIntyre et al., 2006a). These findings are consistent with a previous study on another catabolic state (ageing) in which microvascular skeletal muscle perfusion was not an indicator of blunted muscle protein synthesis in response to feeding (an anabolic stimulus) (Phillips et al., 2015).Muscle wasting may be explained by a variety of other factors or multiple factors combined. Further, existing efforts to develop strategies to ameliorate the haemodynamic consequences of dialysis, such as cooling dialysate (Odudu et al., 2015b) may not work to improve or inhibit skeletal muscle wasting.

Uraemic milieu (a condition of CKD with associated toxins and metabolic disturbances) per se could be a major determinant of muscle wasting, independent of muscle hypoperfusion. Metabolic abnormalities associated with uraemia include oxidative stress, inflammation, abnormalities within muscle structure, or mitochondrial defects linked with the severity of CKD (Thome et al., 2021) and the physical performance of HD patients (Gamboa et al., 2020). Additionally, age-related muscle changes (D'Alessandro et al., 2018) and the neuromuscular system (Russ et al., 2012) might contribute to skeletal muscle wasting in dialysis patients. As per the systematic review in

Chapter 2, no comparable data were found regarding the skeletal muscle microvascular response to dialysis-induced circulatory stress.

However, other possible explanations exist for the negative results of this study. CEUS may not be sensitive to detect skeletal muscle blood-flow change at rest, as with peripheral arterial disease studies (Davidson et al., 2017), in which changes were only detected with stress-perfusion imaging. Alternatively, as CEUS measures are cross-sectional, it is possible that I did not conduct these measures at time of peak haemodynamic stress during dialysis. In a study conducted by Eldehni et al. (2014), the peak HD-induced circulatory stress started in the third quarter of dialysis and lasted till the fourth quarter. As depicted in the BP graphs, the largest reduction in BP in the third quarter is consistent with their findings. However, I performed a CEUS scan in the fourth quarter but not the third. Moreover, the baseline scan was not prior to dialysis, as this was technically impossible, and if stress occurs immediately with dialysis, the current study design (with no predialysis scan) may have introduced bias towards the null hypothesis. In addition, I did not observe changes in extra cellular water on bioimpedance measures, and the rates of IDH were low, suggesting that the haemodynamic stress in the observed dialysis sessions may have been less than in a more general (or hypotension-prone) group. Last, the acute effects of a single dialysis session were measured, which may have averted the chance of day-to-day physiological variations.

Although no change occurred in the collective results, the individual graphs indicate that some patients had a decrement in perfusion

measures during the mid or late CEUS, which was offset by curves of other patients who had an increase. Furthermore, the observation of the decline is based on visual assessment, and small numbers could prevent statistical significance. Our results should not be over-interpreted, particularly with lack of statistical comparisons, but it is possible to speculate that patients who had a visual drop in TIC had higher dialysis vintage, ultrafiltration volume, and intradialytic weight gain (Table 3.2), consistent with the published literature on the association of these parameters with reduced perfusion (Zhang et al., 2015, Polinder-Bos et al., 2018, Jefferies et al., 2011).This study has some limitations. First, this study was a small-scale pilot to assess the approach's applicability to be used in larger-scale studies. Therefore, the study findings should not be generalised, and future research must test the hypothesis generated from this work on a larger data set. Another limitation was that the CEUS scans were performed on patients with different feeding states, which may influence skeletal muscle perfusion. The justification for not asking patients to fast was that it was inconvenient for such a vulnerable patient group, especially because most patients were scanned during the afternoon session. Therefore, it was beyond the scope of this study to assess the effect of eating as a confounder on skeletal muscle perfusion. Lastly, the Doppler technique is associated with some limitations. While the waveform shape provides an acceptable qualitative measure, this has no quantitative value and although velocity provides a reliable measure, it remains indirect. Therefore, combining blood-flow velocity with blood vessel area measure provides a quantitative estimation of

blood flow volume measure. However, this method increases the chance of introducing error (Deane et al., 2013).

3.5.1 Challenges to achieve future studies

Overall, the study ran smoothly with a few challenges. One potential future challenge in conducting larger studies is recruitment from a single centre. This is because each dialysis unit has a certain capacity for regular dialysis patients that cannot be surpassed. Once all available dialysing patients are approached, new patients cannot be included unless they have been on haemodialysis for at least three months (as per the eligibility criteria) to avoid the complications associated with the start of dialysis program. Whilst this study was not meant to measure feasibility measures, the ratio of screened to recruited patients was 13 to 3 (a total of 52 patients was approached from 26.11.2018 to 6.12.2019). Another challenge could be related to the fact that contrast could not run into the line connected to the dialysis machine without the initiation of dialysis, preventing a pre-dialysis CEUS scan. So, future studies may consider a separate cannula for contrast administration. Finally, if a pre-dialysis CEUS scan is to be performed, it should be explicitly explained to both patients and dialysis technician/nurse as usually patients' cannulation of the fistula (needling) for dialysis starts as soon as patients arrive to the dialysis bed, as patients expect dialysis to start immediately to avoid delays.

3.6 Conclusions

In summary, I applied a well-established and validated technique in a new population to assess skeletal muscle blood flow during dialysis. In contrast to our original hypothesis, HD did not result in acute changes to skeletal muscle perfusion, and I suggest that intra-dialytic changes in muscle perfusion are unlikely to be a significant contributor to skeletal muscle wasting that is so commonly observed in this group.

Chapter 4: Hand-Grip Muscle Strength (HGS) in People Receiving Haemodialysis – Relationship to Dialysis-Related Factors

In response to the findings from Chapter 3, I sought alternative approaches to corroborate these results. The work in this chapter presents analyses of data from a prior clinical study where measurements of hand-grip strength (indicator of muscle function) were gathered during haemodialysis at baseline, at six months, 12 months, and 24 months. I aimed to analyse the association of Handgrip strength with dialysis-related measures (including blood pressure and ultrafiltration volume) that are known to induce haemodynamic stress. The change in hand-strength measures throughout the two years was also assessed. Patients were then divided into two groups and quartiles according to the status of their hand-grip strength in two years. The purpose of this categorisation was to further examine the relationship between BP and dialysis details with muscle function, as well as looking at alternative factors such as nutritional measures.

4.1 Abstract

Introduction: Hand-Grip Strength (HGS) is a simple, fast, and reliable tool for muscle function assessment. The hypothesis was if the haemodynamic consequences of dialysis were an important factor contributing to muscle wasting, there would be associations of the haemodynamic parameters of dialysis and longitudinal changes in HGS.

The aims were: 1) to analyse the patterns of HGS in dialysis patients and the change over two years, and 2) to explore the association of dialysis related factors and a deterioration in HGS, as well as nutritional markers.

Methods: This was an analysis of a dataset from a prospective study (Viramontes Horner et al., 2019) that enrolled patients who attended for HD from 2016 to 2018. HGS was measured at baseline, 6 months, one year and 2 years. HD parameters as well as BP data were obtained from the hospital electronic records. Other variables such as age and nutritional factors were obtained from the original study dataset. The relationships between dialysis parameters and BP with baseline HGS were evaluated. Furthermore, change of HGS over the two years was assessed and determinants of HGS fall were identified by analysing associations with dialysis parameters, BP, age, and nutritional factors.

Results: 113 patients were included in this study. Median age (years) was 66 (56 – 75), male/female ratio was 74 / 39, dialysis vintage was 31 (11 – 69) months. Of those, 65 patients had four HGS measurements over two years. No significant statistical difference was found between HGS values over two years (p= 0.43). There was no correlation between baseline HGS and UF or BP variables. There were significant differences in serum albumin, energy intake per ideal body weight and fat intake for the two groups of stable or increased HGS and declined HGS. Also, there was significant difference in systolic BP, serum albumin, and fat intake across HGS quartiles.

Conclusion: This analyses of pre-existing data showed no signs of change in the mean HGS values in the overall group of dialysing patients over two years. Assessment of associations between baseline HGS and dialysis parameters with change of HGS over time indicated that nutritional factors are the predominant contributors to reduced muscle function of patients on dialysis.

4.2 Introduction

Hand-Grip Strength (HGS) is a simple, fast, and reliable tool for muscle function assessment. It is a measure of muscular strength, or maximum force generated by the muscles of forearm. HGS can be used in many conditions to assess and trace various medical conditions and can be used as a screening tool for the measurement of upper body and overall strength (Litchfield, 2013). It has good correlation with lean body mass (Heimburger et al., 2000, Shin et al., 2014, Stenvinkel et al., 2002) as assessed by dual-energy X-ray absorptiometry, the most reliable tool for lean body mass assessment. HGS has been widely used in different clinical settings including CKD and haemodialysis (Heimburger et al., 2000) and is associated with muscle atrophy (Carrero et al., 2008a) and plasma carnitine, an amino acid that is related to muscle function (Constantin-Teodosiu et al., 2002). Dialysis patients have reduced muscle function and lower HGS values compared with controls, with females having lower values than males (Slee et al., 2020, Leal et al., 2011a, Vogt et al., 2016). Several studies revealed that declined HGS may independently predict poor outcomes including inflammation, malnutrition, and higher mortality and morbidity in CKD populations (Leal et al., 2011a, Stenvinkel et al., 2002, Chang et al., 2011). Also, a recent meta-analysis showed significant correlations between low HGS values and all-cause mortality in CKD patients requiring dialysis (Hwang et al., 2019). The relationship of HGS with cardiovascular events was also studied in a large late adolescent cohort and showed inverse associations with vascular disease and arrythmia (Andersen et al., 2015).

Recent work compared HGS measurements taken from 101 patients before and after dialysis and found significant reduction in HGS after dialysis when compared to pre-dialysis measurement (Delanaye et al., 2018).

In the previous chapter (Chapter 3), I demonstrated that there was no acute effect of haemodialysis on skeletal muscle perfusion. Therefore, I looked for further corroborative evidence to assess the relationship of HD-induced circulatory stress on skeletal muscle. A recent study by Viramontes Horner et al. (2019) measured HGS in dialysis patients at baseline, six months, one year and two years, as one of the markers for nutritional assessment. Longitudinal changes in HGS over the two years has not been investigated in this dataset to date. We therefore hypothesised that if the haemodynamic consequences of dialysis were an important factor contributing to muscle wasting, there would be associations of the haemodynamic parameters of dialysis and longitudinal changes in HGS. Therefore, the aim of the present study was twofold: firstly, to analyse the patterns of HGS in dialysis patients and the change over two years. Secondly, to explore the association of dialysis related factors that are known to be responsible for dialysis-induced organ hypoperfusion, including blood pressure and ultrafiltration volume, and a deterioration in HGS, as well as factors that are unrelated to haemodynamic stress (e.g. nutritional markers).

4.3 Methods

The present study is an analysis of a dataset from a prospective study (Viramontes Horner et al., 2019) that enrolled patients who attended for HD in Royal Derby Hospital from 2016 to 2018. Inclusion criteria were patients on HD who were ≥ 18 years and with over a year expected survival. Exclusion criteria were pregnancy or expecting pregnancy, breastfeeding and patients with dark skin tone (one of the main measures in the main study was skin autofluorescence as a marker of advanced glycation end-product deposition). HGS measurements were collected within the first hour of dialysis. HGS was measured at baseline, 6 months, one year and 2 years using Takei 5401 handgrip digital dynamometer (Takei Scientific Instruments Co., Ltd, Tokyo, Japan). Participants were subdivided into those with stable/increasing HGS or decreasing HGS. HD parameters (pre-dialysis weight, post-dialysis weight, and dialysis duration) as well as pre-dialysis and post dialysis blood pressure data for patients who had successful baseline HGS measurements were extracted from the hospital electronic records (Vital Pulse Limited ©, Vital Data Client Version1.7.0.16396). Three sets of these data were extracted: on the day of HGS measurement and readings from the sessions before and after HGS measurement. An average was taken to reduce the effect of outlier measures that do not represent the patient's usual characteristics on HD. In five cases, there were missing pre-dialysis or dry weight entries, in this case, the average of only two readings were extracted. UF rate was calculated as the difference of pre-dialysis weight and post-dialysis weight, divided by dialysis duration. Other variables

such as age and nutritional factors were present in the original study dataset. The relationships between dialysis parameters and blood pressure (which affect intra-dialytic haemodynamics) with baseline HGS in the cohort as a whole were assessed. In addition, change of HGS over the two years was assessed. Then, determinants of HGS decline were identified by analysing associations with dialysis parameters, BP, age, and nutritional factors. Finally, the difference between HGS at baseline and HGS at year 2 was calculated, and the association between HGS and BP, dialysis variables, and nutritional factors was compared across the quartiles of HGS change.

4.3.1 Justification for group analysis

It is now appreciated that a decline in HGS in CKD patients is associated with poor outcomes, including mortality, inflammation, and malnutrition (Hwang et al., 2019, Leal et al., 2011a, Stenvinkel et al., 2002, Chang et al., 2011). We sought to test the hypothesis that changes in haemodynamics during dialysis may contribute to muscle wasting. Therefore, I tested the hypothesis in several methods rather than looking at a single approach to generate robust findings and confirm the results. I looked at associations in the cohort as a whole. Then, the HGS values were categorized into two groups of declined and stable or increased HGS. This approach of grouping has precedent and has been used in prior studies, for example: the association between changes HGS and depression was compared across two groups: decreased and same or increased HGS (Kim et al., 2022). Similarly, patients were divided into

increased functional capacity group and decreased functional capacity groups assessed using 6-min step test, peripheral muscle strength (sit-to-stand test and handgrip strength) (de Almeida et al., 2019). Also, the original study (Viramontes Horner et al., 2020) employed a similar method where patients were divided into normal and abnormal groups based on their nutritional status measured by Subjective Global Assessment. To further explore this, I also compared the association between HGS and BP, dialysis variables, and nutritional factors across the quartiles of HGS change.

4.3.2 Statistical analysis

All data were tested for normality graphically. Data are expressed as mean and standard deviation for normally distributed data, or as median and inter-quartile range (IQR) for non-normally distributed data. Non-normal data were log-transformed to achieve normality for parametric testing. Repeated measures- one-way ANOVA test was performed to assess the change of HGS over two years. Pearson correlation was performed to examine the association between HGS and other factors. Intergroup comparisons were performed using Mann Whitney U test or student's t-test. A p-value of < 0.05 was considered statistically significant. For the comparison of variables across quartiles of HGS, one way ANOVA was used where data were normally distributed, or log transformed or Kruskall Wallis for non-normally distributed variables.

4.4 Results

The number of HD patients that were enrolled in the prospective study was 120 patients, of which six patients had no baseline HGS measurements due to arthritis or fistula in both arms which are contraindicated with HGS test. Moreover, the hospital records for one patient were not accessible and they were excluded from current analysis. Thus, 113 patients remained for inclusion in this study. Of those, 65 patients had four HGS measurements over two years. Figure 4.1 demonstrates a flow chart of the number of patients included in this study.

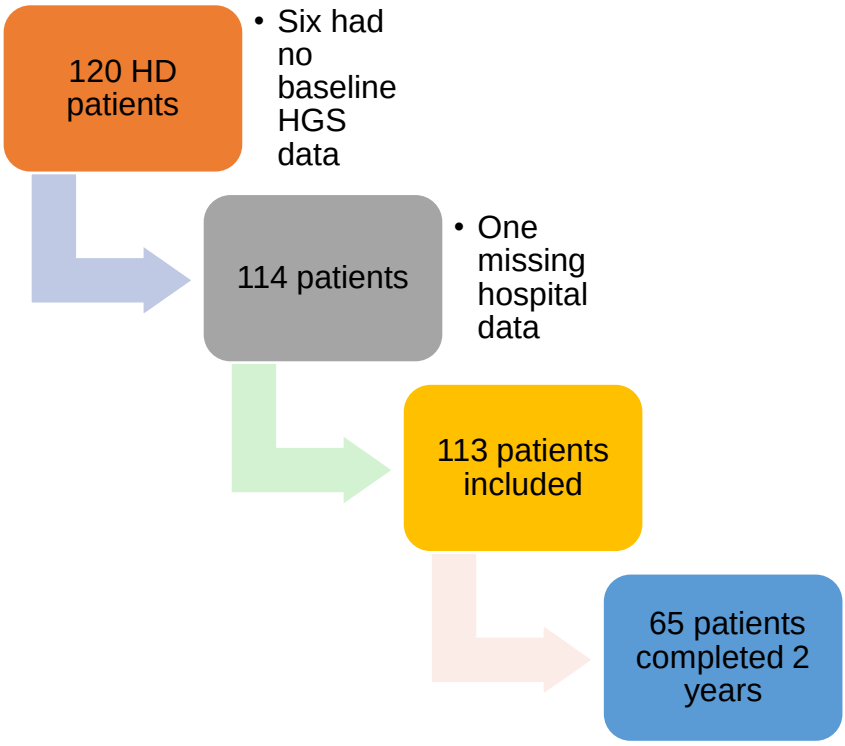


Figure 4.1: Flow chart showing included patients

4.4.1 Patient description

Table 4.1 demonstrates baseline clinical, biochemical, and nutritional characteristics of the enrolled HD patients and the 65 patients who completed 2 years follow-up and was reproduced from Viramontes Horner et al. (2019) study. All patients were dialysed thrice or four times per week for a duration of 3 to 4 hours using one of three dialyzers: high-flux polysulphone, polyarylethersulfone or polyvinylpyrrolidone.

Variable	N= 113 (included at baseline)	N=65 (completed 2 year follow up)
Age (years)	66 (56 – 75)	66 (55 – 75)
Male/female (ratio)	74 / 39	41 / 24
white ethnicity, %	88.5	89
Current smoking, n	17	9
Diabetes, n	47	29
Coronary heart disease, n	48	30
Peripheral vascular disease, n	10	5
Dialysis vintage (months)	31 (11 – 69)	29 (9 – 70)
Dialysis adequacy (Kt/V)	1.2 (1.0 – 1.46)	1.17 (1.00 – 1.44)
Haemoglobin (g/L)	119 (109 – 124)	120 (111 – 125)
Serum albumin (g/L)	33 (30 – 35)	33 (31 – 35)
C-reactive protein (mg/L)	9 (4 – 20)	9 (4 – 20)
Serum creatinine (µmol/L)	627 (493 – 749)	636 (481 – 754)
Serum phosphate (mmol/L)	1.48 (1.21 – 1.81)	1.44 (1.24 – 1.78)
Serum corrected calcium (mmol/L)	2.43 (2.35 – 2.53)	2.43 (2.35 – 2.53)
Serum potassium (mmol/L)	4.7 (4.3 – 5.3)	4.8 (4.3 – 5.3)
Serum sodium (mmol/L)	139 (137 – 141)	139 (138 – 141)
Energy intake (kcal/kg/d)	1317 (1064 – 1702)	1372 (1068 – 1608)
Protein intake (g/kg/d)	58.10 (44.40 – 72.95)	58.10 (46.35 – 68.95)
Fat intake (g/d)	53.6 (37.5 – 71.5)	54.50 (38.05 – 71.65)
Body mass index (kg/m2)	26.3 (23.6 – 31.4)	27.0 (23.6 – 31.4)
Midarm muscle circumference (cm^2)	31.2 (27.2 – 33.6)	25.90 (22.58 – 28.22)
Triceps skinfold thickness (mm)	15.7 (11.9 – 22.0)	15.4 (11.6 – 22.4)

Data are expressed in expressed in median (IQR), ratio, or number.

Table 4.1: Patients demographics, pre-dialysis biochemical, and nutritional characteristics for dialysis patients.

4.4.2 Handgrip strength

Median (IQR) HGS for the dialysis patients who were enrolled in the four timepoints (n= 65) are presented in Table 4.2. No significant statistical

difference was found between HGS values over two years (F= 0.91, p= 0.43, n=65). Figure 4.2 presents a Box and Whisker plot of the difference of HGS (HGS at year 2 – HGS at baseline).

N= 65	Baseline	6months	1year	2year
HGS (kg) Median (IRQ)	21.40 (13.4 – 30.2)	21.60 (15.9 to 29.9)	21.00 (14.7 to 30.0)	20.90 (12.6 to 29.1)

Table 4.2: Hand-Grip Strength measurements for dialysis patients who completed two years.

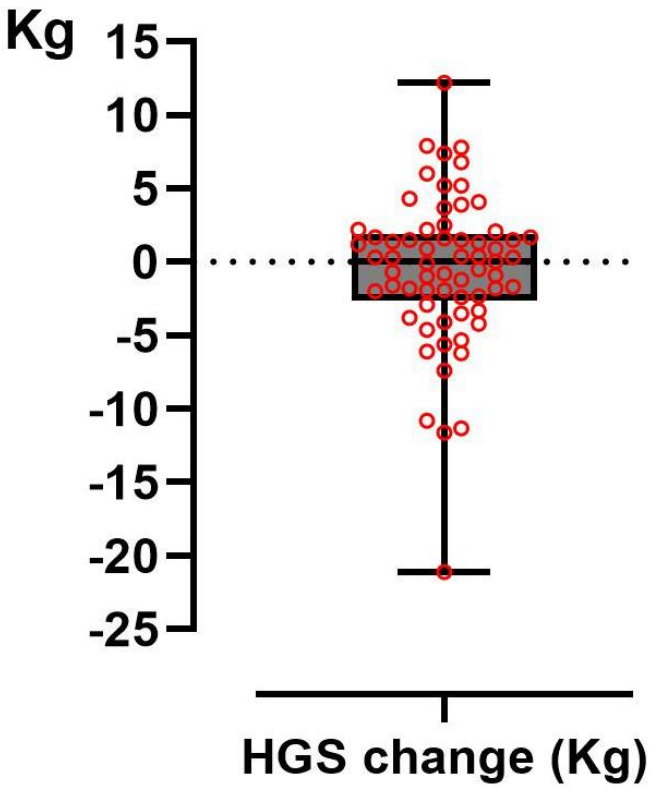


Figure 4.2: Box and Whisker plot displaying the median, lower and upper quartiles along with the minimum and maximum values of the 65 HGS difference values over two years (HGS at year 2 – HGS at baseline).

4.4.3 Association of HGS with BP and dialysis parameters at baseline (n=113)

There was no correlation between baseline HGS and UF or BP variables for the population as a whole as shown in Table 4.3.

Variable	R	P-value
UF volume (mL)	0.06	0.51
UF rate (mL/h/kg)	0.14	0.14
SBP (mmHg)		
Pre-dialysis	– 0.11	0.25
Post-dialysis	– 0.11	0.23
DBP (mmHg)		
Pre-dialysis	– 0.003	0.97
Post-dialysis	0.04	0.70
Post-pre dialysis SBP	0.02	0.88
Post-pre dialysis DBP	0.05	0.58

N= 113; Baseline hand grip strength (kg)

Table 4.3: Pearson's correlation test between HGS and UF and BP.

4.4.4 Analysis of HGS change over time

The 65 patients who completed four HGS measurements over two years were equally grouped into those with declining HGS (51%) and those with stable/increasing HGS (49%). Comparison between the means/medians of baseline BP, dialysis, and nutritional factors between these two groups revealed significant differences in serum albumin, energy intake per ideal body weight and fat intake as presented in Table 4.4.

Baseline Variable	Decline in HGS	Stable/increase in HGS	p-value
Age (years)	61.58 (16.16)	66.28 (11.94)	0.19
Predialysis SBP (mmHg)	135.06 (17.83)	144.03 (22.08)	0.08
Postdialysis SBP (mmHg)	127.71 (18.72)	132.29 (21.74)	0.37
Predialysis DBP (mmHg)	74.22 (10.37)	76.34 (10.13)	0.41
Postdialysis DBP (mmHg)	68.52 (11.11)	70.37 (7.88)	0.44
UF rate (mL/h/kg)	0.30 (0.18 – 0.48)	0.29 (0.12 – 0.46)	0.46
UF volume (mL)	1.17 (0.86)	1.25 (0.82)	0.73
Dialysis vintage (months)	18.00 (9.00 – 79.50)	29.50 (7 – 66.25)	0.95
Dialysis adequacy (Kt/V)	1.22 (0.41)	1.24 (0.31)	0.85
Serum albumin g L^{-1}	32.00 (30.00 – 34.00)	34.00 (32.25 – 36.00)	0.01
Energy intake per IBW (kJ kg-1 per kg m^{-2})	19.50 (15.20 – 21.35)	21.70 (18.95 – 30.03)	0.02
Protein intake per IBW (g kg^{-1} per kg m^{-2})	0.83 (0.25)	0.91 (0.29)	0.23
Fat intake g day^{-1}	49.20 (33.90 – 61.05)	61.30 (47.98 – 84.78)	0.01
Pre-dialysis Haemoglobin g/dl	119.46 (14.35)	116.69 (13.34)	0.42
Pre -dialysis C-reactive Protein mg L^{-1}	8.00 (5.00 – 17.00)	10.50 (3.75 – 24.00)	0.6
Pre-dialysis Creatinine (µmol L^{-1})	642.67 (195.80)	592.59 (146.03)	0.25
Mid arm muscle circumference cm^2	26.22 (3.87)	24.83 (3.88)	0.15

Table 4.4:Comparison between the means/medians of baseline blood pressure, dialysis, and nutritional factors between stable/increasing hand-grip strength and decreasing hand-grip strength groups. HGS: hand-grip strength; SBP: systolic blood pressure; DBP: diastolic blood pressure; UF: ultrafiltration; IBW: ideal body weight.

4.4.4.1 Analysis of BP, dialysis variables and nutritional factors across HGS quartiles

In addition, the association between HGS and BP, dialysis variables, and nutritional factors was compared across the quartiles of HGS change over time (HGS at year 2 – HGS at baseline). Table 4.5 demonstrates the mean/median of BP, dialysis, and nutritional factors in each quartile group consisting of 16 patients. There is significant difference in systolic BP, serum albumin, and fat intake.

Baseline Variable	**Group 1 (-21.1 to -2.9) kg**	**Group 2 (-2.4 to -0.1) kg**	**Group 3 (0.3 to 1.7) kg**	**Group 4 (2.1 to 12.2) kg**	**P-value**
Number of participants	**N= 16**	**N= 16**	**N= 16**	**N= 16**	
Age (years)	66.56 (8.41)	63.69 (14.60)	60.75 (18.10)	64.06 (15.31)	0.73
Predialysis SBP (mmHg)	133.9 (20.28)	133.4 (14.64)	148.2 (23.94)	151.6 (14.24)	= 0.01
Postdialysis SBP (mmHg)	119.4 (16.19)	126.1 (12.72)	135.5 (24.81)	139.8 (19.35)	= 0.01
Predialysis DBP (mmHg)	73.73 (10.07)	72.96 (9.22)	78.85 (11.94)	77.75 (10.19)	0.30
Postdialysis DBP (mmHg)	66.46 (7.49)	67.69 (7.60)	72.69 (11.26)	71.69 (10.04)	0.17
UF rate (mL/h/kg)	0.36 (0.25)	0.60 (1.06)	0.30 (0.17)	0.29 (0.20)	0.38
UF volume (mL)	1.18 (0.87)	1.26 (0.87)	1.27 (0.87)	1.22 (0.80)	0.99
Dialysis vintage (months)	17 (10 – 93)	43 (13 – 106)	26 (3 – 55)	37 (12 – 74)	0.51
Dialysis adequacy (Kt/V)	1.14 (0.95 – 1.36)	1.260 (1.02 – 1.44)	1.27 (0.99 – 1.46)	1.31 (0.98 – 1.48)	0.81
Serum albumin g L^{-1}	32.93 (3.97)	30.88 (2.83)	33.06 (2.52)	34.81 (3.04)	0.009
Energy intake per IBW (kJ kg-1 per kg m^{-2})	19.15 (6.11)	18.73 (4.15)	22.64 (6.40)	24.22 (10.10)	0.08
Protein intake per IBW (g kg^{-1} per kg m^{-2})	0.81 (0.23)	0.84 (0.29)	0.91 (0.20)	0.91 (0.36)	0.69
Fat intake g day^{-1}	50.02 (17.28)	49.64 (20.73)	64.04 (27.41)	76.87 (42.63)	0.03
Pre-dialysis Haemoglobin g/dl	120.00 (14.64)	119.30 (14.89)	117.00 (12.07)	116.40 (14.90)	0.86
Log Pre -dialysis C-reactive Protein mg L^{-1}	10 (3 – 37)	8 (3 – 28)	9 (3 – 22)	9 (5 – 11)	0.96
Pre-dialysis Creatinine (µmol L^{-1})	596.1 (154.4)	631.4 (178.2)	588.7 (204.9)	666.3 (162.0)	0.58

Mid arm muscle circumference cm^2	25.65 (3.95)	25.63 (3.77)	24.43 (4.99)	26.23 (2.85)	0.63

Table 4.5:Comparison between the means/medians of baseline blood pressure, dialysis, and nutritional factors across the quartiles of hand-grip strength change. HGS: hand-grip strength; SBP: systolic blood pressure; DBP: diastolic blood pressure; UF: ultrafiltration; IBW: ideal body weight.

4.5 Discussion

The analyses presented in this chapter were performed to assess the pattern of muscle function and the longitudinal change in HD patients over two years and to look at the relationship between muscle function with clinical, dialysis and nutritional factors. Consistent with the literature, the range of HGS reported in this study were in line with the published values for dialysis patients (Hwang et al., 2019).

When comparing the change of mean HGS values over two years for the collective, muscle function did not change. Moreover, I observed no associations between baseline muscle HGS values with dialysis factors such as blood pressure and ultrafiltration volume. This lack of association persisted when patients were categorised according to the status of HGS over two years into stable or increased HGS and declined HGS and comparisons made between these groups. We, therefore, explored what other factors can contribute to the declining muscle function in our cohort of dialysis patients. I found significant differences in serum albumin, energy intake per ideal body weight and fat intake for the two groups of stable or increased HGS and declined HGS. On the other hand, analysis of baseline BP, dialysis factors, and nutritional factors across the quartile groups of HGS change showed a significant difference in systolic BP, where patients with a greater HGS decline in group 1 (lower quartile) had lower SBP. Whilst this may indicate association with intra-dialytic haemodynamic stress, it should also be noted that pre- and post-SBP measures are not particularly reliable surrogates for intradialytic blood pressure values, or indeed for changes in end-organ perfusion

(Dasselaar et al., 2009). The observation that there were also significant differences in serum albumin and fat intake across the quartile groups, suggests that nutritional factors may be a more prominent determinant of declining HGS in dialysis patients than haemodynamic-related factors, as these associations were seen across more than one analysis.

Our findings are consistent with previous results showing significantly lower values of HGS in malnourished dialysis patients evaluated by the subjective global nutritional assessment (Qureshi et al., 1998, Stenvinkel et al., 2002). Likewise, Viramontes Horner et al. (2019) found higher tissue AGE deposition as measured by skin autofluorescence had significant negative correlation with HGS ($r = – 0.34$, $p<0.0001$), and fat intake ($r= – 0.35$, $p<0.0001$), as well as serum albumin ($r = – 29$, $p= 002$). Similarly, malnourished dialysis patients had lower levels of serum albumin ($r = – 0.61$, $p< 0.001$) in the study by Qureshi (Qureshi et al., 1998) who also found a positive correlation between serum albumin and HGS ($r = 0.42$, $p < 0.001$).

The lack of association between HGS in the declined and stable/increased groups and haemodynamic-related factors such as UF and BP are in accordance with our findings in the previous chapter (Chapter 3) where no acute changes were found in patients' muscle perfusion during dialysis. Furthermore, this seems to be in agreement with previous studies which found no association between HGS and UFR (Delanaye et al., 2018) and BP (Leal et al., 2011b). Whilst one study found results that could be similar to the change in BP across the HGS

quartiles, the associations were weak (r= 0.18, p= 0.03)(Pinto et al., 2015).

Interpretation of the data provided should be with reference to the following limitations. First, dialysis-related factors were only collected during the baseline HGS sessions for the association assessment. The other limitation is related to the nature of the study design where the original study was designed with no consideration of the current research aims. Therefore, there may not have been sufficient haemodynamic-related parameters available to depict associations between haemodynamic-related factors and muscle strength. In addition, the baseline HGS data in our study were already below the normal range of HGS for non-dialysing population (Schlussel et al., 2008). Therefore, the anticipated decline in HGS in HD patients might have already occurred by the time they were recruited. Furthermore, there is currently limited evidence describing the use of HGS for the assessment of longitudinal change in muscle function to accurately compare our data with. However, HGS has been previously used to examine the acute change in muscle function in response to HD. Both Leal et al. (2011b) and Delanaye et al. (2018), designed their studies to compare between HGS data collected prior and after dialysis. However, the findings of the two investigations were contradictory. The study of (Leal et al., 2011b) revealed no change in HGS, whereas the study of (Delanaye et al., 2018) reported significant fall in HGS after dialysis. In the latter study, the cohort was a combination of HD and HDF patients. In addition to that, HGS test was performed on arms with fistula which might have contributed to patients' functional

ability. The relevance of discussing these studies despite the difference in the approach used in the main study (where HGS were collected only once within the first hour of dialysis), is to emphasise the need for a more standardised approach when measuring HGS in the context of dialysis. Furthermore, it could be that the use of HGS for the assessment of the long-term effect of dialysis on muscle function over time requires further validation.

4.6 Conclusions

In summary, this analysis revealed no signs of change in the mean HGS values in the overall group of dialysing patients over two years, but almost half of the group had a decline in HGS over time. Assessment of associations between baseline HGS and dialysis parameters, as well as associations with change of HGS over time indicated that both nutritional factors and SBP could contribute to reduced muscle function of patients receiving HD, although nutritional factors are likely to be more important.

Chapter 5: Application of Contrast-Enhanced Ultrasound in Healthy volunteer Kidneys

This chapter applies CEUS for a different purpose, namely the assessment of kidney perfusion. The chapter is composed of two parts. The first section focuses on the development and optimisation of the methodology and protocols for CEUS perfusion imaging acquisition and analysis performed on the kidneys of ten healthy volunteers. Comparisons were made between the bolus and infusion techniques and between the administration of CA into the antecubital and hand dorsal veins. I also tested the impact of different aspects of image analysis including ROI placement and size, as well as analysis of curve-fitting. The second part of this chapter describes work on ten healthy volunteers, in which I implement the optimised CEUS technique from the first section and assess the repeatability of CEUS-derived perfusion variables.

5.1 Abstract

Introduction: Alterations in renal perfusion play a major role in the pathogenesis of renal diseases. Renal contrast-enhanced ultrasound (CEUS) is increasingly applied to quantify renal cortical perfusion and to assess its change over time, but comprehensive assessment of the technique’s repeatability is lacking.

Methods: Renal CEUS methods were optimised. Then, using the optimised protocol, ten adults attended two renal CEUS scans within 14 days. In each session, five destruction/reperfusion sequences were

captured. One-phase association was performed to derive the following parameters: acoustic index (AI), mean transit time (mTT), perfusion index (PI), and wash-in rate (WiR). Intra-individual and inter-operator (image analysis) repeatability for the perfusion variables were assessed using intra-class correlation (ICC), with the agreement assessed using a Bland–Altman analysis.

Results: The 10 adults had a median (IQR) age of 39 years (30–46). Good intra-individual repeatability was found for mTT (ICC: 0.71) and PI (ICC: 0.65). Lower repeatability was found for AI (ICC: 0.50) and WiR (ICC: 0.56). The correlation between the two operators was excellent for all variables: the ICCs were 0.99 for PI, 0.98 for AI, 0.87 for mTT, and 0.83 for WiR. The Bland–Altman analysis showed that the mean biases (± SD) between the two operators were 0.03 ± 0.16 for mTT, 0.005 ± 0.09 for PI, 0.04 ± 0.19 for AI, and −0.02 ± 0.11 for WiR.

Conclusion: Time-based variable (mTT) had good repeatability and is likely the most reliable measure for future studies in which CEUS is used to assess renal perfusion in patient cohorts and to assess changes in perfusion over time. The large intra-individual variation in (AI) suggest that this parameter may not be suitable for this purpose.

5.2 Section 1: Methods Development and Optimisation

5.2.1 Introduction

A growing body of evidence recognises that alterations in renal perfusion play a major role in the pathogenesis of different renal diseases, including the syndromes of AKI (Harrois et al., 2018, Yoon et al., 2020) and CKD (Garessus et al., 2021, Dong et al., 2014), as well as diabetic kidney disease (Ma et al., 2012, Wang et al., 2015a, Tsuruoka et al., 2010b) and in kidney transplant rejection (Schwenger et al., 2014). Among the current promising available techniques for renal perfusion assessment in humans is arterial spin labelling MRI, which has been validated against alternative measures of perfusion, including contrast agent-based methods (Odudu et al., 2018). However, this is limited by the lack of accessibility, high cost, and challenges of scanning acutely unwell patients. Colour and spectral Doppler ultrasound techniques are widely used for non-invasive assessment of renal blood flow. These techniques provide insight about blood velocity only in major renal vessels due to their inability to detect slow intracortical microvascular blood flow (Schneider et al., 2011). This limitation was improved with the introduction of a new Doppler technique (Microvascular Doppler ultrasound), which has improved the ability to delineate renal microvasculature but does not provide quantitative measures of renal perfusion (Choi et al., 2021).

The interest in microvascular quantification stems from the fact that perfusion alterations can occur at the microcirculatory level without changes in large vessel blood flow (De Backer et al., 2014), and since

most of the blood entering the kidney supplies the renal cortex (Roman, 1990), direct assessment of microvascular perfusion can significantly expand our understanding of regional microvascular blood flow. Doppler-derived resistive index has been the focus of research for years as a potential marker of renal blood alterations but has been shown to be more reflective of systemic haemodynamics. Moreover, Doppler-derived indices were shown to correlate poorly with direct measurements obtained with an implanted flow probe in an experimental study (Wan et al., 2008).

CEUS is an alternative bedside technique for quantifying microvascular perfusion (Schneider et al., 2012). CEUS uses gas-filled microbubbles as an intravascular CA, which makes them suitable for microvascular perfusion assessment. Importantly, CEUS CA are not nephrotoxic and so are suitable for patients with renal insufficiency. With an increasing number of studies reporting the application of CEUS to assess renal perfusion (Garessus et al., 2021, Yoon et al., 2020, Harrois et al., 2018, Li et al., 2017, Schneider et al., 2012, Wang et al., 2015a, Schwenger et al., 2014, Dong et al., 2014, Kihm et al., 2012), it is important to understand the performance of the technique, in particular the repeatability of the different quantitative perfusion measures that are generated. This is essential to supporting its translation to patient-based studies and before it can be introduced into clinical practice for this purpose.

Some studies have adopted standardised acquisition and analysis techniques to try to ameliorate variations in contrast perfusion variables

(Schneider et al., 2013, Schneider et al., 2012, Garessus et al., 2021, Yoon et al., 2020).

One of these studies reported a high correlation coefficient between operators for two different perfusion variables (PI and AI) and moderate correlation for a separate variable (mTT) (Schneider et al., 2013). However, before more studies are conducted in patient cohorts, it is important to establish the repeatability of different CEUS-derived parameters in healthy cohorts, which has not been reported previously. Since there is currently no standardised approach to quantify renal cortex perfusion using CEUS, I also identified the need to optimise the CEUS methods on healthy volunteers (HV) prior to conducting a repeatability study.

5.2.2 Methods

An iterative study protocol was tested on adult HVs to identify potential practical issues and obtain better understanding of how study procedures fit together. A number of potential problems were identified that are not well described in the literature.

These include a problem that emerged when the cannulated left dorsal hand vein resting against the bed was squashed as the participant was lying on their left side to elevate the right flank for posterior kidney scan and subsequently blocked the flow of contrast into the injection line. Also, following initial tests, the ante-cubital fossa was preferred over dorsal hand vein which was less reliable.

Another issue appeared upon the analysis of the acquired files when I found that motion was not well-controlled and yielded suboptimal clips for accurate analysis. Sources of motion were either respiratory or ultrasound probe movement for when adjusting kidney view after breath-hold.

I also identified issues that arose form ultrasound artefacts (e.g. acoustic enhancement or shadowing) which obscured the visualisation of bubbles and had detrimental effect on the quality of reperfusion curves. (Figure 5.1).

All of these problems resulted in images that were not adequate for analysis.

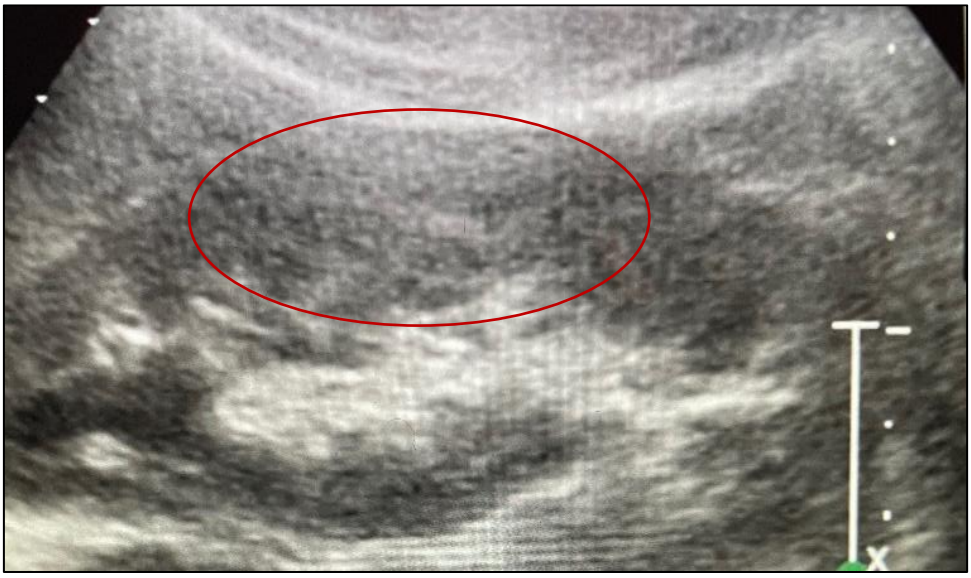


Figure 5.1: Coronal view of right kidney with an acoustic enhancement artefact. Placing ROI at this area does not result in proper reperfusion curve.

When CEUS is used in tumour perfusion quantification studies, a reference perfusion measure is taken from an adjacent healthy area of the same tissue. However, when measuring whole tissue perfusion, this isn't possible. I attempted to find ROIs outside the kidney to provide a reliable reference (to help with normalisation/standardisation of intensity-based measures), but this was not possible due to the difference in the nature of the tissues around or within the kidney.

From these observations, we concluded that the cannula must be placed in the right arm and that motion artifacts must be systematically addressed. This included training participants on how and when to hold their breath and to be familiarised with the instructions/commands beforehand. Moreover, the intensity of inhalation prior to breath-hold should be minor (i.e. breath-hold at mid-inspiration or at end-expiration, as opposed to end-inspiration) to avoid massive kidney displacement from field of view FOV. Also, attention should be paid so that probe is held still particularly during destruction-reperfusion clips. Finally, taking time to perform baseline 2-D scanning to acquire a kidney view that is clear from artefacts is important, with particular focus on the anterior renal cortex (the region closest to the probe) to ensure better quality TICs.

5.2.2.1 Study design

Following this, method development studies were conducted in ten HV to optimise image acquisition and analysis techniques and to gain deeper

understanding of the origin of possible degrading factors of perfusion curves. Inclusion criteria were male or female adults > 18 years with no known CKD, diabetes, or Known allergy to Sonovue. The study was approved by the University of Nottingham, Faculty of Medicine & Health Sciences Research Ethics Committee (403-1910) and all participants provided written informed consent. The study sessions took place in a room with a fully stocked resuscitation trolley and equipped with tools to reach further support. The design of the mechanistic studies is summarised in Figure 5.2. Participants were invited for a single visit to undergo two renal CEUS scans using two different approaches: Intravenous infusion of a CA with disruption-replenishment analysis and bolus injections of CA with TIC analysis. No specific preparation was required from participants prior to the visit. The disruption-replenishment part was performed first. Then, the time for contrast disappearance was observed and recorded before injecting and tracing bolus CA washing into and out from the kidney. Demographic and anthropometric details including age, gender, ethnicity, height, and weight were recorded. All CEUS scans were performed by a single sonographer with CA administration performed by a medically qualified member of the research team. The primary outcome was renal cortical perfusion parameters.

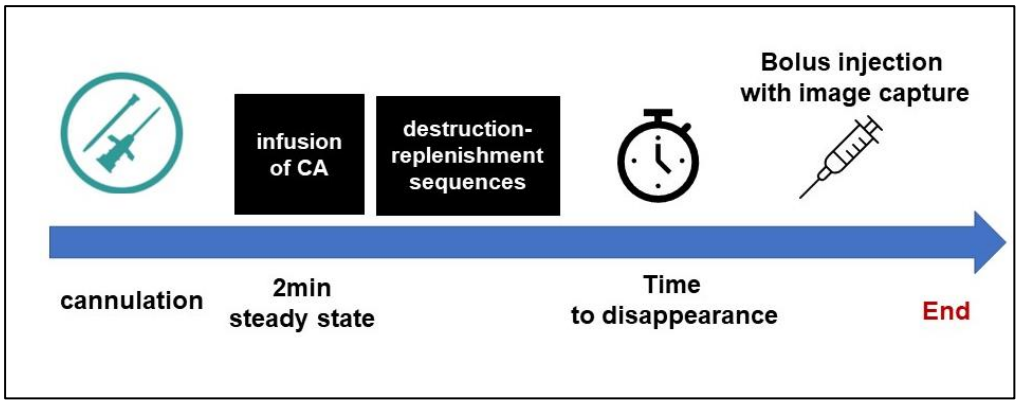


Figure 5.2: Summary of methods development study design.

5.2.2.2 Renal CEUS Technique

CEUS scans were performed using a Philips iU22 ultrasound machine (Bothell, WA, USA), with contrast-specific software and a Philips C5-1 curvilinear transducer. Participants were instructed to lie on their left side, so the right flank was easily accessible for right kidney scanning. A 20 Gauge cannula was placed and SonoVue® (Bracco, Milan, Italy) CA was prepared as per manufacturer's instructions to yield 4.8 mL. This then was further diluted with 15.2 mL of 0.9% sodium chloride solution to yield a total volume of 20 mL. The CA syringe was then inserted into a dedicated infusion pump (VueJect®, Bracco, Milan Italy), which rotates the syringe to keep the contrast dilution agitated, preventing constituted bubbles from separating. The infusion line (length: 91 cm, internal diameter: 0.5 mm) was then primed and connected to the participant's cannula, and the infusion rate was set at 3.3 mL/min.

CA infusion and imaging recording were started simultaneously. A period of two minutes was allowed for the CA to reach steady state, during which

CA arrival to the kidney was visually observed. After this 2 min period, participants were instructed to hold their breath in mid-inspiration for 10s and the transducer was held still in place. Five cycles of destruction/replenishment loops were captured, allowing participants to regain their breath between breath-holds. The destruction/replenishment loops involved a brief high MI ultrasound pulse (flash) that caused complete CA destruction in the imaging FOV, followed by a replenishment phase when contrast re-entered the kidney. Upon completion of each assessment, recorded clips were exported to a hard drive in DICOM format for off-line analysis. Figure 5.3 demonstrates a dual view of the kidney with contrast mode and conventional mode.

Contrast mode	Conventional B-mode

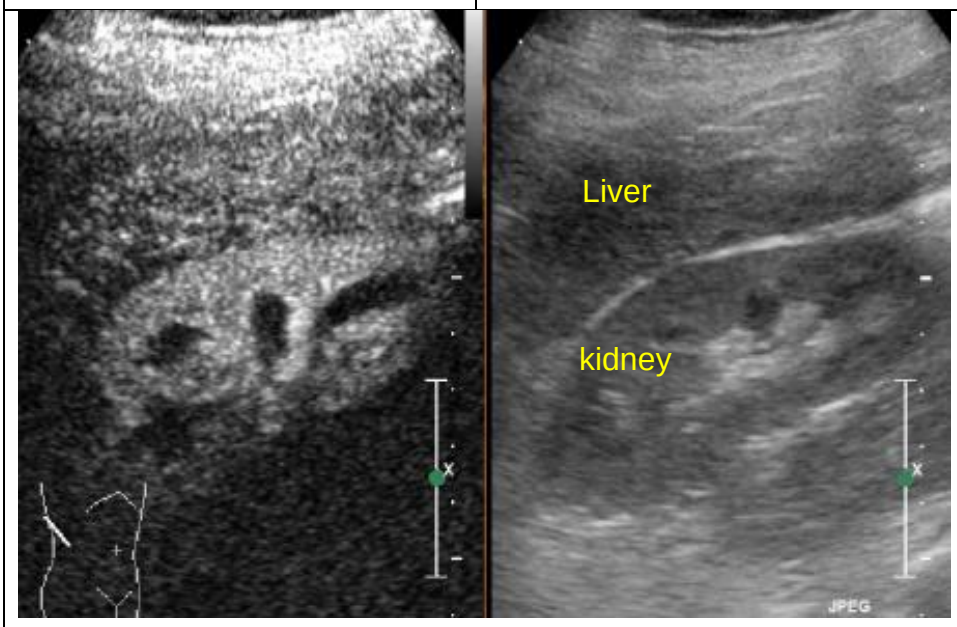


Figure 5.3: Dual screen of a right kidney. Contrast mode on the left displaying a vascular only image reached the steady state with a bright

(increased echogenicity) kidney indicating a visually well-perfused kidney. On the right, conventional image used as a map during scanning

5.2.2.3 Ultrasound Machine Settings

Ultrasound machine settings were optimised at the beginning of each scan. The frame rate was 11 Hz and depth 14 cm. The focus depth was set below the kidney for a more unified beam towards the kidney. Time-gain-compensation was set as follows from top to bottom: 53, 71, 71, 73, 73, 73, 73, and 73. The view displayed was set to dual contrast/B-mode, which was essential as contrast imaging supresses signals from background tissue to enhance the signals from inflowing bubbles. B-mode images were used to guide scanning and transducer position. Contrast resolution (C40) and MI (0.04) were the same on both sides, with higher gain on the contrast (96%) compared to tissue (80%) side. For destruction/replenishment loops, the low MI was 0.04 and the high MI was 1.07.

5.2.2.4 Image Acquisition

A coronal or longitudinal imaging plane of the kidney was obtained. The right kidney was scanned in all patients but one, where the view was not satisfactory, so the left kidney was scanned instead. Analysis was performed using VueBox® (Version 7.2.0.58362) Gastrointestinal Perfusion Package (Bracco, Geneva, Switzerland) with this software calibrated to “Philips IU22, C5-1, C40, Map 2 Chr. Map Off” (scanner

model, transducer, transducer resolution, and chr map) from the acquisition settings.

5.2.2.5 Time to disappearance

At the end of the infusion and to ensure contrast clearance from the field of view before performing the bolus part, I visually observed the clearance by capturing the kidney every six minutes. This was based on the average 12min half-life of SonoVue and the time to disappearance was recorded for each participant. Upon visual disappearance and using the same cannulated vein in infusion (20 Gauge), the remaining contrast was used for bolus injections (depending on the remaining amount of CA). Two subjects had three bolus injections, four had two, and four had single injection.

5.2.2.6 Bolus imaging

Using the same prepared CA from the infusion part of the study, bolus injections were administered, each were rapid bolus of 2.00 ml followed by 5 ml of 0.9% saline at a rate of ~1ml/s avoiding high pressure to prevent bubble destruction. Continuous two-minute loop recording was simultaneously started with bolus CA administration to capture the contrast arrival to FOV (wash-in), arterial peak enhancement and the wash-out. A time (maximum of 5 min) was observed to ensure contrast clearance before introducing subsequent bolus injection. Finally, the cannula was removed as soon as the last renal CEUS scan was completed, and the study was terminated. Upon completion of each

assessment, recorded clips were transferred to a hard drive in DICOM format for analysis.

5.2.2.7 Vascular access

In a subset of two participants, renal CEUS scans were executed twice using dorsal hand vein as well as the antecubital vein in two separate days. We initially used the dorsal vein for contrast administration in one participant. Then, we repeated the study using the antecubital vein and immediately noticed improved enhancement of renal cortex. Therefore, we decided to repeat CEUS for one more participant who similarly had better visual renal enhancement. As a result, I modified the protocol so that antecubital vein was used for all subsequent participants.

5.2.3 Image analysis

5.2.3.1 Destruction-reperfusion analysis

Each of the five recorded loops was analysed individually. First, perfusion model was set to perfusion. Then, using the clip editor, unwanted frames (beyond 10 s) were removed. A systematic approach was then adopted for ROI placement. A single ROI was drawn so that it included the largest possible area of renal cortex perpendicular to the ultrasound beam. In addition, the visualised renal cortex had to be free from any artefacts (acoustic shadow/enhancement), renal interlobar or arcuate vessels (verified by absence of bubbles right after the flash frame), inadequate insonification (flood of ultrasound waves), or excessive out-of-plane

motion. VueBox® measures the average intensity within the drawn ROI, so small ROIs were avoided to minimise local heterogeneities. VueBox then displays TIC and a parametric image (heatmap). From the generated parametric image, the ROI was reassessed and adjusted as necessary to ensure there were detected signals within the drawn ROI for the desired perfusion parameters. Motion compensation settings were not used.

The ROI of the first loop was saved and loaded into subsequent loops for consistency. Minor adjustments to the ROI were performed as necessary, bearing in mind the same cortex depth and kidney pole. The generated data were analysed independently using GraphPad Prism® version 9 (San Diego, CA, USA) so the method of generating the TIC could be specified. The non-linear one-phase exponential decay model $y = (y_0 - Plateau) * \exp(-K * X) + plateau$ was used, with y_0 constrained to $y_0 = 0$. The following perfusion parameters were derived for each loop individually: acoustic index (AI) (or plateau), which is the maximal intensity after reperfusion; mTT, which is the time needed after CA destruction to reach 50% of the maximal intensity; perfusion index, which is the ratio of AI to mTT; and WiR or K, which is the maximum slope. Each destruction-replenishment loop was analysed, and then, the median value was calculated for each parameter. A minimum of three loops with data of sufficient quality for analysis was required.

5.2.3.2 Bolus analysis

Bolus clip was imported into VueBox software, and perfusion model was set to bolus. The clip editor tab was used to view the recorded frames for initial visual assessment. Unlike the short destruction-replenishment clips, bolus clips were recorded over two-minute to include contrast wash-in and wash-out phases and, therefore, breath-holding was not possible. Motion compensation was therefore applied, and a single ROI was placed within the renal cortex before running the analysis. TIC and a number of parameters were generated and exported including peak enhancement (PE) a.u., time to peak (TTP) in seconds, wash-in rate (maximum slope) a.u. and rise time (RT) in seconds.

5.2.3.3 Variability between the individual flash/reperfusion clips

The coefficient of variation (COV) for the perfusion variables obtained from the optimal flash-reperfusion clips (using VueBox perfusion model) was computed between the individual flash-reperfusion curves. The COV was calculated using the equation: *COV % = (standard deviation/ mean) * 100.* Intra-individual repeatability COV was also calculated.

5.2.3.4 Analysis of different analysis methods

5.2.3.5 Region of interest size

Since the analysis was performed to determine the optimal methods for generating renal cortical perfusion curves, and since for every ROI, the software determines mean pixel intensities (proportional to contrast-

agent concentration) and creates a time–intensity curve (TIC), I tested two approaches for placing ROI in the renal cortex: Single large ROI around the visualised part of renal cortex as described in Section 5.2.3.1 versus multiple smaller ROIs placed at upper, mid, and lower parts of the renal cortex perpendicular to the probe. TICs from these two approaches were compared visually.

5.2.3.6 VueBox versus GraphPad Prism curve-fitting model

VueBox automatically performs TIC fitting and generates quantitative measures directly, but I sought to compare this against the raw data from each scanned clip imported into GraphPad Prism ® version 9 (San Diego, CA, USA) software so the method of generating the TIC could be specified. The non-linear one-phase exponential decay model $y = (y_0 \_ Plateau) * \exp(-K * X) + plateau$ was used, with y_0 constrained to $y_0 = 0$. Furthermore, a comparison between the perfusion variables derived directly from Vuebox and variables obtained from GraphPad prism fit model was presented for 10 participants. Comparison of the means generated by each software was performed using paired t-test. A p-value of < 0.05 was considered statistically significant.

This study was performed during COVID-19 pandemic with restrictions imposed in the University of Nottingham, which limited the plan for a larger sample size.

5.2.4 Results

I recruited 10 healthy volunteers (3 male, 7 female). Median (IQR) age was 34.5 years (28.75 – 41.22). Median body mass index (BMI) was 25.35 kg/m2 (22.93-27.3). Median mTT, the time needed after contrast agent destruction to reach 50% of the maximal intensity signal, was 0.82sec (0.71-1.1). Median AI, a measure of the maximal intensity after complete reperfusion, was 19143 a.u. (11239-23699), PI (AI/mTT) was 15750 a.u. (12125-33040), and Wash-in rate was 0.85 a.u. (0.63-0.98).

Figure 5.4 shows the time-intensity curve for the mean and 95% CI for the 10 healthy volunteers included in this analysis and the panel in Figure 5.5 illustrates individual TIC's for each participant.

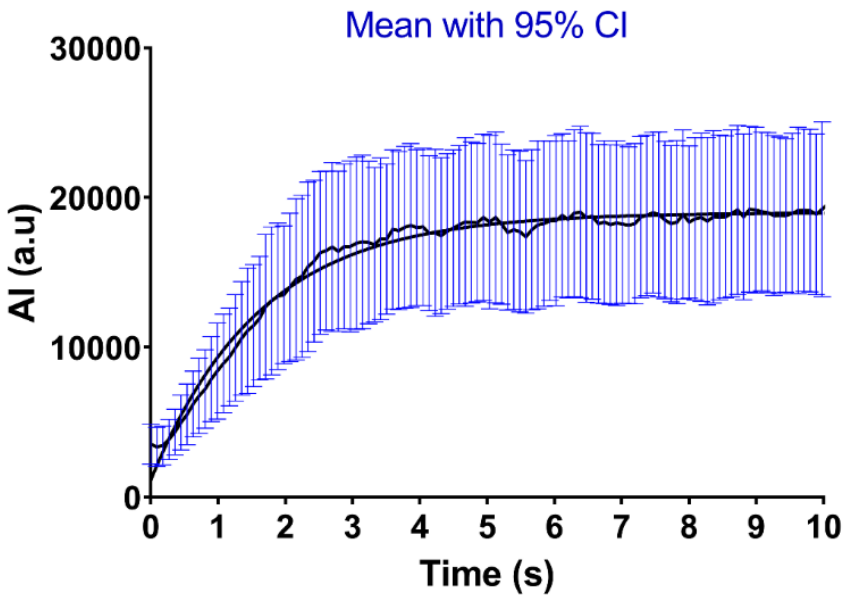


Figure 5.4: Time-intensity curve for the mean and 95% confidence interval for the healthy volunteers included in methods development renal CEUS study.

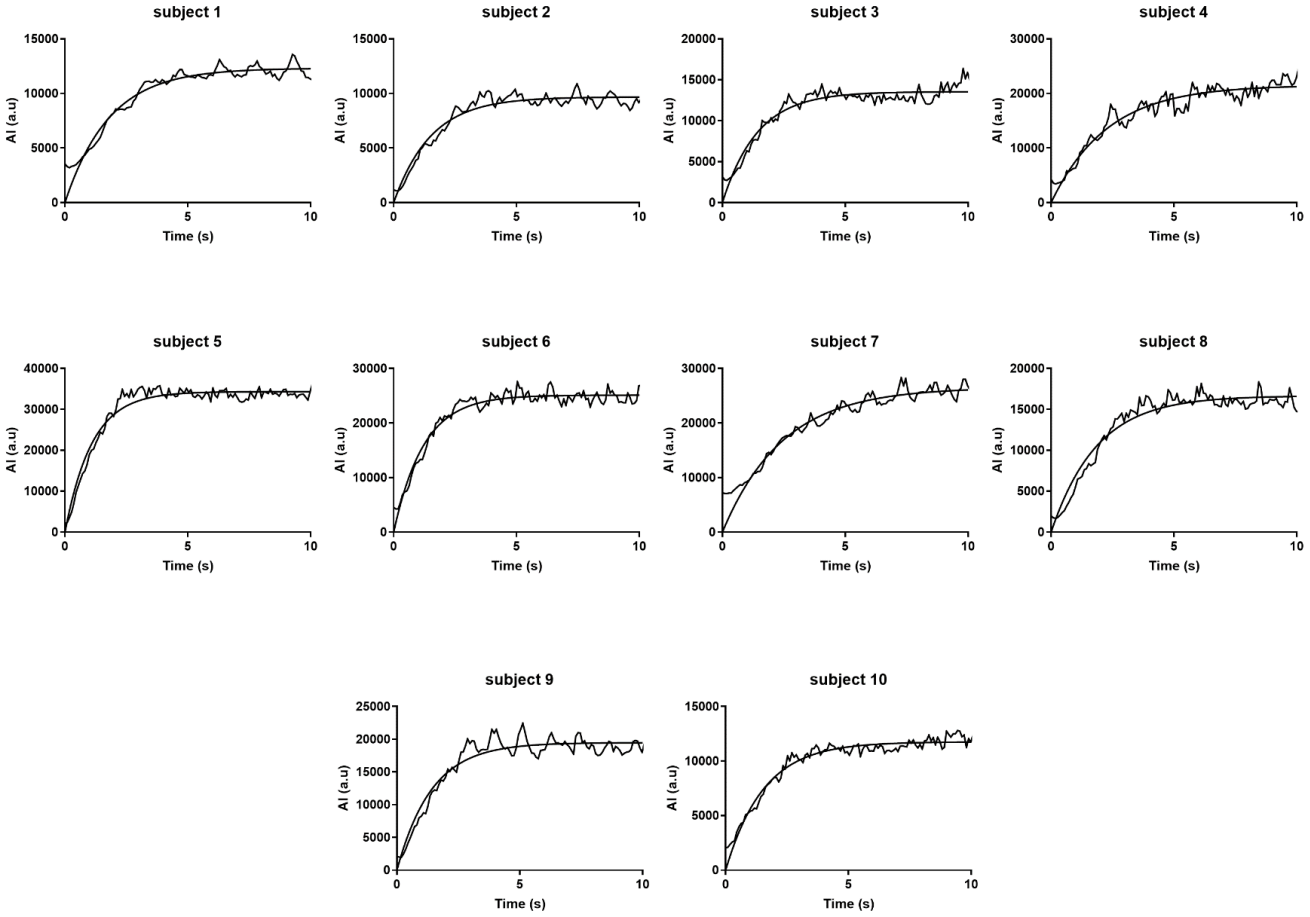


Figure 5.5: Prism-derived mean time-intensity curve for each subject

5.2.4.1 Time to disappearance and bolus injections

Time to disappearance of infused contrast was 12 minutes in seven subjects and six minutes in the remaining three subjects, calculated from the end of the infusion CEUS part and stopping the infusion.

A major challenge with bolus injection approach was respiratory motion during the two-minutes recording. This resulted in significant motion which prevented large ROI. A single small ROI was used for this approach as with observed motion, it was more controlled to contain the renal cortex without including other irrelevant tissue. In addition, images were also not as bright as those obtained from the infusion part, which could be due to fact that diluted rather than neat contrast was used.

All participants had at least one bolus injection. Bubbles were visible in the FOV soon after contrast injection, but with visually poorer intensity compared to that appeared in the infusion part of the study. Analysis of bolus clips was significantly affected by the significant motion from breathing throughout the continuous recording. As a result, analysis was possible for only 4 participants even with VueBox motion compensation applied. Therefore, I found that the quality of the clips did not meet the standards for interpretative analysis. A few examples of the analysis image and the VueBox-generated TIC are displayed in Figure 5.6 in which excessive motion is reflected in the noisy curves.

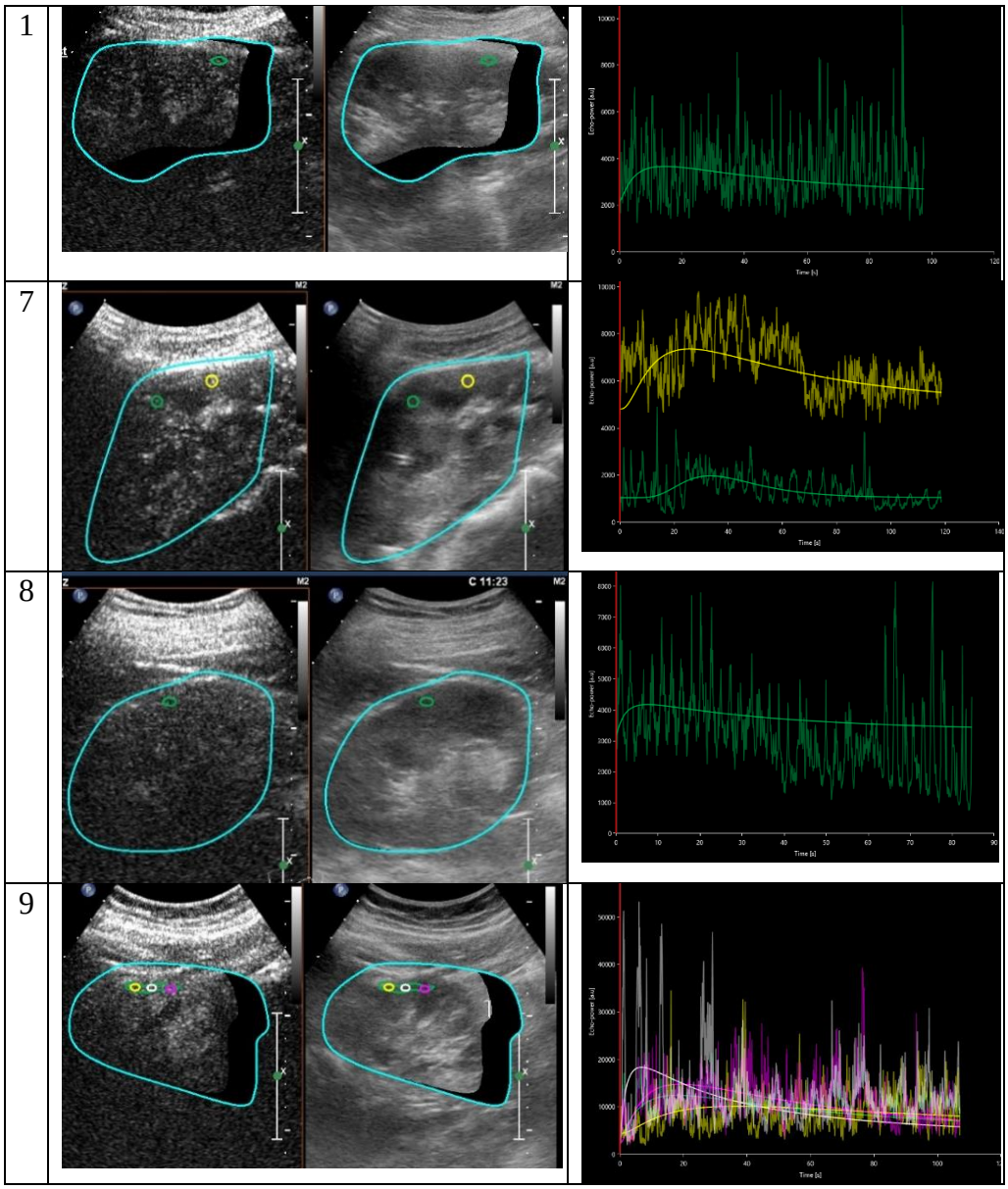
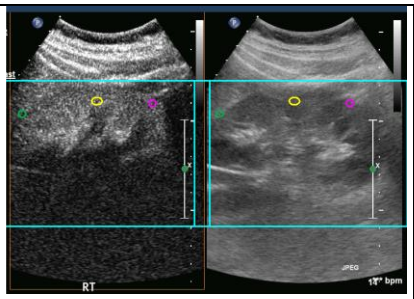
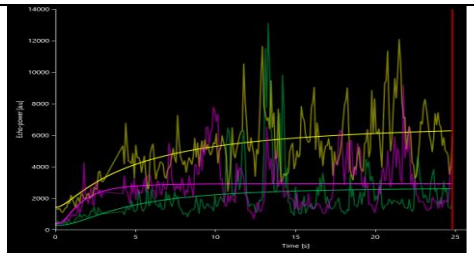
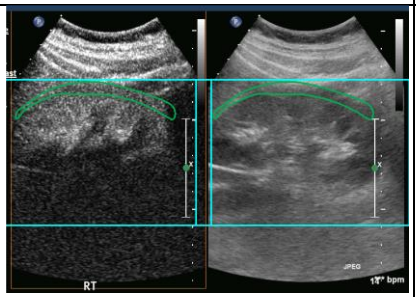
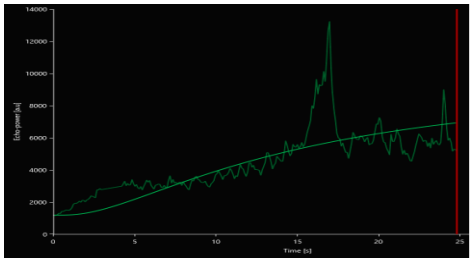
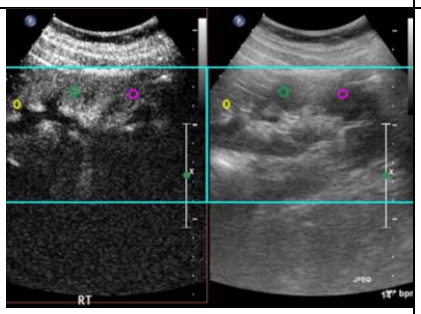
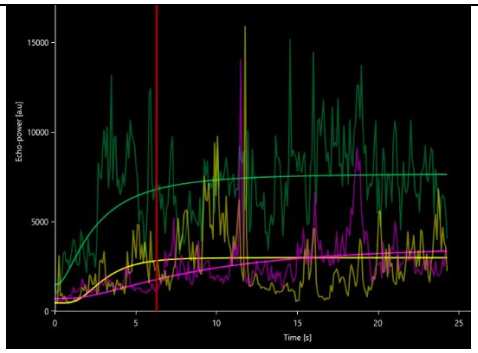


Figure 5.6: Examples images and generated TIC from bolus-based technique.

5.2.4.2 Region of interest

Analyses of clips in which both small ROIs and a single large ROI were simultaneously placed revealed how larger ROI produced smoother curves than those generated from smaller ROIs (Figure 5.7). Additionally, curves generated from the three smaller ROIs placed at different renal

parts were diverse, reflecting the local heterogeneity of renal cortex as ROI representing the average intensities within the drawn ROI which should be averaged and imported into another analysis software for a single averaged TIC. Figure 5.7 displays panel of images from the first participant.

Clip 1: Small ROIs		
Clip 1: Large ROI		
Clip 2: Small ROIs		

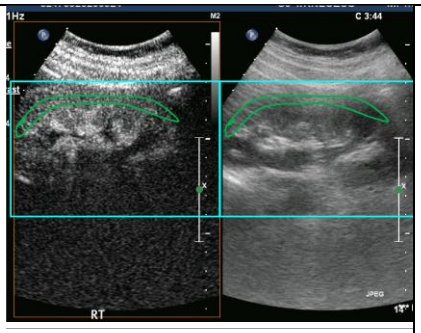
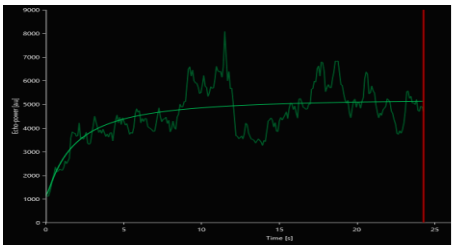
Clip 2: Large ROI		

Figure 5.7: Comparison between multiple small ROIs and a single large ROI analysis for the first participant.

Consequently, large ROIs were used for analyses and the coefficient of variation for each VueBox-derived perfusion variables obtained from 3 – 5 optimal flash-reperfusion clips were computed as summarised in Table 5.1. As a result of the high coefficient of variation percentages, the median rather than the mean was considered to minimise the effect of extreme values.

Subject number	**Acoustic index (a.u.) CV %**	**Mean transit time (s) CV %**	**Perfusion index (a.u.) CV %**
1	11%	12%	15%
2	24%	15%	32%
3	37%	37%	35%
4	16%	16%	31%
5	15%	33%	25%
6	29%	11%	30%
7	27%	26%	20%
8	24%	15%	24%
9	9%	39%	40%
10	19%	10%	22%
Intra-subject CV%	21%	21%	27%

Table 5.1: Coefficient of variation of perfusion variables for each subject.

The graphs in Figure 5.7 also shows that curves do not start from zero due to background noise included within the ROI after bursting the bubbles in the FOV. Therefore, I attempted two further steps: First, using reference ROI for normalisation. Second, using GraphPad Prism curve-fitting model which allow for curves constrain so it starts from zero since VueBox software does not offer a method to control this.

5.2.4.3 VueBox versus GraphPad Prism curve-fitting model

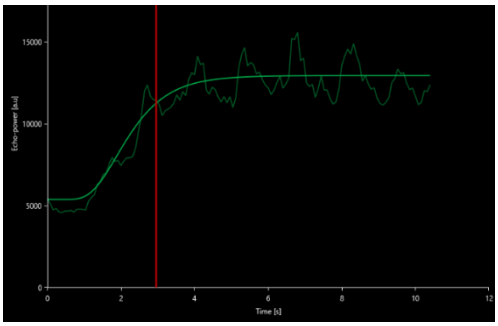
The fitted curves produced by VueBox and GraphPad Prism are observed in Figure 5.8, which shows how the curve does not start from zero, limiting standardised comparison between repetitive clips or subjects. Therefore, the curvefitting generated by GraphPad Prism (B) is constrained to zero so one staring point is achieved.

Figure 5.9 shows a panel of box and whisker graphs comparing perfusion variables derived from GraphPad Prism and VueBox. The means (SD) of each variable obtained from each software are presented in Table 5.2.

	GraphPad Prism	VueBox	P-value
AI	19149 (9005)	15848 (8539)	= 0.001
mTT	0.899 (0.24)	1.879 (0.57)	< 0.0001
PI	23742 (15924)	10200 (8671)	= 0.0005

Table 5.2: Means (SD) of perfusion variables obtained from analysis software. Paired t-test.

A:



B:

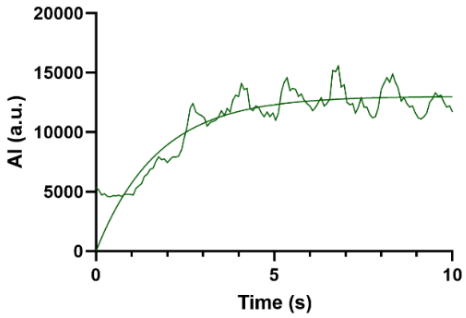


Figure 5.8: Subject 1-clip1. **A**: VueBox-generated fit curve of renal cortex. **B**: GraphPad Prism-generated fit curve constrained to zero.

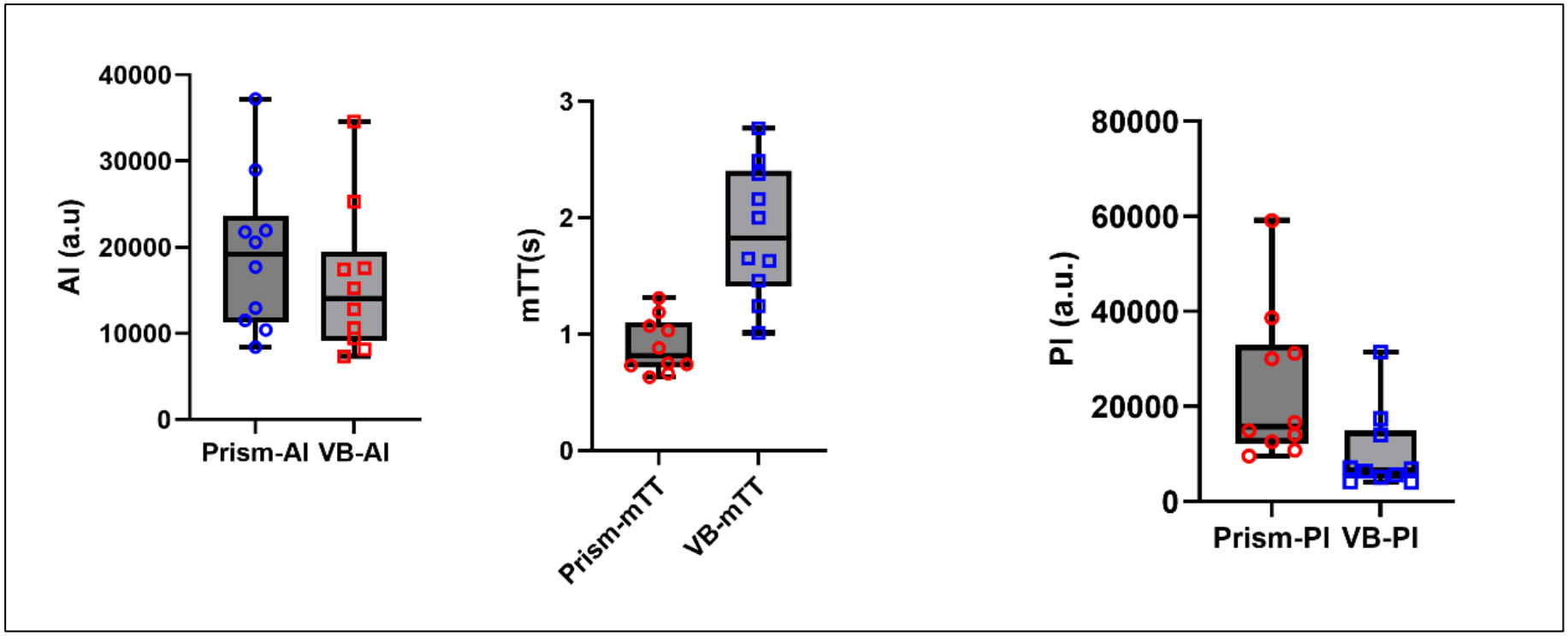


Figure 5.9: A panel demonstrating box and whisker plots with minimum, first quartile (Q1), median, third quartile (Q3), and maximum of perfusion variables obtained from GraphPad Prism fit model versus those obtained from VueBox (VB) perfusion model for the 10 included participants.

5.2.5 Summary of results from methods development and optimisation

The section has shown how assessment of renal perfusion using the infusion technique with destruction-reperfusion sequences performed better than the bolus technique which does not reliably produce images that can be analysed. It was also shown that contrast time to disappearance after the end of infusion occurs by 12mins in most of the HVs. The variability between repeated TICs in the same CEUS examination was high (high COV %) and, therefore, the median was preferred over the mean for data averaging. For image analysis, single larger ROIs in the renal cortex generated smoother curves than small ROIs placed in different areas of the cortex. In addition to that, combining the multiple small ROIs requires additional analysis software for averaged TIC. Moreover, there are differences in the pre-set curve fitting in VueBox versus replotting the raw data in GraphPad Prism; this results in some differences in quantitative variables generated. Whilst either method can be adopted, results should not be combined, and the analysis method clearly stated. Finally, CEUS-derived perfusion measure, half-time, showed tighter interquartile range compared to the intensity-based (AI) measure and the calculated PI.

5.3 Section 2: Repeatability of Contrast-Enhanced Ultrasound to Determine Renal Cortical Perfusion

Having optimised the CEUS protocol and obtained initial data in healthy volunteers, this section now assesses the repeatability of CEUS-derived perfusion measures on the kidneys of ten healthy volunteers.

5.3.1 Aims

The aims of the repeatability study were 1) to determine the intra-individual repeatability of different measures of perfusion from renal CEUS taken under standardised conditions in HV, and 2) to test inter-operator repeatability of the process of image analysis. 3) To assess the relationship between perfusion variables and subjects' characterestics and cardiovascular measures.

5.3.2 Methods

This was a cross-sectional observational study designed to assess intra-individual and inter-operator variability of quantitative CEUS measures of renal perfusion. The study was approved by UoN Research Ethics Committee (403-1910), and all participants provided written informed consent.

5.3.2.1 Participant Characteristics

Ten adults were recruited between February 2021 and April 2021. Inclusion criteria were healthy people above the age of 18 years, with no known kidney disease, diabetes, or hypertension and no known sensitivity to the CA (Sonovue®).

5.3.2.2 Study Procedures

Each participant attended Royal Derby Hospital Centre for two renal CEUS scans within a 2-week period. No specific preparation was required from participants prior to the scan. Demographic and anthropometric details including age, gender, ethnicity, height, and weight were recorded, with BP and heart rate (HR) also measured at each visit. All CEUS scans were performed by a single sonographer with CA administration performed by a medically qualified member of the research team. The primary outcome was intra-subject repeatability of CEUS-derived cortical perfusion parameters.

5.3.2.3 Image Acquisition

Utilising the optimised methods from Section 5.2, and using right antecubital vein, renal CEUS was measured in all subjects on each study day (9 right kidney scanned, 1 left kidney where the view of right kidney was not satisfactory). The same imaging plane used in the first session was used for session 2. This was confirmed by reviewing first visit images and referring to notes on transducer position made at time of the first

scan. The selection of the best view was based on the image quality of the visualised renal cortex that was clear of artefact, e.g., acoustic shadow from ribs.

5.3.2.4 Image Analysis

Image analysis was performed independently by two operators to assess inter-observer variability.

5.3.2.5 Statistical Analysis

Analyses were performed using IBM SPSS® (Version 27, New York, NY, USA). Normality of distribution was tested graphically. Non-normally distributed variables were log-transformed prior to analysis. Data are expressed as median and IQR for non-normally distributed data. A *p*-value of <0.05 was considered statistically significant. The perfusion variables obtained from the two renal CEUS sessions for each participant were assessed for repeatability using an intra-class correlation (ICC) one-way model. Inter-observer variability was assessed using ICC two-way mixed model with absolute agreement. This was interpreted with reference to the criteria by Cicchetti [24], where an ICC of >0.75 is considered excellent, 0.60–0.74 is good, 0.4–0.59 is fair, and <0.40 is poor. Bland–Altman analyses were also performed and expressed as the mean difference, standard deviation and 95% limits of agreement.

The mean difference between the first and second scans was also calculated for each parameter. Associations between perfusion variables and subject characteristics and cardiovascular measures were assessed

using a two-tailed Pearson's correlation for normally distributed data of continuous variables and with independent samples t-test for categorical variables. For non-normally distributed data, Spearman's correlation, and Mann–Whitney tests were used for continuous and categorical data, respectively.

5.3.2.5.1 Sample size justification

This study was performed while COVID-19 restrictions were imposed and only limited participants were allowed to be invited to the study room based in the University of Nottingham. This is an original work and no similar repeatability study for renal CEUS measures has been performed, and sample size was determined on a pragmatic basis. Additionally, sample size is adequate to demonstrate important points regarding the differences in repeatability between intensity-based and time-based measures that have relevance to the design of other studies using CEUS to assess renal perfusion.

5.3.3 Results

I recruited 10 participants (5 male and 5 female), with a median (IQR) age of 39 years (30–46) and BMI of 24.9 kg/m^2 (22.3–25.9).

5.3.3.1 Perfusion Variables

Perfusion variables and the mean and (SD) of the difference between CEUS sessions are shown in Table 5.3. Figure 5.10 illustrates box and whisker plots of each perfusion variable in scan 1 and scan 2. Figure

5.11 shows the TIC for each participant in both visits. A summary description for participants' blood pressure and heart rate in the two CEUS sessions is in Table 5.4.

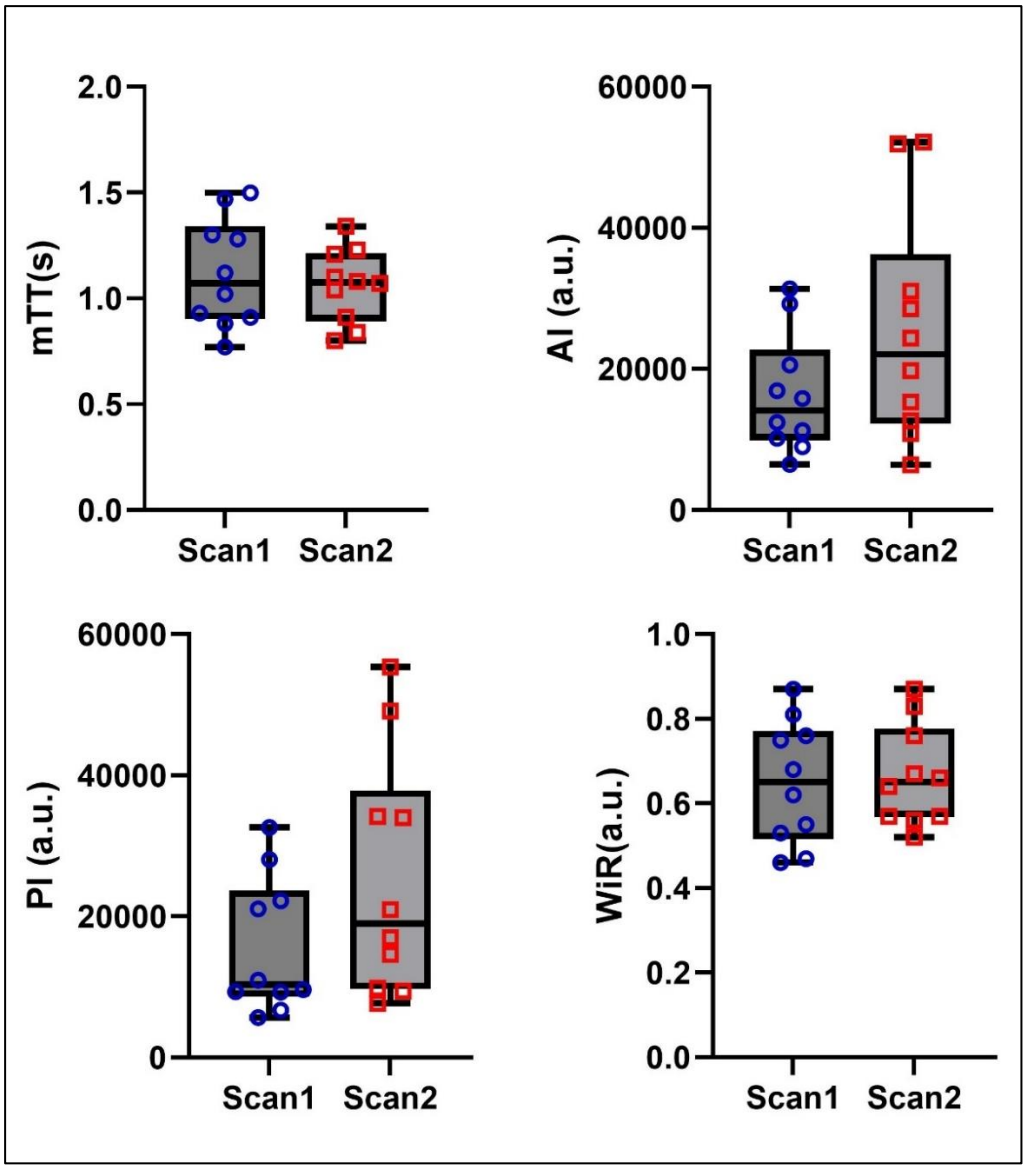


Figure 5.10: A panel of box and whisker plots for perfusion variables in the first and second scan. mTT: mean transit time; AI: acoustic index; PI: perfusion index; WiR: wash in rate.

	Scan1	Scan2
mTT (Second)		
Median (IQR)	1.07 (0.90–1.34)	1.07 (0.89–1.21)
Range	[0.77–1.50]	[0.80–1.34]
Difference mean (SD)	0.06 (0.21)	
AI (a.u.)		
Median (IQR)	14,120.50 (9872.13–22,728.00)	22,083.50 (12,211.13–36,251.62)
Range	[6473.00–31352.00]	[6411.00–52191.00]
Difference mean (SD)	−8993.35 (13,350.64)	
PI (a.u.)		
Median (IQR)	10,302.64 (8647.33–23,680.44)	18,959.96 (9713.19–37,897.76)
Range	[5699.38–32,616.76]	[7682.51–55,390.67]
Difference mean (SD)	−9653.75 (11,940.57)	
WiR (a.u.)		
Median (IQR)	0.65 (0.51–0.77)	0.65 (0.57–0.78)
Range	[0.46–0.87]	[0.52–0.87]
Difference mean (SD)	−0.0150 (0.15)	

mTT = mean transit time; AI = acoustic index; PI = perfusion index; WiR = wash-in rate.

Table 5.3: CEUS-derived perfusion variables for scan1 and scan2; *n* =

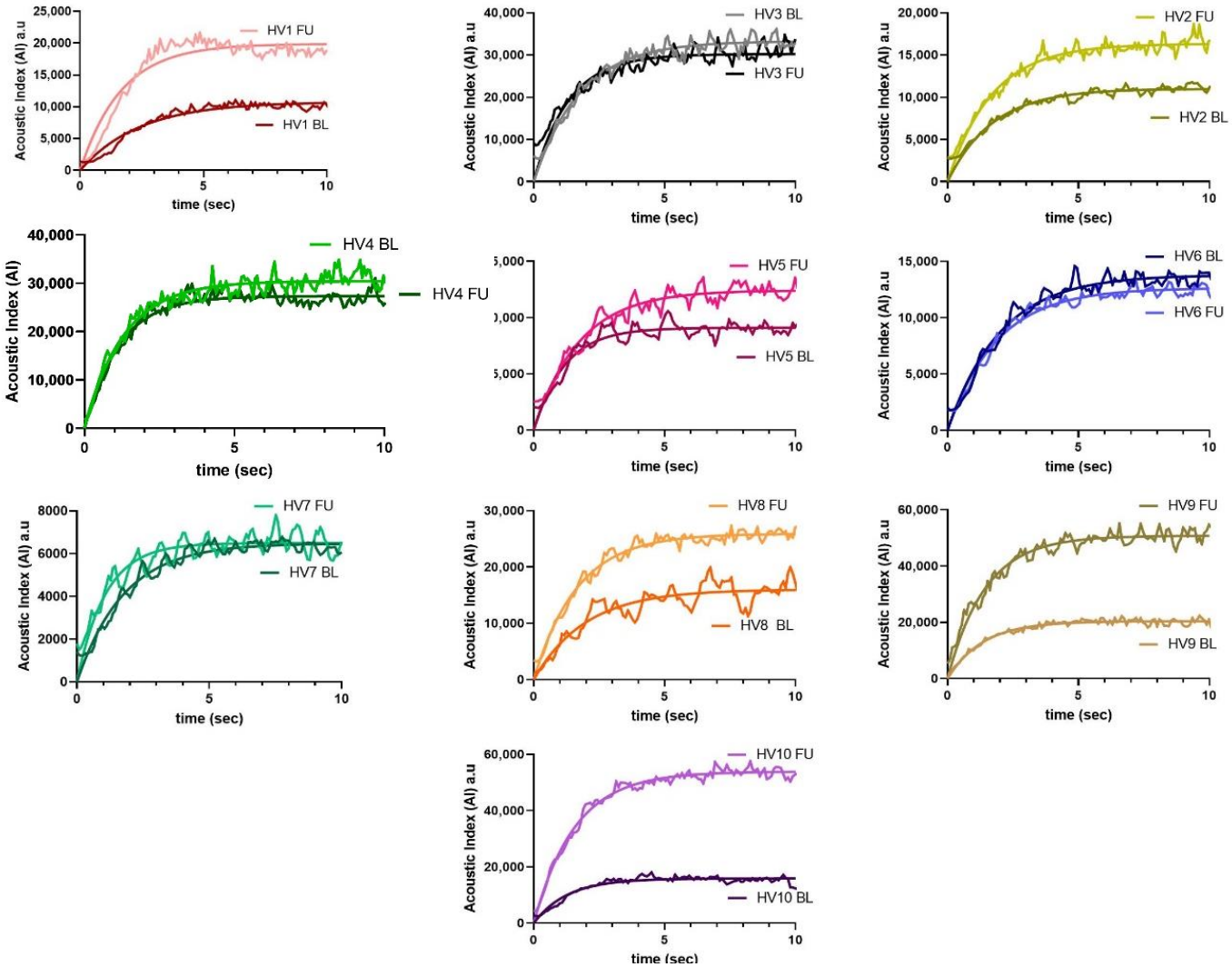

Acoustic Index (AI) a.u
time (sec)
HV1 FU
HV1 BL
HV3 BL
HV3 FU
HV2 FU
HV2 BL
HV4 BL
HV4 FU
HV5 FU
HV5 BL
HV6 BL
HV6 FU
HV7 FU
HV7 BL
HV8 FU
HV8 BL
HV9 FU
HV9 BL
HV10 FU
HV10 BL

Figure 5.11 Time–intensity curves representing contrast replenishment in renal cortex for individual patient

	CEUS1			CEUS2		
	HR (bpm)	SBP (mmHg)	DBP (mmHg)	HR (bpm)	SBP (mmHg)	DBP (mmHg)
Median (IQR)	60.00 (54.50 – 76.75)	120.50 (108.75 – 132.25)	82.00 (76.50 – 86.00)	54.50 (50.75 – 68.25)	122.00 (110.50 – 136.50)	80.50 (72.25 – 86.00)

HR= heart rate; bpm= beats per minute; SBP= systolic blood pressure; DBP= diastolic blood pressure.

Table 5.4: Patients' cardiovascular measures on CEUS sessions; n = 10.

5.3.3.2 Intra-Individual Repeatability

A good degree of repeatability was found for mTT (ICC: 0.71; 95% CI: −0.11 to 0.93; $p = 0.03$). The corresponding value for PI was (ICC: 0.65; 95% CI: −0.34 to 0.91; $p = 0.06$). Only fair, non-significant correlation values were found for AI (ICC: 0.50; 95% CI: from −0.89 to 0.94; $p = 0.15$) and WiR (ICC: 0.56; 95% CI: −0.67 to 0.89; $p = 0.11$). As seen in Figure 5.11, some individuals had TICs that were very similar between study sessions, which indicates good repeatability for both perfusion variables (AI and mTT), but others had clear differences, particularly in maximal intensity, reflecting less repeatability for AI. In these latter cases, the time to reach plateau appeared to vary less, which is consistent with the ICC analyses.

5.3.3.3 Inter-Operator Variability

The correlation between the two operators were excellent for all values. For PI, ICC was 0.99; 95% CI: 0.96 to 1.00; $p < 0.001$. For AI, ICC was 0.98; 95% CI: 0.95 to 0.99; $p < 0.001$. For mTT, ICC was 0.87; 95% CI: 0.65 to 0.95; $p < 0.001$. For WiR, ICC was 0.83; 95% CI: 0.56 to 0.93; $p < 0.001$. Figure 5.12 shows the Bland–Altman plots for inter-observer agreement on each of the obtained perfusion variables. The mean difference between the two observers was close to zero for all variables with no evidence of systematic bias.

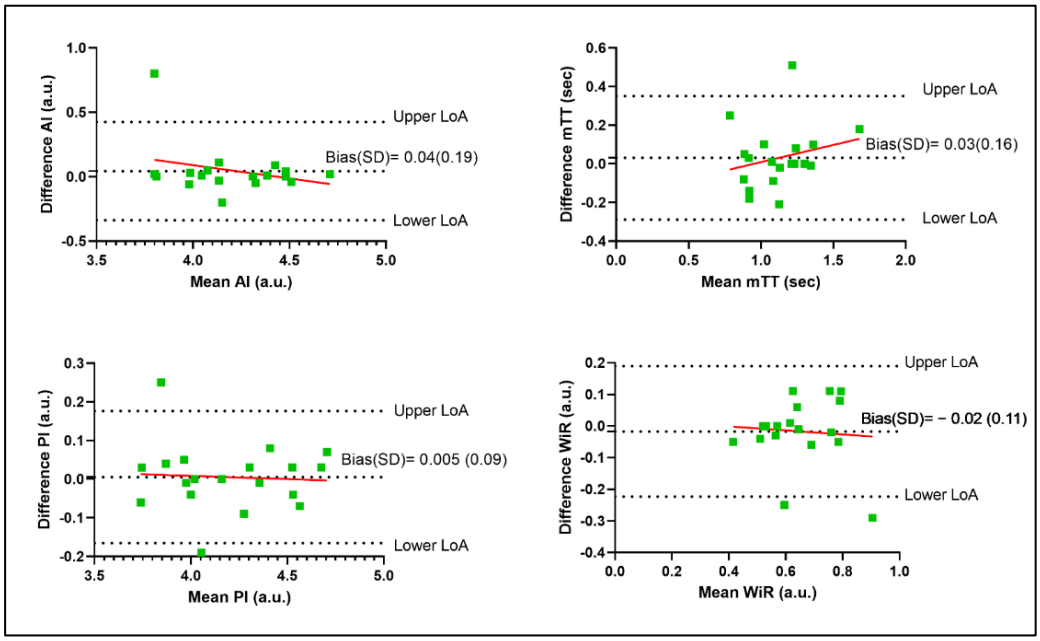


Figure 5.12 Inter-observer variability Bland–Altman plots; with linear regression line (red). Limits of agreement = 95% limit of agreement.

5.3.3.4 Relationship between Perfusion Variables and gender

There was no difference in perfusion values between genders (Table 5.5).

	Mean (SD) Female	Mean (SD) Male	*P*-value
mTT (sec)	1.004 (0.16)	1.232 (0.30)	0.17
AI (a.u.)	20, 279.6 (10,139.62)	12,360 (4254.49)	0.25
PI (a.u.)	20,636.48 (10,284.58)	10,486.44 (6151.23)	0.06
WiR (a.u.)	0.71 (0.101)	0.59 (0.169)	0.21
mTT = mean transit time; AI = acoustic index; PI = perfusion index; WiR = wash-in rate.			

Table 5.5: Association of gender with perfusion variables.

5.3.3.5 Relationship between Perfusion Variables and cardiovascular measures

There was no evidence of association between baseline perfusion variables and baseline systolic BP and heart rate (Table 5.6).

SBP (mmHg)	**R**	**95% CI range**	**P-value**
mTT (s)	– 0.49	[– 0.81 – 0.08]	0.16
AI (a.u.)	0.12	[– 0.60 – 0.8]	0.75
PI (a.u.)	0.26	[– 0.47 – 0.86]	0.47
WiR (a.u.)	0.44	[– 0.35 – 0.78]	0.21
HR (bpm)			
mTT (s)	– 0.27	[– 0.84 – 0.47]	0.45
AI (a.u.)	0.26	[0.54 – 0.71]	0.47
PI (a.u.)	0.38	[– 0.35 – 0.89]	0.28
WiR (a.u.)	0.27	[– 0.47 – 0.83]	0.45
mTT = mean transit time; AI = acoustic index; PI = perfusion index; WiR = wash-in rate; SBP: systolic blood pressure; HR: heart rate.			

Table 5.6: Association between perfusion variables and cardiovascular variables.

5.3.3.6 Relationship of Perfusion Variables with age and BMI measures

There was no evidence of association determined between personal characteristics (age, or BMI) and baseline perfusion variables (Table 5.7)

Age (years)	**R**	**95% CI range**	**p-value**
mTT (s)	0.39	[– 0.47 – 0.94]	0.26
AI (a.u.)	0.12	[– 0.60 – 0.87]	0.75
PI (a.u.)	– 0.15	[– 0.81 – 0.65]	0.69
WiR (a.u.)	– 0.41	[– 0.89 – 0.35]	0.24
BMI (kg/m^2)			
mTT (s)	– 0.26	[– 0.68 – 0.44]	0.47
AI (a.u.)	– 0.37	[– 0.93 – 0.38]	0.29
PI (a.u.)	– 0.2	[– 0.87 – 0.66]	0.58
WiR (a.u.)	0.26	[– 0.47 – 0.69]	0.47

Table 5.7: Association of perfusion variables with age and Body mass index. mTT = mean transit time; AI = acoustic index; PI = perfusion index; WiR = wash-in rate; BMI: Body mass index.

5.3.4 Discussion

In this study, I report intra-subject repeatability data from healthy volunteers for each of the measures of renal cortical perfusion generated from renal CEUS using the destruction/replenishment technique. The time-based measure of mTT had the best repeatability, whilst measures based on image intensity (AI) performed less well. The individual curves for each participant show results consistent with this (Figure 5.11), with some participants having very similar TICs between the two scans but others displaying large variations in AI (plateau) with less effects on mTT (half time). Additionally, I report a high degree of inter-operator agreement for all measures for the analysis process, suggesting that

variation observed within individuals arises from differences in image acquisition. These results suggest that mTT is likely the most reliable measure for future work in which CEUS is used to assess renal perfusion in patient cohorts.

In the present study, I employed a systematic approach to tackle potential factors that could contribute to variations in the acquisition of CEUS perfusion measures. Image acquisition was performed with a standardised approach using consistent machine settings. Parameters obtained from a certain ROI depend on the attenuation properties of the tissues overlying it, through which ultrasound waves propagate. These vary not only with the type of the tissue but also across the population and may also be influenced by pathology (Maklad et al., 1984). Therefore, differences between study sessions that were seen in some individuals could have arisen from differences in the transducer position or scanning plane between study sessions, in addition to effects of breath-holding. Variation could also be attributed to the differences in the participants' physiological status between the two study days, but this seems less likely. In this study, I assessed the relationship between BP, HR, gender, age and BMI with CEUS perfusion variables, and there was no significant correlation. However, other physiological factors that could potentially affect CEUS variables include hydration status (Pruijm et al., 2014), dietary salt intake (Pruijm et al., 2010), and tobacco use (Boss et al., 2009), which previously showed an effect on renal tissue oxygenation.

The analysis was performed with the ROI placed at the same depth and with those of the same size as far as possible. However, this was still subject to a degree of variability between the repeated flash/reperfusion loops. However, I have demonstrated that the analysis technique is repeatable with high inter-operator ICC values, and hence, the analysis process is unlikely to be a major source of variation.

Previous studies using renal CEUS for cortical perfusion quantification have used a variety of ROI sizes/numbers. Three small (5×5 mm^2) ROIs have been used in some studies (Yoon et al., 2020, Schneider et al., 2012, Tiemann et al., 2000), whereas one larger ROI was used in others (Harrois et al., 2018, Garessus et al., 2021, Schneider et al., 2013, Schneider et al., 2014). In our hands, I have found that adopting the largest ROI around visualised renal cortex helps avoid regional heterogeneity in renal perfusion and smooths out the generated TICs. The choice of a larger ROI could potentially average the effect of lateral shift variation, as observed by Ignee et al. (Ignee et al., 2010), as VueBox® calculates the average of intensities in a ROI. The excellent inter-observer agreement for all CEUS perfusion parameters reflects the minimisation of heterogeneity and reinforced our analysis approach. It is worth pointing out that I did not identify a reference ROI for normalisation as with renal tumour perfusion quantification studies, which means that a comparison of intensity-based variables between individuals becomes more challenging. A reference ROI is normally placed within adjacent representative renal tissue at the same depth, an approach that was not possible with this method (Lu et al., 2015, Dietrich et al., 2012).

In accordance with our findings, a study by Harrois et al. (2018) shows differences between time-based and intensity-based CEUS measures of perfusion in clinical settings. They reported that mTT was significantly increased in patients with septic shock who went on to develop severe AKI as compared with those who did not, with mean mTT values of 5.6 s and 3.4 s in the AKI and non-AKI groups, respectively. Notably, both groups had higher mTT ranges than seen in our healthy individuals (0.8–1.5 s), perhaps representative of disease-induced renal cortex hypoperfusion. However, in contrast to that reported for mTT, Harrois et al. (2018) reported no difference in the intensity-based measures of regional blood volume (equivalent to AI) and PI between AKI and non-AKI groups (Harrois et al., 2018). Other studies in animals have reported a higher variability of ultrasound measures of image intensity compared with time-dependent variables. For example, in a recent animal study by Liu et al. (Liu et al. (2019), variation in the repeated renal cortex CEUS perfusion parameters was assessed in healthy dogs using a bolus CA technique. This study showed large COV for intensity-based variables (of ~45%) compared with time to peak, which had a COV of only 14.7%. Similarly, Leinonen et al. (2010) performed renal CEUS on healthy cats using a bolus technique and showed a significant change in the peak intensity variables with different cortical ROI depths and sizes. Taken together, these data suggest that time-based parameters are less susceptible to technical variation than those based on intensity.

There are some limitations to this study. First, the sample size was relatively small, and as this study was performed on healthy volunteers,

the results may not be generalisable to patient cohorts. Future studies should report intra-subject repeatability for patients with kidney disease (e.g., CKD). Other potential physiological factors such as hydration status, dietary salt intake, or tobacco use were not evaluated nor controlled and, although unlikely to have been significantly different within individuals between study sessions, have potential to influence results. Finally, inter-operator variability was only assessed for the analysis process, not for image acquisition, another aspect that should be considered as scope for future work.

5.3.4.1 future clinical utility

CEUS is a promising bedside, fast, and cost-effective tool. Its portability and high safety profile allow for easy implementation in clinical practice and most renal patients are already familiar with the ultrasound scan. It can provide access to unique diagnostic information including real time visualisation of microvascular perfusion as well as quantitative measures post analysis. Such information is useful to address the gap of limited understanding of organ perfusion which can play a vital role in the pathophysiology of kidney disease. I demonstrated through the repeatability study that mTT was the marker that performed the best. Therefore, it could be the preferred measure to guide clinicians on whether, for example, AKI is associated with reduced perfusion or not, or to monitor response to fluid resuscitation or vasopressors.

5.3.5 Conclusions

The current study reports the repeatability of CEUS-derived perfusion variables for renal cortical perfusion. Based on data from healthy individuals we conclude that the time-based variable (mTT) had good repeatability and is likely the most reliable measure for future studies in which CEUS is used to assess renal perfusion in patient cohorts and to assess changes in perfusion over time. The large intra-individual variation in intensity-based measures (AI) seen in some participants suggest that this parameter may not be suitable for this purpose.

Chapter 6: Demonstrating Renal Cortical Perfusion Changes in Acute Kidney Injury

Having established the CEUS protocol for image acquisition and analysis, and studied the repeatability of CEUS in healthy volunteers, this chapter presents a prospective observational study exploring the feasibility of CEUS in patients with AKI.

6.1 Abstract

Introduction: Acute kidney injury is a global health challenge. A reduction in renal perfusion may plays an integral role in many AKI cases, but there is a lack of diagnostic methods that can quantify renal perfusion clinically. The primary aim was to generate descriptive data on the variation in renal microvascular perfusion in AKI.

Methods: This was a prospective, observational pilot study of renal CEUS in adult patients with AKI stage 2/3. Renal cortical perfusion was measured using CEUS at study entry, and once on each of the subsequent four days. CEUS Images were acquired according to the procedure used for healthy volunteers (HV) in (Section 5.1) where a minimum of three flash/reperfusion clips were obtained for the kidney. Analysis was performed using VueBox software. Associations between mTT and clinical variables were assessed.

Results: A total of 12 AKI patients were recruited (11 had AKI stage 3 at time of recruitment, and one had stage 2). Patients were 5 male and 7 female. Median age (years) was 73 (65-78), median BMI (kg/m^2) 25.01 (18.88 – 36.36).

Of all CEUS scans, 63% of all scans were successfully delivered. Five patients had too few serial CEUS measures and were excluded, leaving seven patients for the analysis. CEUS perfusion variables obtained on day 1 were as follows: Mean (SD) mTT (s) was 1.59 (0.72), median (IQR) AI (a.u.) and PI (a.u.) were 4415 (3549 –14188) and 4485 (2384 –10628), respectively. Not all AKI patients had reduced CEUS measures of renal cortex perfusion compared with the HV's range. Five patients had increased mTT across all timepoints compared to the HV. There was a moderate negative correlation between mTT and SBP, but no association with serum creatinine.

Conclusion: Assessment of renal perfusion using CEUS is generally feasible in an acute setting of AKI. Sequential CEUS scans are harder. AKI patients had reduced CEUS perfusion measures, but there was variability at a patient-level. Further clinical studies are necessary to support or refute these preliminary findings, and their design may be informed by this work.

6.2 Introduction

Acute kidney injury is a global health challenge defined as an abrupt deterioration in kidney function or decline in GFR (Lewington et al., 2013). Over the years, AKI classification systems have been developed for use in clinical settings: Risk, Injury, Failure, Loss, End-Stage Kidney Disease (RIFLE), the AKI Network (AKIN) and, most recently, Kidney Disease Improving Global Outcomes (KDIGO) criteria (Kellum and Lameire, 2018, Selby, 2019)**.** KDIGO defines AKI as an increase in serum creatinine (SCr) by 0.3 mg/dL within 48

hours, an increase in SCr by 50% above baseline value within 7 days and/or < 0.5 mL/kg/h of urine production for ≥ 6 h (oliguria) (Kellum et al., 2013).

AKI can occur in a normally functioning kidney or superimposed on chronic kidney disease (Lameire et al., 2021). AKI is common in hospitalised patients, with nearly 500,000 cases per annum in England alone, and is associated with remarkable mortality rates of > 20% and very poor outcomes (Chawla et al., 2014, Hoste et al., 2018). AKI is a diverse syndrome with various aetiologies and outcomes that differ greatly between individuals. These causes can be broadly categorised into pre-renal (perfusion-related), intra-renal, and obstructive causes. The inability to pinpoint the underlying aetiology of AKI often hinders its management and research. In addition, the fact that significant renal impairment can occur by the time SCr levels rise to the point at which they indicate AKI is still a concern (Petrovic et al., 2015), leading to a smaller chance of effective treatment and a worse prognosis. Therefore, exploring additional tools for earlier AKI detection has been the focus of recent research (Petrovic et al., 2015).

The kidney is normally a highly perfused organ. It is traditionally thought that a reduction in renal perfusion plays an integral role in many AKI cases. Common perfusion-related aetiologies include sepsis and intravascular volume depletion, which if severe or uncorrected and associated with prolonged renal hypoperfusion can precipitate acute tubular injury that persists even after the underlying haemodynamic injury subsides (Sharfuddin et al., 2016). However, this is likely an over-simplification, with the causes of AKI traditionally labelled as ‘pre-renal’ likely affecting perfusion in different ways and to different extents. For example, sepsis induces profound changes in microvascular flow in the

entire organism, including the kidney. These alterations are characterised by a decrease in vascular density along with an increment proportion of sluggish or no flow capillaries. This results in increased heterogeneity of flow, a greater diffusion distance for oxygen and areas of tissue hypoxia (Gomez et al., 2014). These changes would not be expected in volume depletion. Importantly, there is a lack of diagnostic methods that can quantify renal perfusion clinically, or that can assess response to supportive AKI treatments such as fluid administration or the use of vasopressors.

In the previous chapter, I demonstrated that CEUS can be used to measure healthy volunteers' renal microvascular perfusion. A series of small studies have measured renal perfusion using infusion-based CEUS among different AKI subsets (postcardiac surgery patients, patients in the ICU and patients with AKI caused by hepatorenal syndrome) and found that whilst perfusion measures were reduced in some patients, there were differences in the degree of change in perfusion between individuals (Schneider et al., 2013, Schneider et al., 2014, Schneider et al., 2015), Harrois et al. (2018), Wang et al. (2019), Yoon et al. (2020). How these individual changes are associated with the onset and progression of AKI needs to be further understood. Therefore, diagnostic tools capable of visualising and measuring the renal microvascular perfusion at the bedside benefit the evaluation of AKI. CEUS is particularly valuable for patients with renal impairment where the contrast is not nephrotoxic (Wang and Mohan, 2016). Further research is still needed to support the translation of CEUS to clinical settings for renal perfusion measurement in patients with AKI.

6.2.1 Aims

The primary aim of this study was to generate descriptive data on the variation in renal microvascular perfusion in the context of AKI, which is essential for the design of future studies. The secondary aims were to conduct exploratory clinical observations of the relationship between CEUS measures of renal cortical perfusion and clinical measures of AKI and its recovery and to determine the feasibility measures of CEUS use in the acute care setting.

6.2.2 Primary outcomes

The primary outcome is the mean and standard deviation or median and interquartile range for renal microvascular blood flow on first assessment <72h after onset of AKI as assessed by CEUS. This will allow robust sample size calculations for future clinical trials involving renal CEUS.

6.2.3 Secondary outcomes

Exploratory clinical outcomes:

- Change in serial CEUS measures over time
- Comparison of CEUS measures with daily serum creatinine (severity and recovery of AKI)
- Relationship of CEUS measures with clinical measures including blood pressure, urine output, the volume of intravenous fluid administration, cause of AKI.

6.3 Methods

6.3.1 Study design

This was a prospective, observational pilot study of renal CEUS in people with AKI. Renal cortical perfusion was measured using CEUS at study entry, and then once on each of the subsequent four days, unless there had been complete recovery of renal function within this time, or CEUS measures were no longer possible for clinical reasons or patient withdrawal.

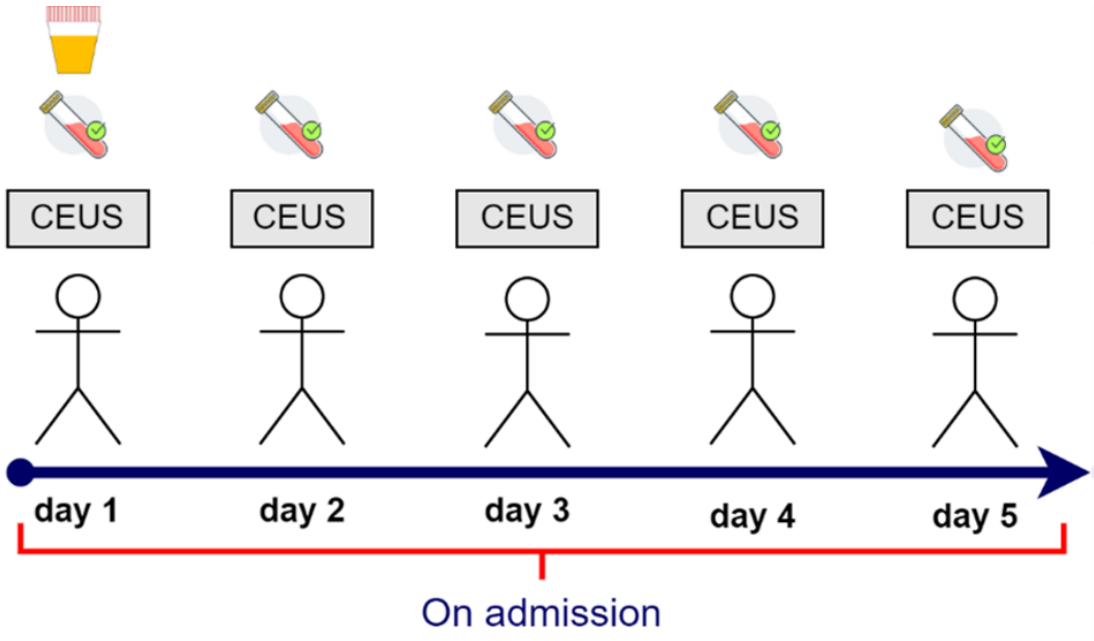


Figure 6.1: Schematic diagram of CEUS in AKI study design including CEUS scans, blood, and urine collection.

6.3.2 Recruitment

The study was approved by the East of England - Essex Research Ethics Committee and the UK Health Research Authority (reference number 21/EE/0027). Patients were identified by members of the research team who

are also members of the clinical teams covering the renal wards and ICU at RDH with reference to the eligibility criteria in Table 6.1. The exclusion criteria were confirmed through the patient's medical records and/or by asking patients/consultee directly.

Inclusion criteria	Exclusion criteria
AKI stage 2/3, as defined by the KDIGO criteria	Patients who cannot communicate in English
Age ≥18 years; no upper limit.	>72h elapsed since the detection of AKI
Male or female	Lack of baseline serum creatinine value within the previous 365 days
Ability to provide informed consent /or in the case of participant incapacity, a consultee who could be (a relative, friend or health care professional) should be available to advise participant participation.	Concurrent co-morbidities as follows: Autosomal dominant polycystic kidney disease, glomerulonephritis receiving immunosuppression, multiple myeloma, obstructive uropathy, solid organ transplant
	Contraindications to the CA: Known allergy to SonoVue CA, recent acute myocardial infarction (<7 days), class iii/iv cardiac failure, right-to-left cardiac shunts, severe pulmonary hypertension, pregnancy, or breastfeeding.

AKI: acute kidney disease; KDIGO: Kidney Disease Improving Global Outcomes; CA: contrast agent.

Table 6.1: Eligibility criteria

6.3.2.1 Informed consent

All patients or consultee were informed of all aspects pertaining to participation in the study before providing written informed consent.

Consent was obtained from the patients themselves in the first instance. However, recognising that in some situations patients' acute illness affected their capacity to provide consent (e.g. sedated and ventilated), appropriateness of entry into the study in this scenario was confirmed by an appropriate independent health care professional. Additionally, a patient consultee (friend or relative), who was provided with complete information about the study and provided with a specific consultee information sheet, was requested to advise about appropriateness for inclusion. Consultee's decision was documented on the consultee declaration form.

6.3.3 Data collection

Patient demographics (gender, age, ethnicity) and medications were collected from electronic patient records. Blood biochemistry and haematology panel was collected as part of routine clinical care and results were collected from electronic patient records for the first CEUS day. SCr value was recorded at baseline, at AKI occurrence and during inpatient stay. Urine ACR was also collected. Cause of AKI was collected from patients' notes. Blood pressure, heart rate, patient weight, urine output, and fluid volume balance were collected from a web-based (Patient Track) software for each day. For ICU

patients, additional data were collected, including noradrenaline dose and ventilation type.

6.3.4 CEUS image acquisition

CEUS Images were acquired according to the procedure used for HV previously detailed in Section 5.1. Some compromises were necessary here, however, to adjust for the clinical setting and patient condition. Such compromises included patient position or cannula site other than ante-cubital fossa. Whilst all healthy volunteers were asked to lie on their side for coronal view of the kidney, some patients were unable to lie on their side due to illness, but all patients were lying supine or supine oblique. The in-situ arm cannula was used but if there was no cannula or when the priming saline would not run through, a 20 Gauge cannula was inserted. Also, in one ICU patient who was intubated but on spontaneous ventilation, CEUS was performed accepting that breath-holding was not possible. In addition, breath-hold was not ideal in some patients, although all patients went through the same breath-hold instruction and rehearsal as per HV protocol. When possible, more than five destruction/reperfusion sequences were captured in these cases so a minimum of three loops with data of sufficient quality would be available for analysis.

6.3.5 CEUS sequences analysis

Data were synthesised using the same method that was detailed for HV, in Chapter 5. However, motion compensation settings were applied as necessary to compensate for motion artefact caused by suboptimal breath-hold.

Associations between mTT and clinical variables were assessed. No assessment of association was performed for AI or PI since I had concerns as to their reliability as shown in the previous chapter.

6.3.6 Statistical analysis

Analyses were performed using IBM SPSS® (Version 27, New York, USA). Normality of distribution was tested graphically. Data are expressed in median and IQR for non-normally distributed data. Graphs were generated using GraphPad Prism. Association between mTT and clinical variables was assessed using Pearson's Correlation.

6.4 Results

A total of 56 patients were screened. Reasons for excluding patients during screening stage were: Mental incapacity with lack of consent from consultee, lack of baseline creatinine, AKI onset >72h (before referral to renal ward/ICU), ESKD on dialysis, pulmonary hypertension, cardiac failure, solid organ transplant, and patient cannot communicate in English. Reasons for refusal as reported by the patients were: involvement in another study, illness, and lack of interest.

A total of 12 AKI patients were recruited, of which nine were recruited from the renal ward while the other three were recruited from the ICU. All patients self-consented except one patient, whose consent was provided by their relatives due to patients' lack of mental capacity in line with ethical permissions. Descriptive data of the cohort are presented in Table 6.2. All patients had AKI stage 3 at time of recruitment except one patient (number 9) who had stage 2.

6.4.1 Patients' characteristics

Variables	n=12
Patient characteristics	
Age (years)	73 (65-78)
Male/female (ratio)	5/7
Ethnicity (%)	White (100%)
BMI (kg/m^2)	25.01 (18.88 – 36.36)
AKI details	
Baseline creatinine (µmol/L)	79 (60 – 99)
Creatinine at AKI onset (µmol/L)	387 (165 – 479)
Cause of AKI	1. Volume depletion 2. Biliary sepsis 3. Tubulointerstitial nephritis 4. Volume depletion 5. AKI on CKD stage 4 due to medication 6. Septicaemia and urinary retention (catheterised) 7. Cause uncertain 8. ICU patient (post operative) 9. ICU patient 10. Volume depletion (increased stoma output) 11. Post chemotherapy ICU patient 12. Volume depletion
CEUS details	
Number of CEUS scans	38
Cannula site at Day1 (ratio)	Antecubital (7/12) Forearm (1/12) Dorsal (2/12) Midline (1/12) Central line (1/12)

Data are expressed as median (IQR) or mean (SD), as appropriate.

Table 6.2: Descriptive data for all recruited patients

6.4.2 Feasibility of the study

Through a reflection on my experience conducting this study, I focused on answering the following feasibility measures: 1) recruitment feasibility, 2)

suitability of the data collection procedure, 3) acceptability and suitability of study design of and 4) resources and ability to manage the study.

6.4.2.1 Recruitment feasibility

56 AKI patients were screened within 197 days from Royal Derby Hospital renal unit and ICU wards, out of whom 12 patients were included in this study (a ratio of 14 to 3). Reasons for not including patients were: patient refusal (12 patient) due to lack of interest, too ill or unwillingness to have five daily CEUS scans. Other reasons were refusal of contrast administration, and unadvised participation by relative for a patient with lack of mental capacity (four cases). The other reasons were patient was too unwell/symptomatic, patient doesn't speak English, no baseline serum creatinine, AKI onset more than 72hrs, existing dialysis patient, pulmonary hypertension, cardiac failure, and solid organ transplant.

These data helped us evaluate the feasibility of the proposed recruitment procedure. The eligibility criteria were suitable and justified for patient safety and study objectives e.g. capturing early perfusion changes before recovery (within 72hrs). Reasons for exclusion were convenient and anticipated and it is likely to have similar criteria when recruiting patients for a larger study. The only limiting factor was the time allocated for the study which would have allowed for replacing the patients who have not successfully completed five days scan.

6.4.2.2 Data collection procedure feasibility

Amount of data collection was appropriate and relatively complete and usable. There was no difficulty completing the demographic and clinical measures in a timely manner with very little missing data. A few exceptions were the urine albumin to creatinine ratio (uACR) at AKI occurrence, which is not always routinely assessed at AKI occurrence. I adapted to this as the project progressed by requesting the test at the first CEUS day. Also, I attempted to assess the relationship of CEUS measures with urine output and the volume of IV fluid administration, but these were recorded in a hospital online system (Patient Track) as volume within 24hr, without recording the exact time, which makes it difficult to collect them in a consistent time frame comparable with CEUS-derived data. Therefore, I decided not to report uACR, IV fluid volume, or urine output.

This study was designed so each patient ideally completed five daily CEUS scans. For the 12 recruited patients, only 38 scans (63%) were successfully delivered. Reasons for not obtaining a scan were either patient-related reasons (73%) (e.g., patient discharge, transfer, or withdrawal), or technical reasons (18%) (e.g., cannula occlusion, patient obesity leading to poor kidney visualisation, or suboptimal analysis). The other reason was staff unavailability (9%). The individual flow of daily CEUS scans is illustrated in Table 6.3 where green cells signify completed scan and red, yellow, and grey cells signify unperformed scans with the reason.

Patient	Day 1	Day 2	Day 3	Day 4	Day 5
1					
2					
3					Discharge
4					Discharge
5					
6					Withdrawal
7			Suboptimal scan in the first 2 days (unclear kidney and contrast visualisation)	Suboptimal scan	
8		Transferred			
9		Transferred			
10		Scan terminated- occluded cannula and inability to insert a new one.			Discharged
11					Suboptimal images for analysis
12		Withdrawal			

■successfully scanned; ■ no scan for patient-related reasons; ■ no scan for technical reasons; ■ staff unavailability

Table 6.3: Individual flow of daily CEUS

6.4.2.2.1 Breath-hold feasibility

All recruited patients understood breath-hold instructions and managed to hold their breath for at least three loops in each CEUS scan. However, one ICU patient was on spontaneous ventilation (patient 11) that could not be paused, affecting the analysis quality.

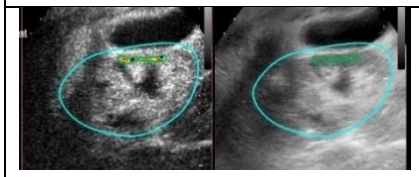
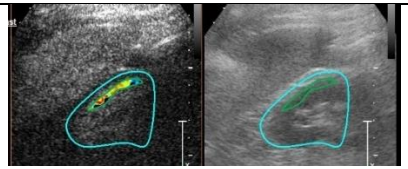
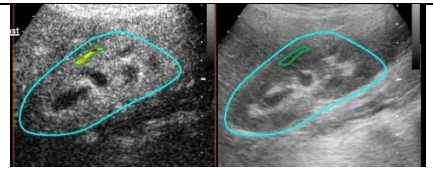
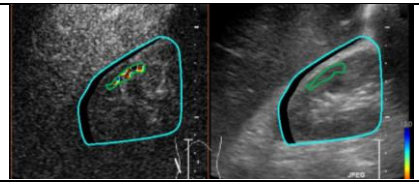
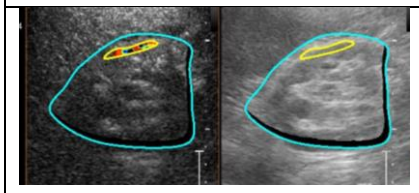
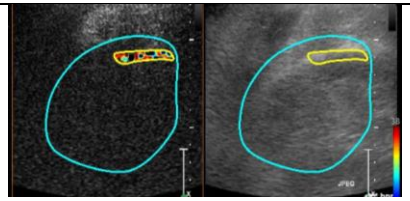
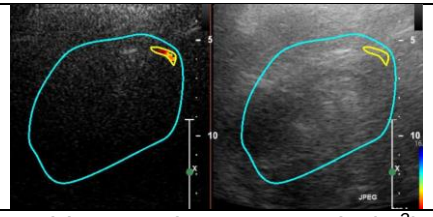
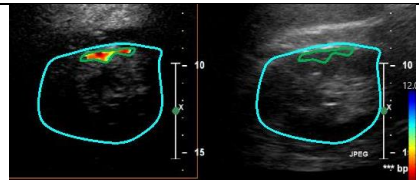
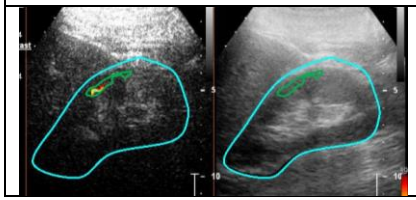
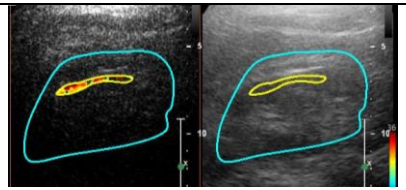
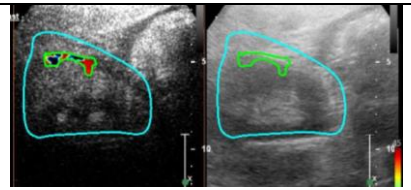
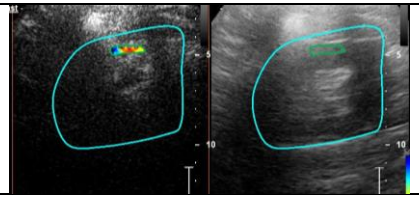
Subject 1; (BMI= 19.62 kg/m^2)	*Subject 2; (BMI= 33.28 kg/m^2)*	*Subject 3; (BMI= 22.31 kg/m^2)*	*Subject 4; (BMI= 17.71 kg/m^2)*
			
Subject 5; (BMI= 58.18 kg/m^2)	*Subject 6; (BMI= 36.75 kg/m^2)*	*Subject 7; (BMI= 35.19 kg/m^2)*	*Subject 8; (BMI= 27.71 kg/m^2)*
			
Subject 9; (BMI= 20.20 kg/m^2)	*Subject 10; (BMI= 41.10 kg/m^2)*	*Subject 11; (BMI= 17.95 kg/m^2)*	*Subject 12; (BMI= 18.63 kg/m^2)*
			

Figure 6.2: A panel providing an example of analysed images for each patient along with the BMI. The blue delimitation ROI is to delimit the processing area. Closed curvilinear-shaped green or yellow ROI are drawn around the visualised renal cortex, that is free from artefacts, renal interlobar or arcuate vessels, inadequate insonification, or excessive out-of-plane motion

6.4.2.3 Acceptability and Suitability of Design

Patients' acceptance of study procedures was not measured by survey feedback, but rather by observation. Instructions (mainly breath-hold instructions) were well-understood by patients with capacity. Scan time was quick and there was no complaint by either wards' staff or patients. There were no unexpected adverse events. In terms of patients' retention, the daily scans were performed on patients' beds while admitted so they did not have to attend for the study. Only two patients (ratio 6:1) opted to withdraw from the study as previously shown in Table 6.3. The other reasons for not completing five days scan were not related to patients' acceptability. Furthermore, five days scan were possible for only one patient, indicating a challenge in performing daily sequential CEUS scans.

Following this feasibility study, future researchers will have to identify strategies to address the challenges noted prior to designing larger studies to evaluate study outcomes more reliably. In the following sections, I report preliminary data on the use of CEUS in AKI settings. I examined the data at the patient level as well as for the group to further assess whether CUES shows promise of being successful and whether the changes of the outcomes are in the expected direction.

6.4.3 Contrast-enhanced ultrasound scans

Patients 7-10 & patient 12 had too few serial CEUS measures and were excluded from grouped analyses, leaving seven patients for the analysis of CEUS parameters over time. Descriptive data and concurrent medications for these patients are detailed in Table 6.4 and 6.5 respectively. Of note, patient 2 was on the ICU and was receiving noradrenaline.

Variables	n= 7
Patient characteristics	
Age (years)	74.71 (3.90)
Male/female (ratio)	2/5
Ethnicity (%)	White 100%
Body mass index (kg/m^2)	22.31 (17.95 – 36.75)
Physiological data	
SBP (mmHg)	121 (98 – 130)
DBP (mmHg)	61.14 (11.81)
Heart rate (beat per minute)	82 (72 – 87)
Fluid balance (within 24h prior to CUES1)	1297 (825 – 2197)
Urine output within 24hr from CEUS1 (ml/kg/hr)	475 (150 to 600)
AKI details	
Baseline creatinine (µmol/L)	92 (47)
Creatinine at AKI onset (µmol/L)	384 (153 – 477)
Creatinine at CEUS day1 (µmol/L)	294 (151 – 448)
Urine Albumin to creatinine ratio	3.5 (1.3 – 11.7)
Laboratory data (at CEUS day 1)	
Potassium (mmol/L)	4.9 (4.0 – 5.4)
Adjusted Calcium (mmol/L)	2.22 (1.90 – 2.38)
Sodium (mmol/L)	137 (132 to 139)
Phosphate (mmol/L)	1.63 (0.71)
Bicarbonate (mmol/L)	18 (6)
Serum albumin (g/L)	20 (13 to 26)
Urea (mmol/L)	22 (18 – 40)
Random Blood Glucose (mmol/L)	5.60 (4.25 – 12.10)

WBC 10^9/L	15.26 (8.14 – 20.09)
RBC 10^9/L	3.73 (0.67)
Haemoglobin g/L	114 (23)
Haematocrit ratio	0.33 (0.29 to 0.36)
Platelet (10^9/L)	277.57 (92.06)
C-reactive protein mg/L	144 (90)
CEUS infusion	
Cannula site at Day1 (ratio)	Antecubital (3/7) Forearm (1/7) Dorsal (1/7) Medline (1/7) Central line (1/7)

Data are expressed as median (IQR) or mean (SD), as appropriate. SBP: systolic blood pressure; DBP: diastolic blood pressure; CEUS: contrast-enhanced ultrasound; eGFR: estimated glomerular filtration rate; CKD-EPI: Chronic Kidney Disease Epidemiology Collaboration; WBC: white blood cells; RBC: red blood cells.

Table 6.4: Descriptive data for the seven patients included in CEUS analysis

Patient	Medication
1	Beclometasone, Cyclizine, Cinnarizine, Betahistine, Quinine Sulphate, Omeprazole.
2	Quinine Sulphate, Noradrenaline in Glucose, Meropenem, Aspirin, Enoxaparin, Metoclopramide, Fluticasone Propionate with Salmeterol, Lansoprazole, Creon, Salbutamol, Morphine Sulphate, Ondansetron, Paracetamol, Cyclizine, Furosemide, Metoclopramide
3	Clopidogrel, Enoxaparin, Gabapentin, Atorvastatin, Citalopram, Glyceryl Trinitrate, Famotidine.
4	Co-Amoxiclav, Enoxaparin, Warfarin, Ondansetron, Paracetamol, Piperacillin with Tazobactam.
5	Co-Magaldrox, Gliclazide, Co-Amoxiclav, Gliclazide, Insulin Aspart, Bisoprolol, Amoxicillin.
6	Ondansetron, Enoxaparin, Levothyroxine, Liquid Paraffin with Benzalkonium, Chlorhexidine & Isopropyl Myristate-Dermol (As Required), Darbepoetin Alfa-Aranesp.
11	Enoxaparin, Omeprazole, Piperacillin, Ramipril, Nifedipine

Table 6.5: Concurrent medications (n=7).

6.4.4 Contrast-enhanced ultrasound perfusion parameters

The primary outcome was CEUS-derived perfusion parameters obtained at day 1 from the included seven patients. These are summarised in Table 6.6 and illustrated in Figure 6.4. Clinical variables summarised in Table 6.7.

Variable	Mean (SD)/median (IQR)	Range
Mean transit time (s)	1.59 (0.72)	0.78 – 2.94
Acoustic index (a.u.)	4415 (3549 –14188)	2708 – 59526
Perfusion index (a.u.)	4485 (2384 –10628)	2026 – 20275

Table 6.6: Contrast-enhanced ultrasound perfusion variables on the first CEUS day; n=7

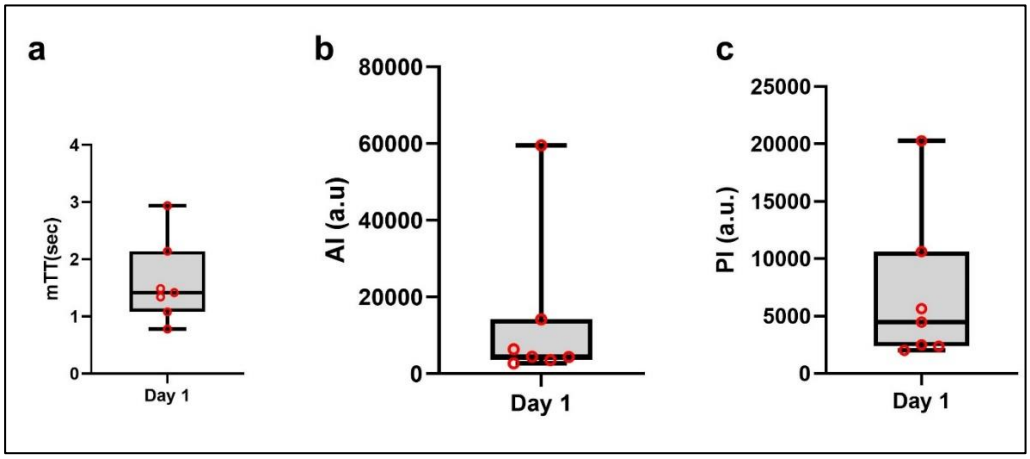


Figure 6.4: CEUS-derived perfusion parameters obtained at day 1 from seven patients.

Variable	Mean (SD)/median (IQR)
SCr (µmol/L)	315 (177)
HR (beats per minute)	82 (72 to 87)
Systolic blood pressure (mmHg)	116.6 (21.53)

Table 6.7: Clinical parameters on the first CEUS day; n=7

Serial CEUS measures over time were assessed for seven patients by observing the change at individual level. Figures 6.5 and 6.6 show Spaghetti plots of individual CEUS perfusion variables and SCr and SBP respectively for each patient over the five inpatient study days. We can see from these figures that not all AKI patients had reduced CEUS

measures of renal cortex perfusion compared with the healthy volunteers' range (shaded bar) from data presented in the previous chapter.

Closer inspection of the graphs shows that there were five patients that had increased mTT (slower perfusion) across all timepoints compared to the HV range. Four of these five patients had hypoperfusion-related causes for AKI, including volume depletion, sepsis, and medication. The other patient had AKI post-chemotherapy. Also, five patients had low PI and five had low AI, but these were not the same patients or timepoints. The proportion of patients who had reductions in more than one perfusion measure at the same timepoint was: 10% for AI and mTT, 24% for AI and PI, and 34% for mTT and PI.

Most patients had an improvement in serum creatinine results over the first five days, and in parallel there was generally a fall in mTT. The most obvious exception was patient 2, who was receiving noradrenalin in ICU, and whose day 5 creatinine was similar to their creatinine value on day 1.

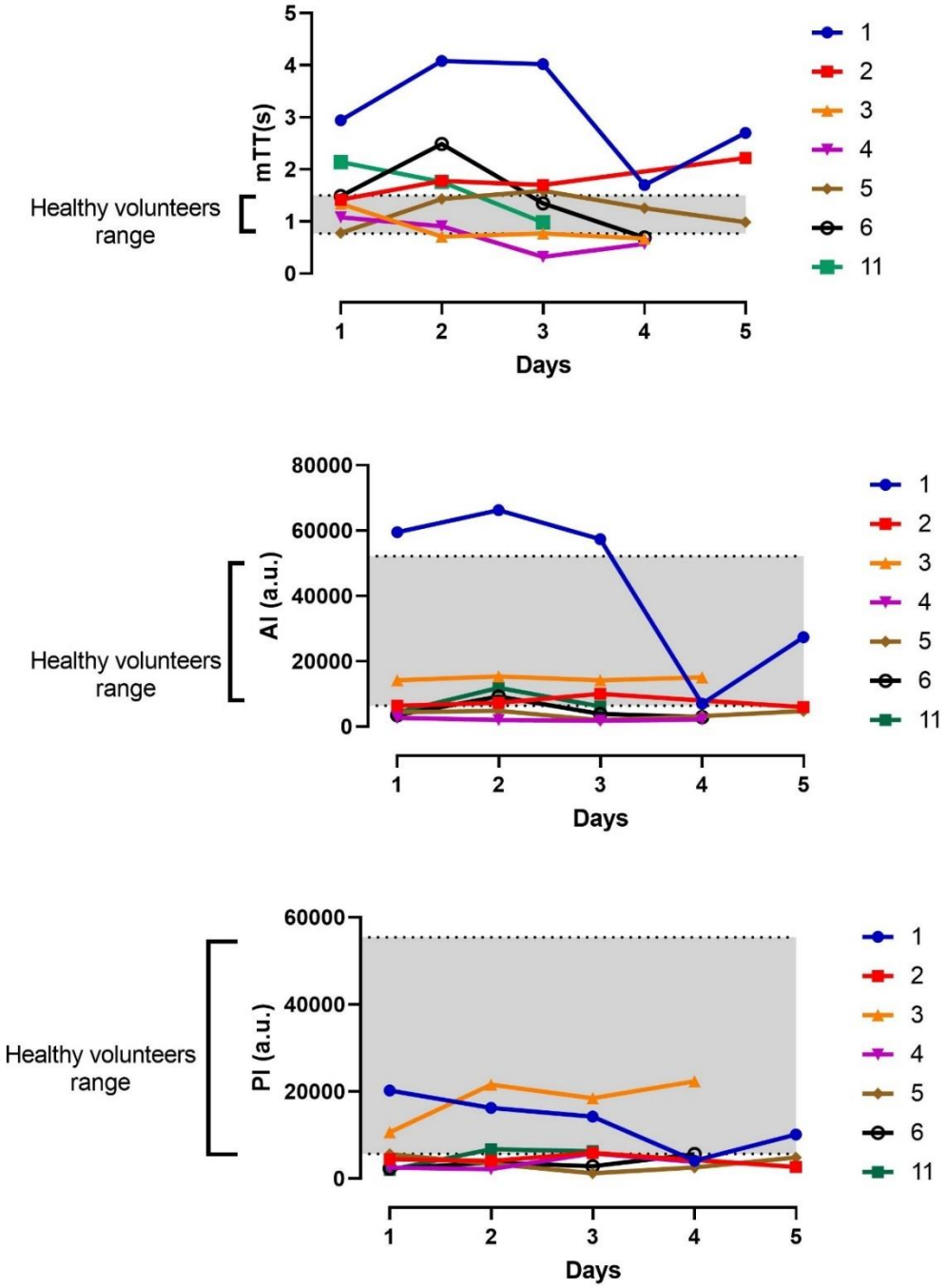


Figure 6.5: Spaghetti plots illustrating individual tracing of mean transit time, acoustic index, and perfusion index.

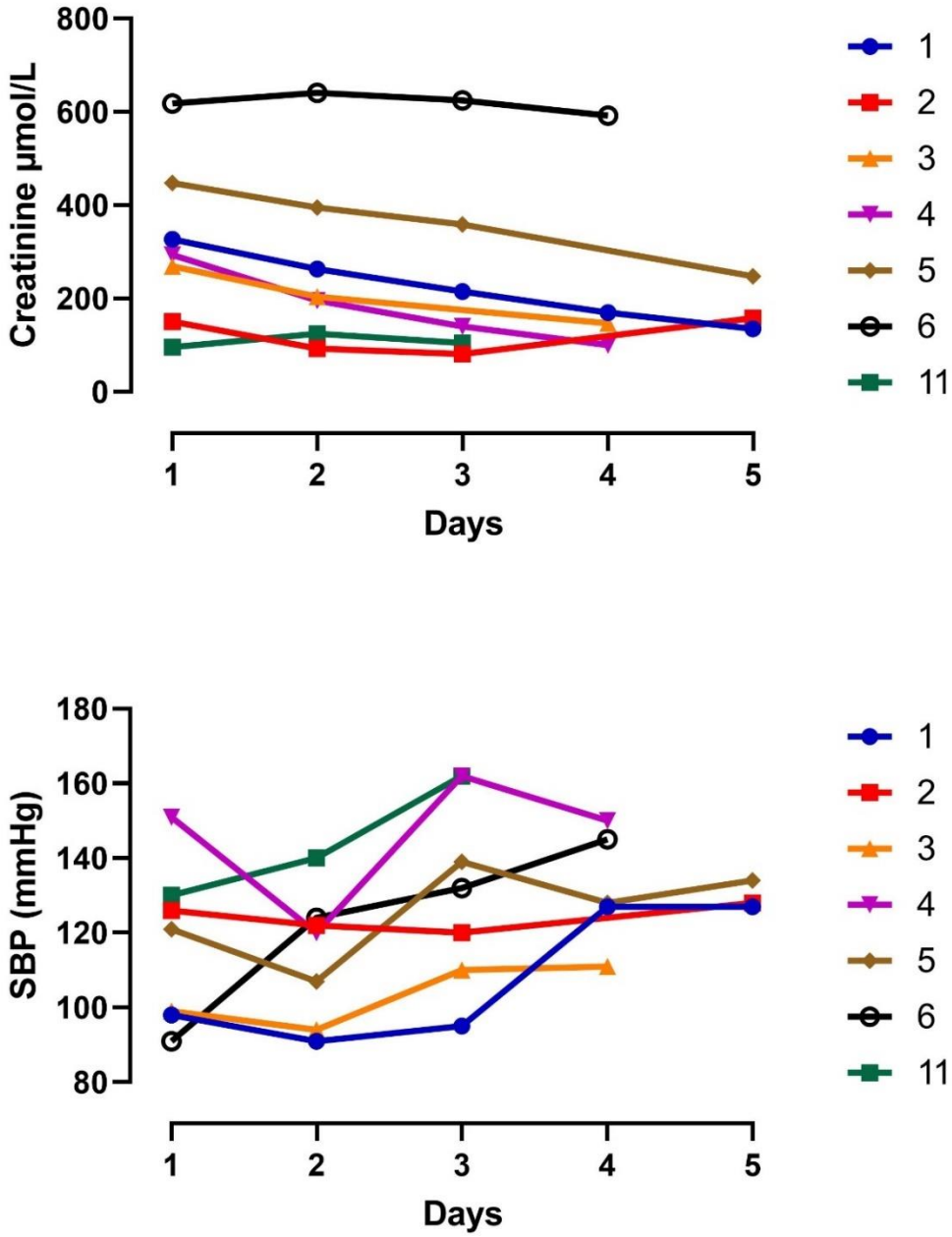


Figure 6.6: Spaghetti plots illustrating individual tracing of serum creatinine and systolic blood pressure.

6.4.5 Association between CEUS perfusion variables and clinical variables

The association in the whole group between mTT with SBP and SCr was performed (Figure 6.7). There was a moderate negative correlation between mTT and SBP. The associations between AI and PI with clinical variables were not assessed since I showed in the previous chapter (Chapter 5), that they had less repeatability compared to mTT. Also, association with urine output and intravenous fluid was not performed as collection of these measures was not possible.

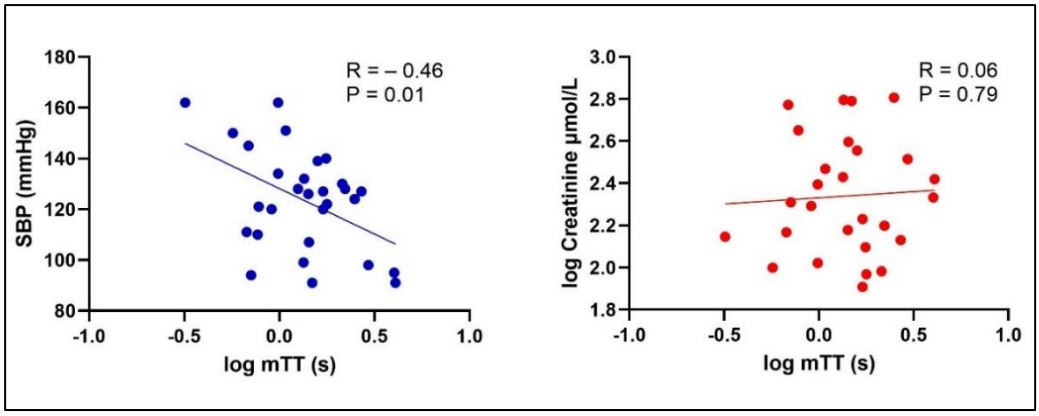


Figure 6.7: Association between mean transit time and clinical variables. mTT: mean transit time; SBP: systolic blood pressure

6.5 Discussion

In the observational study presented in this chapter, I used CEUS to assess perfusion parameters obtained from renal cortex of AKI patients within 72h of AKI onset and for up to four subsequent days. The main findings of this investigation can be divided into two aspects: feasibility-related findings and the variable measures of perfusion in an AKI cohort.

I assessed the feasibility of recruitment, data acquisition and analyses, breath-hold capability, and the feasibility of study management. There are challenges to the study of acutely unwell patient cohorts. Firstly, the unpredictable nature of AKI onset means that regular screening is required to identify potentially eligible patients to recruit. I was able to recruit 12 patients, and this required screening of at least 56 patients in total over a period of five months and 12 days. This is important information to help the planning of future, similar studies.

Whilst I performed baseline CEUS assessments in all participants, it was challenging to perform daily assessments subsequently. This resulted in only 63% of the intended scans being acquired. In the majority of cases this was because of clinical reasons, some of which is to be expected in an acute setting. There were however also some technical challenges experienced, including difficult or suboptimal vascular access, and difficulties in positioning of the critically ill patients. Another possible reason for the unsuccessful scans/analyses might be partly due to patients' high BMI, which is widely known to limit ultrasound imaging in general. The extent to which high BMI limits renal CEUS is important to explore. The other possible factor could be the superimposition of AKI on CKD as with patient 5 and patient 7, whose b-mode ultrasound images also demonstrated loss of corticomedullary differentiation. Therefore, CKD could have had confounding changes in perfusion and future studies might consider excluding AKI on top of CKD. Despite these challenges, images obtained were of sufficient quality to draw cortical

ROI, even if larger ROI were not possible in some cases (as seen in the example in Figure 6.2).

Patients' ability to hold their breath during the flash/reperfusion acquisition was a minor issue (except for one ICU patient who was on spontaneous ventilation whose analysis was degraded by massive breath induced motion artefact). Minor motion in the clips of the other patients was compensated for either by analysing only the clips with minimal motion (minimum of three clips), and by applying motion compensation function in the analysis software. Indeed, breath-motion is a limitation that is always associated with abdominal imaging and is unlikely to be completely eliminated.

The most obvious finding to emerge from this study is that renal perfusion of AKI patients measured by CEUS at study entry (day 1) was reduced compared to the HV range. This is evidenced by the higher mTT (time to half reperfusion plateau time) and lower AI (increased contrast intensity) values, as well as lower PI. Whether such observed perfusion difference is clnically meaningful requires further clarificaiton.

A closer look at individual data in the present study reveals marked variation i.e., not all patients have reduced perfusion. In fact, some patients with AKI have perfusion values in the HV range. In general, most patients showed an improvement in renal function (as evidenced by a fall in serum creatinine) to some extent over the study period and in general, this was mirrored by a fall in mTT (improved perfusion) from the first scan

to the last. However, this wasn't a universal pattern, but in one patient with a static pattern in mTT (patient 2) AKI had not recovered at day 5. Considering patients' clinical picture, specifically the cause of AKI for each patient in our cohorts, I found that the AKI in all but one patient with greater mTT (slower perfusion) were caused by prerenal (hypoperfusion-related) reasons. Whilst promising, it is important that these results are not over-interpreted, and the small sample size precludes any firm conclusions as to whether CEUS assessments can differentiate aetiologies or mechanisms of AKI. Our results also demonstrate that there are some analytical challenges about how best to combine data from patients with AKI in an acute setting, when the clinical trajectory of individual patients may differ. I have adopted the approach of reporting individual level patient data as well as grouped analyses.

Such heterogenous patient-level patterns have been demonstrated in previous small studies e.g., Schneider et al., 2014 and Schneider et al., 2015, where renal cortical perfusion of ICU-admitted patients on noradrenalin (Schneider et al., 2014) and patients with type-1 Hepatorenal syndrome (Schneider et al., 2015) were assessed. In both studies, perfusion was mainly expressed by CEUS-derived PI.

In the current study, patients' mTT had wider range and dispersion than the HV, which could be attributed to the heterogeneity induced by acute illness. This observation corroborates with the findings of Harrois et al. (2018) who, in line with our methods, employed an infusion technique for contrast administration, and used the same analysis software (VueBox). Harrois et al. (2018) examined renal cortical perfusion using CEUS in 20

patients with sepsis in the intensive care unit (ICU) compared with 10 controls. In this well-executed study, there was a spectrum of mTT values between patients with septic shock on an individual level. Importantly, mTT was significantly higher (slower) in patients with severe AKI compared with those who did not develop AKI. In contrast, PI showed no difference between the two groups. In their study, they also reported an wide range of mTT values for septic shock patients. However, their range of mTT was markedly higher than that reported in the present study ranging from 2.8–19 s; with a median (IQR) of 5.1 (3.4–6.1) s at day 0. This variation is probably due to the greater severity of acute illness in their cohorts versus ours. Their criteria included septic-AKI patients recruited from ICU, whereas the cause of AKI in our patients was diverse and most of our cohorts were recruited from the renal ward. The other possible reason for the discrepancy between the mTT ranges could be simply related the preliminary nature of our data. Moreover, their controls were also ICU patients and had slightly higher mTT range than our HV, ranging from 2.2–3.7 seconds, with a median of 2.9 (2.6–3.1) seconds on day 0. This may also indicate a variation in technique or analysis between their study and ours. As shown in Chapter 5, VueBox mTT values are higher than those from TIC fitting in Prism, the method that I used here.

In a more recent study by Liu 2021, renal CEUS was performed in septic AKI patients as well as controls; however, this time using bolus technique for contrast administration. This was performed only once with three averaged ROI in the upper, middle, and lower parts of the kidney and

analysed using Qontraxt analysis software. The time-related variable derived from the bolus TIC that can be compared with the mTT from infusion is the time to peak (TTP), i.e. time to peak enhancement, where the half of the TTP should be, in theory, comparable with mTT (previously defined as the half-time). In this study, septic AKI patients also had a wider range of TTP values of 22.8–48.3 seconds, with a median of 32.2 (27.6–39.2) than the range for controls of 19.4–29.12 seconds, with a median of 25.9 (21.6–27.1); ($p < .001$) (Liu et al., 2021). Clearly, even half of the value of the smallest limit of the TTP range (half TTP = 11.4 seconds) for the AKI patients was still larger than our AKI patients range of mTT. In addition, half the TTP value for the controls (9.7 seconds) was larger than our mTT values.

Similarly, Yoon et al. (2020) performed CEUS using a bolus technique on 48 AKI cases induced by various aetiologies. The most common aetiologies were intrarenal (71%; including medication, infection or glomerular disease), followed by prerenal (25%; including gastrointestinal loss, alcohol and hypotension). CEUS perfusion variables were compared between patients with various AKI aetiologies and showed no significant differences. However, the time of CEUS scan was not standardised between the patients with the time between peak SCr and CEUS ranging from 0 to 14 days, which could have potentially affected the results. The average TTP of the patients with AKI was 45.08 ± 11.95 seconds but ranged from 18.54 to 73.56 seconds, which is also noticeably larger than our mTT values for AKI patients. Such variation in

mTT range is likely to be related to the differences between the TIC generated from infusion and bolus techniques.

In the previous chapter, I showed that the intensity-based CEUS perfusion variable (AI) was less repeatable than mTT, and I discussed a number of possible reasons for this. Thus, in this study, I interpret the findings from AI and PI cautiously and did not look for associations between these variables and clinical variables.

6.5.1 Limitations and implications

There are a few limitations to this study. First, the small sample size impedes conclusive findings. In addition, the control data I had were not age-matched with the current study cohorts. The intubated patient on spontaneous ventilation, which could not be paused, was not able to hold their breath, resulting in a difficulty in scan and analyses. Such patients may not be suitable for future renal CEUS perfusion imaging. Furthermore, the limitations related to patient recruitment (e.g. patient transfer, withdrawal, or discharge) should be noted. Future study designs should take this probability into consideration to specify larger sample sizes than are needed to compensate for possible withdrawals or patient unavailability.

6.6 Conclusions

It is feasible to recruit patients with AKI in an acute setting to research studies in which CEUS is used to assess renal perfusion. It is harder to

reliably perform daily, sequential CEUS scans, which suggests that more targeted application (e.g. on admission, and potentially before-after an intervention) may be more appropriate. Our pilot data showed that AKI patients had reduced CEUS perfusion measures, but that there was variability at a patient-level. There was a suggestion that mTT values tended to fall over time in parallel with improving renal function; whilst these data are certainly not conclusive, they are consistent with results from Chapter 5 (in which mTT (as a time-based perfusion variable) had best intra-individual reliability). Further clinical studies using CEUS to assess patients with AKI are necessary to support or refute these preliminary findings, and their design may be informed by this work.

Chapter 7: Conclusions and Contribution

I have argued through this thesis that progress in ameliorating the morbidity in patients with ESKD requiring haemodialysis and AKI is partially hampered by a limited understanding of organ perfusion. I have aimed to optimize and expand the applications of CEUS perfusion imaging to novel clinical scenarios in kidney disease. In particular, CEUS has been applied for the assessment of perfusion in the skeletal muscles of ESKD and the kidneys of AKI patients.

Chapter 2 provided a comprehensive and systematic review of the existing literature on the HD-induced changes in metabolism, perfusion, and function on the skeletal muscle. The purpose of the chapter was to establish the gap that existed in the understanding of the acute effect of HD on muscle perfusion. Most studies have focused on the short-term effects on the metabolism of skeletal muscle. A few attempts have been made to assess the change on muscle function and scant evidence was available on the effect on muscle perfusion. I critically synthesised available evidence and the most obvious finding to emerge from the review was the consistent acute effect of haemodialysis on muscle metabolism, demonstrated by catabolism and increased inflammation. For muscle function assessment, various muscle groups were tested with conflicting findings i.e., while some patients had improved muscle function, others had decline or no change. Finally, the review revealed a significant gap in our current understanding of the underlying mechanisms and confirmed the lack of evidence on acute changes in muscle perfusion in response to HD.

Building on the conclusions from **Chapter 2**, a clinical study was designed to assess intra-dialytic changes in skeletal muscle perfusion **in Chapter 3**. Employing recognised methods, muscle CEUS was performed on patients' quadriceps during haemodialysis for the first-time. I was unable to detect changes in skeletal muscle perfusion during HD. In parallel, no changes were found in the thigh macro-haemodynamics measured by Doppler ultrasound. Intra-dialytic measurements of BP collected throughout the dialysis session demonstrated peak decrement in BP detected at the third quarter of dialysis, suggesting peak stress during that period. Despite the limited number of patients, the data from this study adds some insights into the scarce literature on muscle microvascular perfusion during HD and offers evidence on technique feasibility as well as tolerance among dialysis patients.

Moving forward with the results I had from **Chapter 3**, I searched in **Chapter 4** for corroborating evidence by exploring the relationship between haemodynamic factors and hand grip strength which is a reliable tool for muscle function measurement. I performed an analysis of pre-existing data from a clinical study where baseline HGS measurements were collected within the first hour of dialysis and follow-up measures were collected in six months, 12 months, and two years. I then analysed the alteration in HGS over the two years. Nearly half of the included patients had a decline in HGS values although comparison of the mean values did not detect an overall change. Thus, I performed a group analysis where patients were divided into two groups: a) patients with stable/increased HGS over two years and b) patients with a decline in

HGS. Then, I investigated how dialysis factors and BP, and nutritional factors that are linked with muscle deterioration differ between the two groups. The results revealed no associations between dialysis parameters and BP (that reflect intra-dialytic haemodynamics) with deteriorating skeletal muscle function over time; however, markers of poor nutrition were more common in those with a fall in HGS over time. Additionally, I compared the same variables across HGS quartiles and showed significant difference in systolic BP, serum albumin, and fat intake between the quartile groups. This suggests that both nutritional factors and dialysis-related haemodynamic factors could potentially contribute to muscle function deterioration in dialysis patients, although nutritional factors had stronger associations across several different analyses.

Chapter 5 consists of two sections: The first part included method development studies to optimise renal CEUS technique and generate a dataset of renal perfusion variables from HVs. The second section contained an assessment of intra-individual repeatability of CEUS-derived perfusion variables to establish their reliability that is crucial for interpretation of results in clinical studies.

My approach has proved useful in expanding our understanding of sources of variability of CEUS on HV renal cortex. I have shown that the application of CEUS on the kidneys is associated with variability and hence, must be delivered in a systematic approach. Training and experience are crucial. Also, interpretation should be made with limitations of the technique in mind. For purposes of analysis

optimisation, I explored the difference between large and single small ROI in the Section 1. I also covered how infusion-based technique is more accurate than bolus-based technique. In addition to that, I proposed to use the median rather than the mean of the measurements obtained from the repeated reperfusion clips to minimise the effect of single extreme values. Lastly, for a more standardised TIC, I used GraphPad Prism fit-model where the fit-curve starts from the same point, and hence eliminating the background noise. Whilst VueBox data are similar, there are differences so the two graph fitting approaches should not be used inter-changeably.

In Section 2, I presented original research on the repeatability of CEUS-derived parameters obtained from HV to determine renal cortical perfusion and established that time-based measures (mean transit time) performed better than the intensity-based parameter (acoustic index) and the computed perfusion index.

Armed with the experience acquired in Chapter 5 and using the optimised methods, renal CEUS was employed in a pilot clinical study in AKI patients in **Chapter 6**. Aspects pertaining to the feasibility of the CEUS perfusion imaging on AKI patients was discussed, which paves the way for future study designs in similar settings. In addition to that, the work in this chapter has expanded our perceptions on the understudied area of microvascular perfusion for the kidneys of patients with early onset of AKI. Overall, CEUS parameters measured on the first study day demonstrated reduced perfusion measures in some patients with AKI, but with larger dispersion compared to HV values, indicating more

variable individual perfusion in AKI. In addition to that, results hinted at patterns of improvement that occurred over the first five days of AKI in mTT in parallel with serum creatinine values.

7.1 Future work

This thesis contained a series of observational studies intended to optimise the technique and analysis of CEUS-perfusion imaging in kidney disease. Limitations of each study was previously discussed. Here, I present implications for future research.

Data obtained from muscle studies suggest that a greater focus on HD-induced metabolic changes may produce interesting findings that account for muscle wasting in dialysis patients in addition to the proposed hypothesis about acute haemodynamic stress of dialysis. However, if the debate about haemodynamic consequences of dialysis is to be moved forward, future work could explore whether pre-dialysis (using a separate cannula) or third-quarter CEUS scans could yield different results. In addition, study designs where CEUS is used to study intra-dialytic skeletal muscle microvascular changes in response to a stimulus such as a nutrition or an exercise right before capturing the flash/destruction loops (stress-perfusion imaging) may be more sensitive to detect perfusion changes and examine whether this approach may detect a difference in perfusion changes over the dialysis session. Importantly, CEUS was proved applicable during dialysis, which opens the door for future larger intra-dialytic studies to be performed.

Another important avenue for future work would be validating renal CEUS on healthy volunteer's kidneys against ASL-MRI, the gold standard for renal perfusion quantification. Furthermore, intra-subject repeatability study could be performed to determine the effect of physiological factors of hydration status, dietary salt intake or smoking on CEUS perfusion variables.

With respect to the use of CEUS in AKI patients and given the observed varying clinical trajectories and heterogenous aetiologies of AKI, larger studies should be carried out to allow for sufficient group analysis. Furthermore, future study designs could explore whether limiting the inclusion criteria to AKI patients who had the onset within 24 hours would capture more marked early microvascular changes before recovery with clinical care, fluid administration or vasopressors. With regards to the serial scans, this could be limited to those who had blood flow reduction in the first scan compared to the established healthy volunteer's data or it could be limited to admission day and discharge day only. It would be intriguing to examine more closely the links between the potential causes of AKI and perfusion changes in CEUS in a larger dataset. Indeed, CEUS appears to be promising as a monitoring tool to determine the effectiveness of emerging therapies targeting renal perfusion, given its feasibility on critical care settings and real-time capabilities.

Further research could apply the methods of renal CEUS developed here on a renal transplant where the transplanted kidney is more superficial and there is a lack of respiratory motion, both which may improve image quality.

Imaging techniques to assess patients with kidney disease remain under-developed and under-utilised in clinical practice. Future research to better establish the utility of CEUS-based measures of organ perfusion may be one approach to help address this important clinical need.

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Appendix A: Database search strategy

Boolean line-by-line search

PubMed and Medline databases

1. Exp Renal Insufficiency, Chronic/
2. Exp Renal dialysis/
3. “End stage kidney disease” OR “End-Stage Kidney Disease” OR “End-Stage Renal Disease” OR “End Stage Renal Disease OR Chronic Renal Failure OR “Chronic Kidney Disease OR “Chronic Renal Failure” [tw]
4. “Chronic renal insufficiency” [tw]
5. (ESKD OR ESRD OR CKF OR CKD OR CRF OR CRD) [tw]
6. Specific for Medline: End ADJ2 Renal.tw. OR End ADJ2 Kidney.tw. OR Chronic ADJ2 Renal.tw. OR Chronic ADJ2 Kidney.tw. OR Chronic renal insuf?iciency.tw.
7. 1 OR 2 OR 3 OR 4 OR 5 (OR 6)
8. Renal Dialysis/
9. Exp Renal Replacement Therapy/
10. Hemodiafiltration/
11. 8-10/adverse effects
12. (Haemodialysis OR haemodialyses OR hemodialysis OR hemodiayses OR Renal Dialysis OR Renal Dialyses) [tw]
13. (RRT OR HDF OR HD) [tw]
14. Specific for Medline: H?emodialysis.tw. OR H?emodiafiltrat$.tw.
15. 8 OR 9 OR 10 OR 11 OR 12 OR 13 (OR 14)

16. Exp Muscle, Skeletal/
17. “Voluntary Muscle” [tw]
18. “Skeletal muscle” OR “Skeletal muscles” [tw]
19. “Skeletal musle” [tw]
20. Specific for Medline: Skeletal muscle?”.tw.
21. 16 OR 17 OR 18 OR 19 (OR 20)

PERFUSION:

22. Perfusion Imaging/
23. Exp Blood Circulation/
24. (“Blood-flow” OR Bloodflow OR BF) [tw]
25. (“Micro-circulation” OR Microcirculations OR “Micro circulation”) [tw]
26. Hemodynamics/ OR Hemodynamics[tw]
27. Microvessels/ OR Microvascula*[tw]
28. 22 OR 23 OR 24 OR 25 OR 26 OR 27
29. 21 AND 28

METABOLISM

30. Metabolism/
31. “Metabolic OR “Metabolic Process” [tw]
32. Anabolism OR anabolsm[tw]
33. Catabolism OR catabolsm[tw]
34. 30 OR 32 OR 33
35. 21 AND 34

FUNCTION

36. Physiopathology/

37. “Physical function” OR Function OR Functions [tw]
38. “Physical activity” [tw]
39. 36 OR 37 OR 38
40. 21 AND 39

Embase database

1. Chronic Kidney failure/
2. End stage renal disease/
3. (end-stage renal or end-stage kidney or endstage renal or endstage kidney).mp.
4. (ESRF or ESKF or ESRD or ESKD).mp.
5. 1 OR 2 OR 3 OR 4
6. renal replacement therapy/
7. hemodialysis/
8. h?emodialys?s.mp.
9. hemodiafiltration/
10. H?emodiafiltrat$.mp.
11. RRT OR HD OR HDF.mp.
12. 6 OR 7 OR 8 OR 9 OR 10 OR 11
13. 5 AND 12
14. Skeletal muscle/
15. Voluntary Muscle.mp.
16. Skeletal muscle OR Skeletal muscles.mp.
17. Skeletal musle.mp.
18. 14 OR 15 OR 16 OR 17
19. 13 AND 18

PERFUSION:

20. Limb perfusion/ or muscle perfusion/ or tissue perfusion/ or perfusion/

21. Blood flow/
22. Blood-flow or Bloodflow or BF.mp.
23. Micro-circulation OR Microcirculations OR Micro circulation.mp.
24. Hemodynamics/
25. Microvasculature/
26. Microvascula*.mp.
27. 20 OR 21 OR 22 OR 23 OR 24 OR 25 OR 26
28. 19 AND 27

METABOLISM:

29. Metabolism/ or muscle metabolism/ or protein metabolism/
30. Metabolic or Metabolic Process.mp.
31. Anabolism OR anabolsm.mp.
32. Catabolism OR catabolism.mp.
33. 29 OR 30 OR 31 OR 32
34. 19 AND 33

FUNCTION:

35. muscle function/ or musculoskeletal function/
36. Physiopathology/
37. “Physical function” OR Function OR Functions.mp.
38. Physical activity.mp.
39. 35 OR 36 OR 37 OR 38
40. 19 AND 39

CENTRAL Cochrane Library

1. MeSH descriptor:[Kidney Failure, Chronic], this term only
2. MeSH descriptor: [Renal Insufficiency, Chronic], this term only
3. Hemodiafiltrat* or Haemodiafiltrat*:ti,ab,kw
4. (ESKD or ESRD or CKF or CKD or CRF or CRD): ti,ab,kw
5. (End-stage NEXT kidney):ti,ab,kw or (end-stage NEXT renal):ti,ab,kw
6. 1 OR 2 OR 3 OR 4 OR 5
7. MeSH descriptor: [Renal Replacement Therapy], this term only
8. MeSH descriptor: [Renal Dialysis], this term only
9. MeSH descriptor: [Hemodialysis], Home, this term only
10. MeSH descriptor: [Hemodiafiltration],this term only
11. (HD or HDF or RRT):ti,ab,kw
12. h*modialysis: ti,ab,kw
13. 7 OR 8 OR 9 OR10 OR 11 OR 12
14. 6 AND 13
15. MeSH descriptor: [Musculoskeletal System], this term only
16. MeSH descriptor: [Muscle, Skeletal], this term only
17. 15 OR 16
18. 14 AND 17

PERFUSION:

19. MeSH descriptor: [Perfusion Imaging],this term only
20. MeSH descriptor: [Regional Blood Flow],this term only

21. MeSH descriptor: [Microcirculation], this term only
22. 19 OR 20 OR 21
23. 18 AND 22

METABOLISM:

24. MeSH descriptor: [Metabolism], this term only
25. MeSH descriptor: [Proteolysis], this term only
26. Metaboli*: ti,ab,kw
27. Anabolism OR Catabolism: ti,ab,kw
28. 24 OR 25 OR 26 OR 27
29. 18 AND 28

FUNCTION

30. Function OR functions: ti,ab,kw
31. Physiopathology: ti,ab,kw
32. 30 OR 31
33. 18 AND 32

Web of science and Scopus

(do not use controlled vocabularies)

1. “Chronic Kidney Disease”
2. Chronic Renal Failure
3. Chronic Kidney Failure
4. “Chronic renal insufficiency” OR “Chronic renal insuficiency”
5. Renal dialysis
6. “End stage kidney disease” OR “End-Stage Kidney Disease” OR “End-Stage Renal Disease” OR “End Stage Renal Disease OR Chronic Renal Failure”.
7. (ESKD OR ESRD)
8. (CKF OR CKD OR CRF OR CRD)

a. End Near/2 Renal	A. End W/2 Renal
b. Chronic Near/2 Renal	B. Chronic W/2 Renal
c. End Near/2 Kidney	C. End W/2 Kidney
d. Chronic Near/2 Kidney	D. Chronic W/2 Kidney

9. 1 OR 2 OR 3 OR 4 OR 5 OR 6 OR 7 OR 8 (OR a OR b OR c OR d)
10. 1 OR 2 OR 3 OR 4 OR 5 OR 6 OR 7 OR 8 (OR A OR B OR C OR D)
11. Renal Dialysis
12. Renal Replacement Therapy
13. Hemodiafiltration
14. Haemodiafiltration

15. Hemodiafiltrated OR Haemodiafiltrated
16. Hemodiafiltrating OR Haemodiafiltrating
17. (Haemodialysis OR haemodialyses OR hemodialysis OR hemodiayses OR Renal Dialysis OR Renal Dialyses)
18. (RRT OR HDF OR HD)
19. 11 OR 12 OR 13 OR 14 OR 15 OR 16 OR 17 OR 18
20. 9 AND 19 (WOS) 10 AND 19 (Scopus)
21. “Skeletal Muscle” OR “Skeletal Muscles”
22. “Voluntary Muscle” OR “Voluntary Muscles”
23. “Skeletal Musle” OR “Skeletal Musles”
24. 21 OR 22 OR 23
25. 20 AND 24

PERFUSION:

26. “Perfusion Imaging” OR Perfusion
27. “Blood-flow” OR Bloodflow OR BF
28. “Blood Circulation”
29. (Microcirculation OR Micro-circulation OR Microcirculations OR “Micro circulation”)
30. 26 OR 27 OR 28 OR 29
31. 25 AND 30

METABOLISM:

32. Metabolism
33. “Metabolic Process”
34. Anabolism

35. Catabolism
36. 32 OR 33 OR 34 OR 35
37. 25 AND 36

FUNCTION:

38. Physiopathology
39. Function OR Functions
40. 38 OR 39
41. 25 AND 40

Appendix B: Studies Selection Checklist

Title, abstract Review stage:

Table 1: Title, Abstract checklist

Checklist	Yes	No	Not clear
ESKD patients receiving HD			
Human			
Adults			
In-centre HD or HDF			
Reported acute effect of HD on either skeletal muscle perfusion, metabolism, or function			
Compared the outcome before and after or before and during dialysis			
Primary study (not a review)			
Published in English			

Entries for each field can be yes, no or unclear.

If no, study will be excluded from the review.

If not clear, study shall be considered for further full text review.

For full text review stage,

Same checklist in Table 1 will be used for unclear answers in the title, abstract stage.

Additional questions for studies that include more than one group are listed in Table 2 below

Table 2 : Checklist for studies that include subgroups

Question	Yes	No	Not clear
A. If it includes both adults and children, can adult data be extracted separately?			
a. Is it examined as a sub-group analysis?			
B. If it includes both Human and animal data, can human data be extracted separately?			
a. Is it examined as a sub-group analysis?			
C. If it includes HD and any other form of RRT, can HD data be extracted separately?			
a. Is it examined as a sub-group analysis?			
D. If it includes in-centre and home HD, can in-centre HD be extracted separately?			
a. Is it examined as a sub-group analysis?			
E. If it includes both acute and chronic effects of HD, can acute effect data be extracted separately?			
a. Is it examined as a sub-group analysis?			

F. If it includes patients with other disease than CKD such as acute kidney injury, can CKD data be extracted separately?			
a. Is it examined as sub-group analysis?			

Entries for each field can be yes, no, or unclear.

If no, study will be excluded from the review.

If not clear, two attempts will be made to contact authors

Appendix C: Systematic Review Data Collection Form

Article details

First author	
Publication year	
Publication type	
Author contact details	
Reviewer name	
Date form completed	

Study details

Study design	
Number of participants	
Is there a sub-group?	
Study primary objective	
measurement method	
Delivered by?	
Muscle/ area of interest examined	

Measurement timing in relation to HD? i.e. pre, intra or post HD	

Patient demographics

Patient age (mean)	
Patient gender (%)	
Ethnicity	
BMI	
Cause of ESKD	
Co-morbidity	

Dialysis details

Vascular access	
Dialysis vintage (mean)	
Frequency of dialysis	
Specific type of dialysis (HD or HDF?)	

Outcomes

Definition of outcome	
Result	
Statistical analysis results	
Missing participants	
Study quality notes (Ethical approval, sample size calculation, etc.)	

Appendix D: MUSHD Study Protocol

Muscle Stunning in HaemoDialysis

Final Version 2.0

2.10.2018

Short title: *MUSHD Study*

IRAS Project ID: 244334

TRIAL / STUDY PERSONNEL AND CONTACT DETAILS

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SYNOPSIS

Title	Muscle Stunning during HaemoDialysis
Short title	MUSHD Study
Chief Investigator	Dr Nick Selby
Objectives	To assess for feasibility of D2O tracer as a tool to measure muscle protein turnover and the profile of change during haemodialysis (study 1) To determine if haemodialysis results in changes in muscle perfusion. (study 2)
Trial Configuration	Two stage observational study
Setting	Secondary care
Sample size estimate	This is a pilot study therefore power calculations are not appropriate.
Number of participants	1. 4-8 patients will complete study 1 2. 12 patients will complete study 2
Eligibility criteria	Age between 18yrs and 80yrs Able to give informed consent CKD5 on chronic haemodialysis for >90days Average interdialytic weight gains > 0.45 kg
Description of interventions	Conventional haemodialysis D2O drink Blood samples
Duration of study	Study 1: 24hrs preceding and duration of a single haemodialysis session. Study 2: 30 min preceding, duration of a single haemodialysis session, and 30 min just after the dialysis session.
Outcome measures	Study 1: Albumin turnover and body water enrichment of D_2O and the profile of change during haemodialysis Study 2:

	The change in muscle perfusion between baseline and peak stress during haemodialysis
Statistical methods	All continuous variables will be tested for normality. The primary and continuous secondary endpoints will be compared between the two modalities using a paired t test or Wilcoxon signed rank test as appropriate. The association between categorical variables will be tested using the Chi squared or Fischer's Exact test. An alpha error at 0.05 will be judged as significant.

ABBREVIATIONS

AE	Adverse Events
BIA	Bioimpedance
BP	Blood pressure
CI	Chief Investigator
CKD	Chronic Kidney Disease
CKRI	Centre for Kidney Research and Innovation
D2O	Deuterium Oxide
DEXA	Dual Energy X-Ray Absorptiometry
HD	Haemodialysis
IDWG	Intra-dialytic weight gain
MPS	Muscle protein synthesis
PIL	Patient information leaflet
RDH	Royal Derby Hospital
REC	Research Ethics Committee
RRT	Renal replacement therapy
SAE	Serious adverse event
TMF	Trial Master File
U&Es	Urea and electrolytes

TRIAL / STUDY BACKGROUND INFORMATION AND RATIONALE

Functionally significant skeletal muscle wasting in haemodialysis patients is highly prevalent, progressive and predisposes this vulnerable patient population to increased risk of complications, poor quality of life, frailty and premature death (Stenvinkel et al., 2015). Among established treatment strategies, the benefit of resistance exercise and endurance training are increasingly recognized as being effective. However, this approach to treating muscle wasting is difficult and often unsuccessful in this typically old, frail, and largely sedentary patient group and novel treatment strategies are urgently needed. Such strategies should be informed by better understanding of the aetiology of muscle catabolism in this particular context. The process is multifactorial and includes changes in amino acid and lipoprotein metabolism, the effects of inflammation, anorexia and endocrine dysfunction, altered muscle intracellular signaling, and defective myocyte regeneration (Garibotto et al., 2012). In addition, previous preliminary studies have suggested that the process of haemodialysis itself may be associated with muscle catabolism (Lim et al., 1993). The latter has previously been attributed to increased dialysate amino acid loss, although recent understandings related to the circulatory stress of dialysis may now suggest an alternative explanation.

A significant body of evidence shows that the circulatory stress exerted by haemodialysis can induce hypoperfusion in vulnerable vascular beds, in particular the coronary and cerebral circulations (McIntyre, 2010b). When this insult occurs repeatedly during the course of thrice weekly dialysis schedules, it results in end organ ischaemic injury: left ventricular systolic dysfunction, cortical white matter change and cognitive function decline (Burton et al.,

2009a, Eldehni et al., 2015b, Odudu et al., 2015a). It Is not currently known whether similar processes may contribute to skeletal muscle atrophy and dysfunction; whilst it is known that muscle perfusion and metabolism are negatively impacted by episodic hypoxia it is also recognised that muscle is more resistant to hypoxia than other tissues (Favier et al., 2015). Thus, I propose to conduct a study to test if haemodialysis alters skeletal muscle microvascular flow during the course of a single dialysis treatment. This process would then be potentially amenable to dialysis-based therapeutic interventions.

Muscle protein synthesis has been measured traditionally using stable isotope tracers of amino acids (AA) such as heavy carbon (13C), deuterium (2H), or nitrogen (15N)(Gasier et al. 2010). Protein synthesis is measurable by following the incorporation of either a bolus-administered or infused labelled AA into protein(Belloto et al. 2007).

Deuterium “heavy water” tracer has overcome certain limitations of other tracers. For instance, it can be administered orally overcoming the need for costly sterile IV infusion. Moreover, it can provide chronic measures as it can last for several weeks rather than 8-12 hours using 13C-AA(Dufner et al. 2018). Also, it is less restrictive and could be performed on free-living participants(Robinson et al. 2011). D2O has been regarded as a promising valid tracer in healthy individuals, but its validity for use in patients undergoing haemodialysis is still to be demonstrated(Wilkinson et al. 2014).

TRIAL / STUDY OBJECTIVES AND PURPOSE

PURPOSE

The purpose of this study is to determine whether the circulatory stress of haemodialysis results in hypoperfusion of skeletal muscle that has injurious metabolic and physiological consequences.

PRIMARY OBJECTIVE

To ascertain in detail, for the first time, changes to muscle perfusion in response to haemodialysis.

SECONDARY OBJECTIVES

To assess the effect of haemodialysis on femoral artery blood flow.

To assess if there is any link between patients' history or/and dialysis variables with skeletal muscle perfusion.

To assess body water enrichment of D_2O and the profile of change during haemodialysis. This will form the basis for future studies in which this tracer can be used to measure muscle catabolism in tandem with measures of muscle perfusion.

DETAILS OF PRODUCT(S)

Description

Deuterium Oxide(D2O) is an orally administered stable isotope tracer used to measure changes in metabolism in human.

Manufacture

It is produced by Isowater (Canada)

Packaging and labelling

D2O is provided at 70 Atom Percent

Storage, dispensing and return

It is stored at room temperature within the clinical physiology labs, which is a secure laboratory only accessible to assigned personnel. It is dispensed according to need for each study, amounts are dependent on weight of individuals.

Known Side Effects

Potential side effects are rare, but can include nausea or dizziness, however this is avoided by providing small regular doses rather than large single boluses.

TRIAL / STUDY DESIGN

TRIAL / STUDY CONFIGURATION

Two stage observational study, each to include a single dialysis session and for study 1, the 24hrs beforehand. The two stages are included in the same protocol because each are essential pieces of pilot work that together will inform the design of subsequent studies. These will necessitate the combined use of contrast enhanced ultrasonography to measure perfusion and tracers to measure muscle turnover, to definitely answer the research question posed.

Study 1

Between 4 and 8 chronic haemodialysis patients will be recruited to assess body water enrichment of D_2O and the profile of change during haemodialysis, which will determine the efficacy of D_2O tracer for muscle protein turnover measurement using albumin measurements as a proxy. A minimum of 4 patients will be studied, with a maximum of 8 depending on the number of the required protocol refinements to the administration and observation of D2O. This is necessary as the small molecular size of tracers means they will be removed by dialysis from the vascular compartment**.** The D2O technique has several advantages, notably being less invasive than the traditional Substrate specific stable isotope traces (i.e. 13Cleucine or Phenylalanine) and obviating the need for an infusion during dialysis. In addition, one patient will have a single CEUS measurement taken to demonstrate measurement parameters are similar to those taken outside of dialysis treatment. The single participant who undergoes CEUS will receive a full 274ottingham274 of this and will provide explicit consent to this procedure.

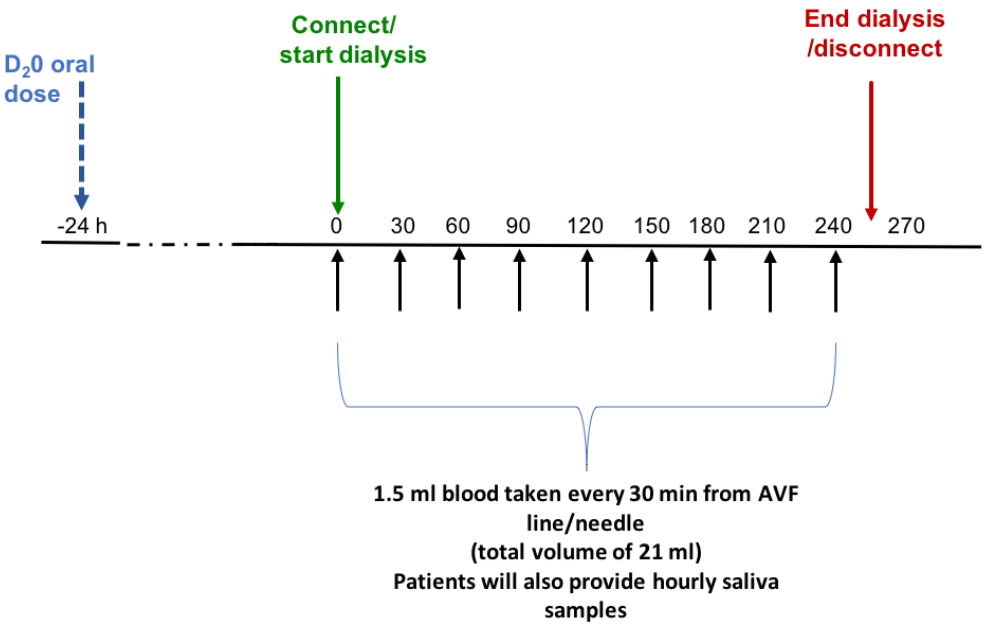

Figure 1: Summary of Study 1 Design

Recruited participants will be administered oral dose of D_2O 24hrs prior to the dialysis session. Then, patients will be asked to provide saliva sampling every hour during the 4hr dialysis session to be used in measuring dilution of D_2O across time. Also, 1.5 ml blood will be taken every 30 min from AVF line/needle (total of 21 ml).

Primary endpoint

D2O enrichment levels at baseline and during dialysis.

Safety endpoints

Frequency of adverse events

Discontinuation due to adverse events

Study 2

12 prevalent haemodialysis patients will be recruited from the chronic dialysis programme at the Royal Derby Hospital. An observational study over a single dialysis session will be performed to determine the impact of dialysis upon skeletal muscle perfusion. The study design is summarised in figure 2.

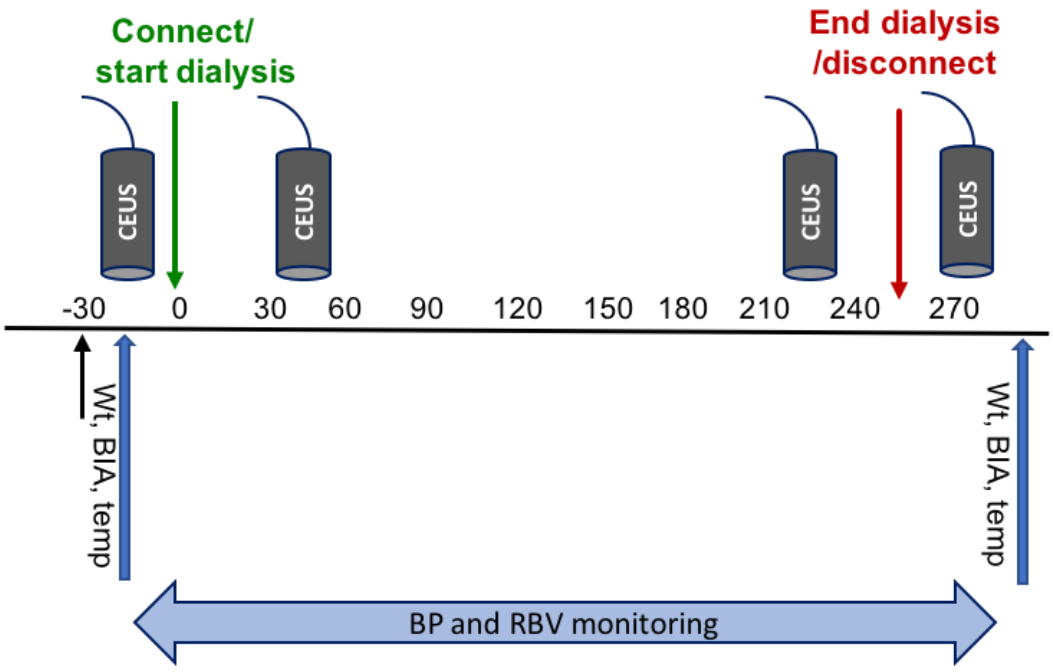

Figure 2: Summary of Study 2 Design.

A number of measures will be taken prior to, during and after dialysis. These include measures of muscle perfusion (contrast enhanced ultrasound), non-invasive measures of blood pressure, and bioimpedance measures to

determine body composition and total body water content. In addition, details about the dialysis treatment and the results of laboratory blood tests from routine clinical care will be recorded. Additional blood sample (277otting.10ml) for storage of plasma and serum samples will be collected.

Primary endpoint

Change in muscle perfusion between baseline and peak stress during dialysis

Secondary endpoints

Correlation between change in muscle perfusion and clinical/dialysis treatment variables

Effect of age and dialysis vintage on outcome measures to inform patient selection for subsequent clinical studies.

To determine the effect of hemodialysis on femoral artery blood flow

Safety endpoints

Frequency of adverse events

Discontinuation due to adverse events

Stopping rules and discontinuation

Patients may withdraw from the study at any time or without giving any reason. If the reason is given this will be recorded in the TMF and confirmed in writing with a copy for the patient, the hospital notes. Abrupt termination of the study would not affect a subject's safety and will not affect their usual medical care.

Patients may be excluded from the study after recruitment if it is not possible to obtain CEUS data of sufficient quality to perform meaningful analysis, or if they develop conditions which fall within the exclusion criteria. Anonymised data obtained until the point of withdrawal will still be included in the analysis. Further information will not be collected from patients who have withdrawn from the study. Once subjects have completed the study session there will be no further follow-up.

RANDOMISATION AND BLINDING

Owing to the nature of interventions in this trial, randomisation and blinding are not part of the study design.

TRIAL MANAGEMENT

The Chief Investigator has overall responsibility for the study and shall oversee all study management with support from the named co-investigators. Given the pilot nature of this study, there will be no Trial Steering or Management Group.

The data custodian will be the Chief Investigator.

DURATION OF THE TRIAL / STUDY AND PARTICIPANT INVOLVEMENT

Study 1 duration will last from the time of D2O administration 24hr before dialysis and end 28hrs later with the completion of dialysis. Total time is 28 hours.

Study 2 duration will be 30 min preceding, duration of a single haemodialysis session, and 30 min just after the dialysis session. Total time will be 5 hours.

For study 1 participants will be take part in the study for 28hrs (24hrs prior to commencing dialysis plus 4hrs of dialysis treatment). For study 2, the study will encompass a single dialysis session (4hrs) in addition to 30min before and another 30min just after the dialysis session.

There will be no run-in or follow up period as part of this pilot study. Once consent has been taken, the study session will be planned. The period between consent and the dialysis study visit will not exceed 4 weeks.

Enrolment will begin in June 2018 and cease after all patients have completed the study. We anticipate that this will take 12 months in total.

End of the Trial

The end of the study will be the last study visit of the last participant.

SELECTION AND WITHDRAWAL OF PARTICIPANTS

Recruitment

Subjects will be recruited from the renal unit at the Royal Derby Hospital. Potential participants will be identified by the clinical care team with reference to the inclusion and exclusion criteria. An initial face to face explanation of the study will be accompanied by an information sheet and subjects will be given until their next dialysis session to consider whether they wish to participate in the trial. At this time, the information sheet will be discussed with them, any questions answered and a member of the research team will obtain informed consent if they are willing to enter the study. Potential participants will be approached for each stage separately, so that they will have a choice as to whether they wish to participate in either stage or both stages.

The renal clinical team will be informed of the patient's participation in the study. A copy of the consent form will be given to participants, a copy filed in their personal health records as documentation of participation, and the original consent will be kept in the Trial Master File (TMF).

It will be explained to the potential participant that entry into the trial is entirely voluntary and that their treatment and care will not be affected by their decision. It will also be explained that they can withdraw at any time but attempts will be made to avoid this occurrence. In the event of their withdrawal it will be explained that their data collected so far cannot be erased and we will seek consent to use the data in the final analyses where appropriate.

Eligibility criteria for study 1 & 2

Inclusion criteria

- Age between 18yrs and 80yrs
- Able to give informed consent
- CKD5 on chronic haemodialysis for >90days

Interdialytic weight gains > 0.45 kg on average

- **Exclusion criteria**
- Unable/unwilling to provide informed consent
- Participants involved in another research study
- Pre-existing myopathy
- Pregnant
- Mental incapacity to consent
- Active infection
- Severe COPD or active inflammatory bowel disease
- Malignancy
- Known sensitivity to SonoVue, ensuring that the participant has sufficient time to consider participating or not. The Investigator will answer any questions that the participant has concerning study participation. The information sheet and the study protocol has undergone review by the CKRI Patient and Public Involvement group that has informed the nature and presentation of information.

Informed consent will be collected from each participant before they undergo any interventions (including physical examination and history taking) related to the study. One copy of this will be kept by the participant, one will be kept by the Investigator, and a third will be retained in the patient’s hospital records.

Should there be any subsequent amendment to the final protocol, which might affect a participant's participation in the trial, continuing consent will be obtained using an amended Consent form which will be signed by the participant.

TRIAL / STUDY TREATMENT AND REGIMEN

Once participants have given their informed consent, the dates for study commencement will be arranged.

After consent, data will be collected as below at the Royal Derby Hospital:

- Collection of personal characteristic data
- Demographics
- Primary aetiology of disease
- Co-morbidities
- Cardiac status – risk factors, vascular history, ECG findings, coronary angiography
- Medication
- Alcohol and smoking history
- Standard nutritional assessment
- Usual exercise tolerance
- Dialysis background
- Date of commencement of RRT
- Target dry weight
- Average UF volume
- Most recent Qa
- Location and type of access

Study dialysis sessions will be midweek, not directly after the long break period.

Patients for study 1 will be coming in for an extra visit 24 hrs before the dialysis session to have D2O drink.

Participants will have the following information recorded at study dialysis session:

- Weight
- Blood pressure (pre, during dialysis at 15min intervals, post)
- Duration of dialysis
- Medication changes

Review for presence of exclusion criteria.

Study sessions will be supervised at all times by a medical practitioner and usual dialysis nurses. Dialysis will be conducted at RDH using Gambro Artis dialysis monitors as per standard clinical practice; no modifications to the dialysis procedure will be undertaken as part of this study. Dialysate flow will be >500mls/min with blood flow exceeding 300mls/min. The type of dialyser will be the same as for routine care. Temperature will be set according to each patient's dialysis prescription. Ultrafiltration volumes will be calculated according to the patient's IDWG and target weight. All dialysis specifications will be recorded in the CRFThe following study procedures will be used:

Administration of stable isotope tracers

Study 1: D2O will be administered as an oral dose; a total dose of 400ml will be administered 24 hrs prior to dialysis study session. The additional fluid volume associated with D2O administration will be assessed on an individual patient basis and adjustments made to ultrafiltration volume of preceding dialysis session as necessary. This will be supervised by Dr Selby or other medically trained members of the research team. The 400ml will be administered in 4 x100ml doses separated by 30-45min to avoid potential side effects of dizziness/nausea that are occasionally reported with larger doses; the first will be taken under medical supervision in renal dialysis unit with subsequent 3 doses being taken at home at hourly intervals. We 0.22um filter D2O before giving it to volunteers.

Blood and saliva tests

Blood samples (10ml additional to the routine sample) will be taken for standard biochemical and haematological tests pre and post dialysis as per standard care. These will include urea, creatinine, sodium, potassium, calcium, magnesium, phosphate, albumin, bicarbonate, glucose, lactate, prothrombin time, Troponin T, nTproBNP, full blood count and liver function tests. Samples will be collected from the dialysis port needle once it is sited. Plasma and serum will be collected and stored at -80^{0}C for subsequent analysis.

In study 1, additional 1.5ml blood samples will be taken in the same way every 30min (total 21ml) to measure plasma, D20 levels, and venous pH (blood gas

analyser) along with saliva samples every hour to measure salivary D2O levels.

Contrast enhanced ultrasound (CEUS)

This will be performed in Study 2 (all patients), plus one patient in study 1 who could be either going through both studies or only through study 1. CEUS (using the Phillips iU22) is a safe and widely used technique in medical diagnostics in cardiology, renal, hepatic and respiratory medicine and in oncology. When CEUS is applied to the imaging of blood flow in muscle, an ultrasound visible contrast agent (SonoVue®) is infused as per manufacturer's instructions into the blood stream as a multitude of inert gas filled "microbubbles". For the current study (study 2), this will be achieved via the venous port in the dialysis circuit. The CEUS system can be set up so there is no ultrasound penetration of microbubbles in thick walled vessels but only in capillaries. The microbubbles in the field of interest (i.e. a thigh muscle) are burst by means of a high frequency sound wave, whereupon the repopulation of the field with bubbles from elsewhere in the circulation gives a measure of the perfusion. Pre-clinical studies conducted by the study team have shown that this technique is unaffected by the dialysis procedure. Four CEUS exams will be performed: one before the start of dialysis session, the second between 30 and 60 minutes of dialysis, the third just before the end of dialysis session (210-240min) and the last 30minutes after the termination of dialysis session. The contrast agent will be prescribed and administered by a medically trained member of the research team.

Monitoring of blood pressure

Measurement of blood pressure will be performed as standard clinical practice every 15 minutes throughout dialysis.

Bioimpedance (BIA) measures

Study 2 only: Body composition and hydration state will be recorded by bioimpedance analysis before and after dialysis. As bioimpedance is contraindicated for patients with pacemakers, it will not be performed in these patients but this will not exclude participants from other aspects of the trial. Two adhesive skin electrodes are placed on the hand and foot on one side, typically the right side of the body and connected to the instrument by an electrode cable set. A non-susceptible current is entered into the body through the first pair of hand-foot electrodes. A second electrode pair on the contralateral limbs is used to determine the voltage drop caused by the body water dependent impedance or total resistance. Simultaneously the phase shift of the alternating current is measured, indicating the cell mass dependent capacitive resistance or reactance. For analysis of test data and interpretation of body composition an evaluation program is used.

Compliance

Compliance with the study involves completion of a single study session plus compliance with the tracer administration protocols. These visits will be continually supervised and therefore there is no need for an assessment of

participant compliance with the study protocol. Should the participant not attend the study session, this will be classed as a withdrawal rather than non-compliance (see Section **Participant Withdrawal**) as their eligibility for participation will be void. Responsibility for compliance otherwise will lie with the research team. Adherence to the study protocol by the research team will be documented at each study visit. Deviation from the protocol will also be clearly documented and reviewed by the chief investigator. Participants may then be withdrawn at the discretion of the CI if necessary.

Criteria for terminating study

Owing to the nature of this research, overall study termination cannot occur early on the basis of interventional impact. In the event of major safety concerns, new information, or issues with study conduct (e.g. poor recruitment, loss of resources), termination of the study will be considered by the sponsor. In the event of study termination, unused study resources will be reallocated as appropriate.

TRANSPORT AND STORAGE OF THE TISSUES

Blood samples will be taken at the Royal Derby Hospital renal dialysis unit. Standard laboratory samples will be sent to the local NHS laboratory at the Royal Derby Hospital for testing of cardiac markers and renal, liver and haematological function. These samples will be labelled according to NHS requirements. These samples will then be stored in a dedicated research freezer in the NHS laboratory under a local agreement.

Studies research blood samples will be stored as plasma and serum after being centrifuged at 3,500rpm for 15 minutes, in aliquots, at -80 degrees centigrade in a secure freezer at the University of Nottingham, Derby Site. These blood samples will be stored in linked anonymised format at the University of Nottingham (Derby Site) for the duration of the study and labelled using study number to permit accurate linkage to clinical data and the consent form. The master database of results will be held in a password encrypted file.

Each participant will be asked to provide a saliva sample collected hourly throughout the dialysis session. These are to be collected in sterile plastic tubes and kept refrigerated. Upon receipt of saliva samples, they will be immediately cold centrifuged to remove any debris that might have been present; then they will be aliquoted into glass vials and frozen at until analysis.

LABORATORY ANALYSES

Blood samples will be analysed in a local NHS laboratory at the Royal Derby Hospital that is serviced and managed according to national standards and governance frameworks. D2O samples will be analysed within the Clinical, metabolic and molecular physiology research group labs at the University of Nottingham.

Samples will be stored by the University of Nottingham at the Clinical Sciences Building, Derby Campus, for possible use in future studies, some of which may be carried out by researchers other than the current team who ran the first study, including researchers working for commercial companies. Any samples

or data used will be anonymised, and participants will not be identified in anyway.

STATISTICS

Methods

Statistical analysis will be done using SPSS version 22 (IBM, Chicago, Illinois, USA). All continuous data will be tested for normality using the Shapiro Wilk test. Data from study 2 will be compared between baseline and during HD or between baseline and after HD. Parametric continuous data will be tested using the t test whereas non-parametric data will be tested using the Mann Whitney U test. Associations between categorical data will be tested using the Chi squared or Fischer's exact test as appropriate. An alpha error of equal to or less than 0.05 will be judged to be significant.

All data will be analysed on University of Nottingham computers and backed up regularly.

Sample size and justification

The acute effects of dialysis on muscle perfusion has never been assessed previously so this study will be a pilot to explore new methodologies and determine whether there is a biological signal. The study has not been powered for analysis of the data with inferential statistics. Consequently, the sample size has been selected on a pragmatic basis and is comparable with published norms.

Assessment of safety

This study is not aimed at primarily assessing safety as the study procedures are largely all within routine clinical care or been well established in other patient groups. However, safety endpoints will be as follows:

Participant reported tolerability of study day procedures

Procedures for missing, unused and spurious data

All missing data will be explained. If a space on the CRF is left blank because the procedure was not done or a question was not asked, “N/D” will be recorded. If the item is not applicable to the individual case, “N/A” will be recorded. All entries will be printed legibly in black ink. If any error is made in data entry, to correct the error, a single straight line will be drawn through the incorrect entry and the correct data entered above it. All such changes will be initialled and dated. Errors will not be erased or altered by any other method. For clarification of illegible or uncertain entries, the clarification will be printed above the item, initialled and dated. Correction fluid will not be used.

Definition of populations analysed

The study will analyse the following populations:

Safety set: All participants who receive at least one treatment.

Full Analysis set: All participants, who participated in at least one study day and for whom at least one post-baseline assessment of the primary endpoint is available.

Per protocol set: All participants in the Full Analysis set who are deemed to have no major protocol violations that could interfere with the objectives of the study.

Efficacy will be assessed on both the full analysis set and the per protocol set. Safety summaries will be performed on the safety set

ADVERSE EVENTS

The occurrence of an adverse event as a result of participation within this study is not expected and no adverse event data will be collected

ETHICAL AND REGULATORY ASPECTS

ETHICS COMMITTEE AND REGULATORY APPROVALS

The trial will not be initiated before the protocol, informed consent forms and participant information sheets have received approval / favourable opinion from the Research Ethics Committee (REC), the respective National Health Service (NHS) Research & Development (R&D) department, and the Health Research Authority (HRA) if required. Should a protocol amendment be made that requires REC approval, the changes in the protocol will not be instituted

until the amendment and revised informed consent forms and participant information sheets (if appropriate) have been reviewed and received approval / favourable opinion from the REC and R&D departments. A protocol amendment intended to eliminate an apparent immediate hazard to participants may be implemented immediately providing that the REC is notified as soon as possible and an approval is requested. Minor protocol amendments only for logistical or administrative changes may be implemented immediately; and the REC will be informed.

The trial will be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki, 2013 the principles of Good Clinical Practice, and the Department of Health Research Governance Framework for Health and Social care, 2005.

INFORMED CONSENT AND PARTICIPANT INFORMATION

The process for obtaining participant informed consent will be in accordance with the REC guidance, and Good Clinical Practice (GCP) and any other regulatory requirements that might be introduced. The investigator or their nominee and the participant shall both sign and date the Informed Consent Form before the person can participate in the study.

The participant will receive a copy of the signed and dated forms and the original will be retained in the Trial Master File. A second copy will be filed in

the participant's medical notes and a signed and dated note made in the notes that informed consent was obtained for the trial.

The decision regarding participation in the study is entirely voluntary. The investigator or their nominee shall emphasize to them that consent regarding study participation may be withdrawn at any time without penalty or affecting the quality or quantity of their future medical care, or loss of benefits to which the participant is otherwise entitled. No trial-specific interventions will be done before informed consent has been obtained.

The investigator will inform the participant of any relevant information that becomes available during the course of the study, and will discuss with them, whether they wish to continue with the study. If applicable they will be asked to sign revised consent forms.

If the Informed Consent Form is amended during the study, the investigator shall follow all applicable regulatory requirements pertaining to approval of the amended Informed Consent Form by the REC and use of the amended form (including for ongoing participants).

RECORDS

Case Report Forms

Case Report Forms will be the primary data collection instrument. All data requested on the CRF will be recorded. Data collected includes inclusion and exclusion criteria, demographics, co-morbidities, aetiology of disease, concomitant medication.

Each participant will be assigned a trial identity code number, for use on CRFs other trial documents and the electronic database.

CRFs will be treated as confidential documents and held securely in accordance with regulations. The investigator will make a separate confidential record of the participant's name, date of birth, local hospital number or NHS number, and Participant Trial Number (the Trial Recruitment Log), to permit identification of all participants enrolled in the trial, in accordance with regulatory requirements and for follow-up as required

CRFs shall be restricted to those personnel approved by the Chief or local Principal Investigator and recorded on the 'Trial Delegation Log.'

CRFs are used to record clinical trial data and are an integral part of the study and subsequent reports. The CRFs, therefore, must be legible and complete.

All paper forms shall be filled in using black ballpoint pen. Errors shall be lined out but not obliterated by using correction fluid and the correction inserted, initialled and dated.

The Chief or local Principal Investigator shall sign a declaration ensuring accuracy of data recorded in the CRF.

Sample Labelling

Each participant will be assigned a trial identity code number for use on the samples, consent forms and other study documents and the electronic database. The documents and database will also use their initials (of first and last names separated by a hyphen or a middle name initial when available).

Samples for NHS pathology analysis will be labelled in accordance with local NHS procedures.

Source documents

The source documents for each patient will consist of the demographic, co-morbidities and concomitant medication data collected from the patient's health care records. This will be transferred into a case report form for that particular participant. Paper copies of haematological and biochemical laboratory test results from the electronic results system and will be retained in the TMF as source documents. Complex recorded data from the other study procedures will only be recorded by ID code and stored as electronic source documents.

Source documents shall be filed at the investigator's site and may include but are not limited to, consent forms, current medical records, laboratory results

and records. A CRF may also completely serve as its own source data. Only trial staff as listed on the Delegation Log shall have access to trial documentation other than the regulatory requirements listed below.

Direct access to source data / documents

The CRF and all source documents, including progress notes and copies of laboratory and medical test results shall made be available at all times for review by the Chief Investigator, Sponsor's designee and inspection by relevant regulatory authorities.

DATA PROTECTION

All trial staff and investigators will endeavour to protect the rights of the trial's participants to privacy and informed consent, and will adhere to the Data Protection Act, 1998. The CRF will only collect the minimum required information for the purposes of the trial. CRFs will be held securely, in a locked room, or locked cupboard or cabinet. Access to the information will be limited to the trial staff and investigators and relevant regulatory authorities (see above). Computer held data including the trial database will be held securely and password protected. All data will be stored on a secure dedicated web server. Access will be restricted by user identifiers and passwords (encrypted using a one way encryption method).

Information about the trial in the participant's medical records / hospital notes will be treated confidentially in the same way as all other confidential medical information.

Electronic data will be backed up every 24 hours to both local and remote media in encrypted format.

QUALITY ASSURANCE & AUDIT

INSURANCE AND INDEMNITY

Insurance and indemnity for trial participants and trial staff is covered within the NHS Indemnity Arrangements for clinical negligence claims in the NHS, issued under cover of HSG (96)48. There are no special compensation arrangements, but trial participants may have recourse through the NHS complaints procedures.

The University of Nottingham as research Sponsor indemnifies its staff, research participants and research protocols with both public liability insurance and clinical trials insurance. These policies include provision for indemnity in the event of a successful litigious claim for proven non-negligent harm.

STUDY CONDUCT

Study conduct may be subject to systems audit of the Trial Master File for inclusion of essential documents; permissions to conduct the trial; Trial Delegation Log; CVs of trial staff and training received; local document control procedures; consent procedures and recruitment logs; adherence to

procedures defined in the protocol (e.g. inclusion / exclusion criteria, correct randomisation, timeliness of visits); adverse event recording and reporting; accountability of trial materials and equipment calibration logs.

STUDY DATA

Monitoring of study data shall include confirmation of informed consent; source data verification; data storage and data transfer procedures; local quality control checks and procedures, back-up and disaster recovery of any local databases and validation of data manipulation. The Trial Coordinator, or where required, a nominated designee of the Sponsor, shall carry out monitoring of trial data as an ongoing activity.

Entries on CRFs will be verified by inspection against the source data. A sample of CRFs (10% or as per the study risk assessment) will be checked on a regular basis for verification of all entries made. In addition, the subsequent capture of the data on the trial database will be checked. Where corrections are required these will carry a full audit trail and justification.

Trial data and evidence of monitoring and systems audits will be made available for inspection by REC as required.

RECORD RETENTION AND ARCHIVING

In compliance with the ICH/GCP guidelines, regulations and in accordance with the University of Nottingham Research Code of Conduct and Research Ethics, the Chief or local Principal Investigator will maintain all records and

documents regarding the conduct of the study. These will be retained for at least 7 years or for longer if required. If the responsible investigator is no longer able to maintain the study records, a second person will be nominated to take over this responsibility.

The Trial Master File and trial documents held by the Chief Investigator on behalf of the Sponsor shall be finally archived at secure archive facilities at the University of Nottingham. This archive shall include all trial databases and associated meta-data encryption codes.

DISCONTINUATION OF THE TRIAL BY THE SPONSOR

The Sponsor reserves the right to discontinue this trial at any time for failure to meet expected enrolment goals, for safety or any other administrative reasons. The Sponsor shall take advice from the Trial Steering Committee and Data Monitoring Committee as appropriate in making this decision.

STATEMENT OF CONFIDENTIALITY

Individual participant medical information obtained as a result of this study are considered confidential and disclosure to third parties is prohibited with the exceptions noted above.

Participant confidentiality will be further ensured by utilising identification code numbers to correspond to treatment data in the computer files.

Such medical information may be given to the participant's medical team and all appropriate medical personnel responsible for the participant's welfare.

Data generated as a result of this trial will be available for inspection on request by the participating physicians, the University of Nottingham representatives, the REC, local R&D Departments and the regulatory authorities.

PUBLICATION AND DISSEMINATION POLICY

The results of this study will be submitted to peer-reviewed journals for publication as soon as data analysis is completed. The results will also be presented at conferences. Participants will not be identified in any publications. However, participants will be informed of the results of the study via a departmental research newsletter which is made available to all patients.

USER AND PUBLIC INVOLVEMENT

Our dialysis population at the Royal Derby Hospital are regularly involved in research. Our group produce a research newsletter biannually which publicises the various studies being conducted and results from completed studies. Our PPI group has informed the study design and content of the patient information leaflets (minutes of PPI meetings available separately that evidence this). The changes that we have incorporated are:

STUDY FINANCES

Funding source

Local agreements are in place.

Participant stipends and payments

Participants will not be paid to participate in the trial. Travel expenses will be offered for any hospital visits in excess of usual care.

SIGNATURE PAGES

Signatories to Protocol:

Chief Investigator: (name)_____

Signature:_____

Date: _____

Co- investigator: (name) _____

Signature :_____

Date : _____

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Appendix E: MUSHD Study Patient Information Sheet

University of Nottingham
UK | CHINA | MALAYSIA

Derby Hospitals NHS
NHS Foundation Trust

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Participant Information Sheet

(final version 3.0: 17/10/2018)

IRAS Project ID: 244334

Title of Study: Muscle Stunning in Haemodialysis

Name of Chief Investigator: Dr Nick Selby

Name of Researchers: Dr Nick Selby, Dr Daniel Wilkinson, Shatha Al Mushayt

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have. Talk to others about the study if you wish. Ask us if there is anything that is not clear.

What is the purpose of the study?

Dialysis patients often find that their muscle size and strength falls over time. The purpose of this study is to see if there are changes during dialysis that may contribute to muscle wasting. In particular, we want to study whether the amount of blood delivered to muscles changes during dialysis. If we do find changes, then this would suggest ways that we could improve the dialysis treatments in the future.

Why have I been invited?

You are being invited to take part of this study because you are a patient who has been treated with haemodialysis for more than 3 months. We are inviting 12 participants like you to take part.

Do I have to take part?

It is totally up to you to take part of this study or not. The research team are available to answer any question that concerns you. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to participate, you still can withdraw from the study at any time without giving a reason. Withdrawal or refusal to participate will have no effect on the medical service you receive at any way.

What will happen to me if I take part?

Once you agree to participate, you will be asked to sign a consent form.

The study will be performed during one of your usual dialysis session visits; we will ask you to attend a little earlier than usual. We will measure

blood flow to the muscle in your thigh using ultrasound scan. Ultrasound involves using a probe that is placed on your skin, and then uses sounds waves to take pictures of the muscle. This is combined with contrast, a fluid, to show up the blood flow to the muscles for better diagnosis, the contrast can be given via the dialysis machine. This process is commonly and safely used in clinical practice. Ultrasound will be performed, before, during and after dialysis.

Expenses and payments

You will not be paid to participate in the trial.

What are the possible disadvantages and risks of taking part?

You will be closely observed during the entire study time.

If you have pacemaker, you can still take part in the study, but one of the measurements will not be possible (body composition measure). Please let us know if you have a pacemaker and we will ask about this again before the study day.

What are the possible benefits of taking part?

We cannot promise the study will help you but the information we get from this study may help understand causes of muscle weakness, which may help us develop the current haemodialysis procedure for better quality of life for future haemodialysis patients.

What happens when the research study stops?

There may be unforeseen circumstances in which your participation in the study may be terminated by the research team. However, the

information collected before the termination cannot be erased and this information may have already been used in some analyses and may still be used in the final study analyses. After taking part in the research study, your treatment will revert back to your normal care and no additional follow up would be required after the research study. A copy of the results will be available upon request. Participants will be informed of the results of the study via a departmental research newsletter which is made available to all patients. You will also be asked to consent for your contact details to be held by the research team.

What if there is a problem?

Should you feel unwell outside the study hours or experience side effects, it is advisable that you seek advice from your GP. If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. The researchers contact details are given at the end of this information sheet. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital PALS department (01332 340131). The normal National Health Service complaints mechanisms will still be available to you.

Will my taking part in the study be kept confidential?

We will follow ethical and legal practice and all information about you will be handled in confidence.

If you join the study, we will record in the hospital notes that you are taking part in this study. Your GP will be notified about your participation in the

study. The data collected for the study and some parts of your medical records will be looked at by authorised persons from the University of Nottingham who are organising the research. They may also be looked at by authorised people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

All information which is collected from you and your medical records during the course of the research will be kept **strictly confidential**, stored in a secure and locked office, and on a password protected database at the University of Nottingham. Under UK Data Protection laws the University is the Data Controller (legally responsible for the data security) and the Chief Investigator of this study (named above) is the Data Custodian (manages access to the data). This means we are responsible for looking after your information and using it properly. Your rights to access, change or move your information are limited as we need to manage your information in specific ways to comply with certain laws and for the research to be reliable and accurate. To safeguard your rights we will use the minimum personally – identifiable information possible.

You can find out more about how we use your information and to read our privacy notice at:

https://www.nottingham.ac.uk/utilities/privacy.aspx.

Where possible information about you which leaves the University will have your name and address removed (anonymised) and a unique code will be used so that you cannot be recognised from it, however sometimes we need to ensure that we can recognise you to link the research data with your medical records so in these instances we will need to know your name and date of birth.

Your personal data (address, telephone number) will be kept for 12 months after the end of the study so that we are able to contact you about the findings of the study (unless you advise us that you do not wish to be contacted). This information will be kept separately from the research data collected and only those who need to will have access to it. All other data (research data) will be kept securely for at least 7 years or longer if required. After this time your data will be disposed of securely. During this time, all precautions will be taken by all those involved to maintain your confidentiality, only members of the research team will have access to your personal data.

In accordance with the University of Nottingham's, the Government's and our funders' policies we may share our research data with other Universities and researchers, including those in other countries, for research in health and social care Sharing research data is important to allow peer scrutiny, re-use (and therefore avoiding duplication of research) and to understand the bigger picture in particular areas of research. Data sharing in this way is usually anonymised (so that you

could not be identified) but if we need to share identifiable information we will seek your consent for thisand ensure it is secure You will be made aware then if the data is to be shared with countries whose data protection laws differ to those of the UK and how we will protect your confidentiality.

Although what you say to us is confidential, should you disclose anything to us which we feel puts you or anyone else at any risk, we may feel it necessary to report this to the appropriate persons.

What will happen if I don't want to carry on with the study?

Your participation is voluntary and you are free to withdraw at any time, without giving any reason, and without your legal rights being affected. If you withdraw we will no longer collect any information about you or from you. However, if you withdraw then the information collected so far cannot be erased as we are not allowed to tamper with study records and this information may have already been used in some analyses and may still be used in the final study analyses. To safeguard your rights, we will use the minimum personally-identifiable information possible.

What will happen to any samples I give?

Blood tests in this study are all part of routine care. However, additional blood sample (approximately 10ml) for storage of plasma and serum samples will be collected.

Consent will be sought for the samples to be stored by the University of Nottingham at the Clinical Sciences Building, Derby Campus, for possible use in future studies, some of which may be carried out by researchers other than the current team who ran the first study, including researchers working for commercial companies. Any samples or data used will be anonymised, and participants will not be identified in anyway.

What will happen to the results of the research study?

The results of this study will be submitted to journals for publication as soon as data analysis is completed. The results will also be presented at conferences. A copy of the published results will be available upon request. Also, results are to be written up as part of a PhD degree. Participants will not be identified in any publications. However, participants will be informed of the results of the study via a departmental research newsletter which is made available to all patients.

Who is organising and funding the research?

This research is being organised by the University of Nottingham.

Who has reviewed the study?

All research in the University of Nottingham is looked at by a group of independent people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by Research Ethics Committee.

The East of Scotland Research Ethics Service REC 2, which has responsibility for scrutinising all proposals for medical research on

humans, has examined the proposal and has raised no objections from the point of view of research ethics. It is a requirement that your records in this research, together with any relevant medical records, be made available for scrutiny by monitors from the University of Nottingham and NHS <<Royal Derby Hospital>>, whose role it is to check that research is properly conducted and the interests of those taking part are adequately protected.

Further information and contact details

If you would like more information or to discuss any part of the research, please do not hesitate to contact any of the following:

Shatha Al Mushayt
Research fellow

Dr Nick Selby
Associate Professor of Nephrology
(Chief Investigator)
Professor Maarten Taal
Professor of Medicine

Contact:
Department of Renal Medicine
Royal Derby Hospital
Tel: 01332 789344 (direct line)
To find out more about the regulation of Research within the NHS visit:

www.hra.nhs.uk

Appendix F: MUSHD Study Participant Consent Form

PARTICIPANT CONSENT FORM

(Final version 2.0: date: 2/10/2018)

Project title: Muscle Stunning in Haemodialysis

IRAS Project ID: 244334

Researcher's name: Shatha Al Mushayt

Supervisor's name: Dr Nick Selby, Dr Daniel Wilkinson

Please read and initial each of the following statements:	Initial
I confirm that I have read and understand the information sheet final version number 3.0 dated 17.10.2018 for the above study and have had the opportunity to ask questions.	☐
I was given time to think and ask questions about the study and my involvement in it.	☐
I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. I understand that should I withdraw then the information collected so far cannot be erased and that this information may still be used in the project analysis.	☐

I understand that relevant sections of my medical notes and data collected in the study may be looked at by authorised individuals from the University of Nottingham, the research group and regulatory authorities where it is relevant to my taking part in this study. I give permission for these individuals to have access to these records (including my contact details) and to collect, store, analyse and publish information obtained from my participation in this study. I understand that I will not be identified and my personal results will remain confidential.	☐
I understand and agree that blood samples will be taken for analysis as stated in the patient information sheet.	☐
I agree to my GP being informed about my participation in this study	☐
Consent for storage and use in possible future research (Optional) I agree that the samples I have given and the information gathered about me can be stored by the University of Nottingham at the Clinical Sciences Building, Derby Campus, for possible use in future studies. I understand that some of these studies may be carried out by researchers other than the current team who ran the first study, including researchers working for commercial companies. Any samples or data used will be anonymised, and I will not be identified in anyway.	☐
I agree to take part in the above study.	☐

_____ _____ _____

Name of Participant Signature Date

_____ _____ _____

Name of Principal Investigator Signature Date