Exploring Mechanisms of Intestinal Microbiota Transplantation on Prevention of Invasive Disease in Patients with Intestinal Colonisation of Multidrug-resistant Organisms

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Statement of Originality:

I certify that this thesis, and the research to which it refers, are the product of my own work. Any ideas or quotations from the work of other people, published or otherwise, are fully acknowledged in accordance with the standard referencing practices of the discipline.

All clinical and laboratory work was carried out by me, except for what was explicitly described in the text.

- MiSeq Illumina Sequencing, which was carried out by Dr Nathan Danckert, Imperial College London.
- Complex modelling on metabonomic which was carried out by Dr Pantelis Takis and Dr Jesús Miguens Blanco, Imperial College London.
- Metagenomic sequencing were provided by Sanger Institute, Cambridge
- Clinical samples were processed by Imperial College Healthcare NHS Trust.

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Abstract

Background

The intestinal microbiome has an important function in the defence against infectious diseases. Intestinal colonisation with multidrug-resistant organisms (MDROs) is a risk factor for invasive disease which leads to higher morbidity than drug-sensitive infections. Intestinal Microbiota Transplantation (IMT) is a modality to restore the gut microbiome. We utilised IMT in MDRO colonised patients and observed both clinical outcomes and effect on microbiota diversity, gene expression, metabolites expression and markers of gut barrier function.

Methods

A retrospective study was performed to identify at risk groups and a literature review was performed to refine the IMT process for MDRO colonised patients. IMT was performed on patients with risk factors for invasive disease who had known intestinal MDRO colonisation and clinical outcomes were assessed 6 months pre- and post-IMT. Metataxonomic profiles were performed on these patient stool samples. Additional stool from donor, pre-and post-IMT samples underwent metagenomic and metabonomic testing.

Results

Renal transplant and pre-hematopoietic cell transplant patients were identified as the highest risk of developing invasive MDRO disease with a worse outcome profile. Guidelines for the use of IMT for MDRO colonised patients were constructed with careful consideration of administration and donor stool preparation. Post-IMT patients had fever bloodstream infections, shorter hospital admissions and reduced antibiotic usage. IMT increased intestinal microbiota alpha diversity and reduced the burden of *Enterococci* but not Gram-negative pathobionts. *Blautia producta* and *Coprococcus catus*, two commensal bacteria, increased post-IMT. The short-chain fatty acid, valerate was higher in concentration in stool in patients who did not develop invasive infection post-IMT. Serum levels of TNF-α and IL-8, proinflammatory cytokines associated with gut barrier permeability both reduced post-IMT.

Conclusion

The microbiome may play a role in protection against infection, and manipulation of the composition may be a key facet of preventing infection in at risk groups.

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List of acronyms

ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
AMP	Antimicrobial peptide
AMR	Antimicrobial resistance
AMS	Antimicrobial stewardship
ARG	Antibiotic resistance gene
ATP	Adenosine triphosphate
BC	Blood culture
BMI	Body Mass Index
BRAKEN	Bayesian Reestimation of Abundance with KrakEN software
BSI	Bloodstream Infection
CARD	Comprehensive Antibiotic Resistance Database
CDC	Centers for Disease Control
CDI	Clostridioides difficile infection
CFU	Colony-forming unit
CML	Chronic myeloid leukemia
CMV	Cytomegalovirus
CPE	Carbapenemase-producing Enterobacteriaceae

CPMG	Carr–Purcell Meiboom–Gill
CRE	Carbapenem resistant Enterobacteriaceae
CTX-M	Cefotaximase-Munich
DNA	Deoxyribonucleic acid
EBMT	European Group for Blood and Marrow Transplantation
EBNA	Epstein Barr Virus Nuclear Antigen
EBV	Epstein-Barr Virus
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunoassay
ESBL	Extended Spectrum Beta-Lactamase
ESKAPE	Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species
FABP	Fatty Acid-Binding Protein
FDA	Food and Drug Administration
FDR	False Discovery Rate
FMT	Faecal Microbiota Transplantation
GES	Guiana extended-spectrum β-lactamase
GI	Gastro-intestinal
GNB	Gran-neagtive bacilli

GPR	Gram-negative rods
HCT	Haematopoietic Cell Transplantation
HIV	Human Immunodeficiency Virus
HPLC	high performance liquid chromatography
HSCT	Haematopoietic Stem Cell Transplantation
IBD	Inflammatory Bowel Disease
IBS	Irritable bowel syndrome
ICHNT	Imperial College Healthcare NHS Trust
ICU	Intensive care unit
ID	Infectious diseases
Ig	Immunoglobulin
IL	Interleukin
IMI	Imipenem-hydrolysing Carbapenemase
IMP	Imipenemase
IMT	Intestinal Microbiota Transplantation
INF	Interferon
IQR	Inter-quartile range
IV	intravenous
KPC	Klebsiella pneumoniae carbapenemase

KPS	Karnofsky Performance Status
LLOD	lower limit of detection
LoS	Length of stay
MAMP	Microbial-associated molecular patterns
MBL	Metallo-betalactamases
MDR	multidrug-resistant
MDRO	Multidrug-resistant Organism
MDS	Myelodysplastic syndrome
MGE	mobile genetic elements
MRSA	Methicillin Resistant Staphylococcus aureus
NDM	New Delhi metallo-beta-lactamase
NG	Naso-gastric
NHS	National Health service
NJ	Naso-jejunal
NMC	non-metalloenzyme carbapenemase
NMDS	Non-metric multidimensional scaling
NMR	Nuclear Magnetic Resonance
OPAT	Outpatient parenteral antibiotic therapy
OR	Odds ratio

OTU	Operational taxonomic unit
OXA	Oxacillin-hydrolysing
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PD	Peritoneal dialysis
PE	Pulmonary embolism
PEG	Percutaneous endoscopic gastrostomy
PK	Pharmacokinetics
PPI	Proton pump inhibitor
QR	Quartile range
rCDI	Recurrent Clostridiodes difficile infection
rUTI	Recurrent urinary tract infection
RCT	Randomised Control Trial
RNA	Ribonucleic Acid
SARS	Severe acute respiratory syndrome
SCFA	Short-chain Fatty Acid
SCT	Stem cell transplant
SD	Standard deviation
SDD	Selective Digestive Tract Decontamination

SHV	Sulfhydryl variant
SMolESY	Small Molecule Enhancement Spectroscopy
SOD	Selective Oropharyngeal Decontamination
SOP	Standard operation procedure
SOT	Solid organ transplantation
sp	Species
STAMP	Statistical Analysis of Metagenomic Profiles
STEC	Shiga Toxin-producing Escherichia coli
TB	Tuberculosis
TEM	Temorina Escherichia coli mutant
TJ	Tight junctions
TNF	Tumour Necrosis Factor
UK	United Kingdom
USA	United States of America
USD	US dollars
UTI	Urinary Tract Infection
VCA	Viral capsid antigen
VIM	Verona integron-mediated metallo-β-lactamase
VL	Viral load

VRE	Verona integron-mediated metallo-β-lactamase
WHO	World Health Organisation

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Chapter 1: Introduction

The aim of this introduction is to explain the interplay of antimicrobial resistance (AMR) within the intestinal microbiome and its impact on health. It will then go on to explain the potential utility of Intestinal Microbiota Transplantation (IMT) on patients colonised with multidrug-resistant organisms (MDROs) from a clinical and biological point of view and the gaps in knowledge that this project aims to address.

1.1 Antimicrobial Resistance

1.1.1 Epidemiology of multidrug-resistant organisms (MDROs)

Antimicrobial resistance (AMR), the ability of microorganisms to counteract the action of antimicrobial agents, is a major global health threat which the World Health Organisation (WHO) has reported carries significant global economic and security implications(1). Multidrug-resistant organisms (MDROs) are defined as organisms with non-susceptibility to at least one antimicrobial in three or more classes, based on antibiotic susceptibility testing *in-vitro*(2). Drug resistance of the ESKAPE pathogens (*Enterococcus* spp*., Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Escherichia coli*) are recognised to harbour the greatest impact on healthcare associated infections(3). Globally it was predicted in 2019 that there were an estimated 4.95 million death associated with bacterial AMR(4). In Europe, in 2015, 671,689 MDRO infections were recorded, with most being associated with health care settings (64%) and contributing to 33,110 attributable deaths. Deaths from carbapenem-resistant *Klebsiella pneumoniae* infections in particular were noted to increase from a factor increase of 6.16 between 2007 and 2015 (5).

1.1.2 Economic impact

International surveillance of AMR infections does not exist to evaluate the global economic impact. In low to middle income countries, due to lower resources, there is a lack of high quality, patient-level microbiology data linked to clinical outcomes (6). In the United States alone, it is estimated that each year 2 million AMR infections accounts for an attributable

health care cost of over 4.7 billion USD (7). Similarly in Europe, each year, over 33,000 deaths and 874,000 disability-adjusted life years are ascribed to hospital and community acquired AMR which accounts for $1.5 billion in indirect and direct costs (8). In one trust an outbreak of outbreak of carbapenemase-producing *Enterobacteriaceae* cost €1.1m over 10 months(9). By 2050 AMR is projected to result in 10 million deaths at cumulative a cost of 100 trillion USD (10).

1.1.3 Aetiopathogenesis of multidrug-resistant organisms

MDRO intestinal colonisation is strongly associated with MDRO invasive infection such as bloodstream infections (BSIs)(11,12). MDROs that colonise the intestine can translocate across the intestinal barrier and cause invasive disease resulting in worse morbidity and mortality outcomes than drug-sensitive infections (13). These outcomes are due to the delay to commencing appropriate therapy (14), and the worse side effect profile and reduced efficacy of second line therapy (15). They often require combination therapy which adds to the cost and side effect profile (16). The progression from intestinal colonisation with MDROs to invasive disease is higher in patients with impaired immune function and prolonged hospitalisation, particularly intensive care, and previous exposure to broad-spectrum antibiotic use (17,18).

1.1.4 Predictive scores

Predictive scores developed to identify patients most at risk from adverse events from CPE BSIs have identified the following characteristics as elevating CPE colonised patients more at risk of developing BSIs, which can help target which patients require early intervention.

- Gastrointestinal injury (19,20) including abdominal invasive procedures (21)
- Tigecycline use (19)
- Increased resistance to carbapenems (19)
- CPE intestinal colonisation (22–25)
- Severe neutropenia (20,23)
- Chemotherapy or radiotherapy (20,21)

- Admission to intensive care (21) as well as invasive mechanical ventilation within previous 30 days of onset of BSI (23)
- Number of additional colonisation sites (21)
- Prolonged hospitalisation (>10 days) (24)
- Previous antibiotics >7 days (24)
- Hypoalbuminemia (20)

1.1.5 Mechanisms of resistance

Acquired multidrug resistance is caused by the interaction of various resistance mechanisms either from the acquisition of external mobile genetic elements (MGEs) such as plasmids or by spontaneous mutations(26). The mechanisms of resistance are generally categorised into either alteration of the antibiotic target site, modification or destruction of the antibiotic molecule or inhibition of antibiotic binding to the target site through elimination method (27). Resistance tends to spread in humans by clonal expansion of resistant species or the transfer inter- or intra-species of pre-formed resistance genes on mobile genetic elements such as transposons or plasmids (28). The density of patients likely to be colonised in healthcare settings increases the likelihood of plasmid transferral across patients and in these settings vulnerable populations are more likely to develop invasive infection(29). Studies demonstrate that colonization with an organism can independently predict development of a systemic infection (22–25). Commensal bacteria within the intestinal microbiota are also able to acquire antibiotic resistance genes (ARGs) and shield pathogens from the bactericidal effects of antibiotics(30), and potentially transfer back ARGs to sensitive pathobionts(31). In one study, following a seven-day treatment course with clindamycin, increased numbers of clindamycin-resistant *Bacteroides thetaiotaomicron* persisted for two years post-treatment. Discussion of significant resistance mechanisms of pathogens that colonise the human gastrointestinal tract is as follows.

1.1.5.1 Extended-spectrum beta lactamase (ESBL) producing Enterobacterales.

Extended-spectrum β-lactamases (ESBLs) are the predominant cause of resistance in Gram-negative bacilli (GNB) against commonly used beta-lactam antibiotics such as penicillin and

cephalosporins. They act by hydrolysing and inactivating the beta-lactam ring of antibiotics. They consist of three groups of enzymes: TEM (Temorina *Escherichia coli* mutant), SHV (Sulfhydryl variant), and CTX-M (Cefotaximase-Munich). ESBLs transfer between bacteria by horizontal transfer through plasmid transmission(32).

1.1.5.2 Carbapenem-resistant Enterobacterales (CPE)

Carbapenemases are a group of beta-lactamases that enzymatically inactivate a large group of beta-lactam based drugs including carbapenems, the first line beta-lactam antibiotics for resistant organisms. Carbapenems are normally insensitive to the effects of β-lactamases because of the setting of the side chain in the trans position instead of the cis position(33). They are based on the Ambler classification system which classifies them according to their molecular structure, they are classified as A, B or D carbapenemases. Class A and D require serine at their active site, which Class B, known as the metallo-betalactamases (MBL) require zinc. (34). Class A carbapenemases include members of *Serratia marcescens* enzyme, imipenem-hydrolysing carbapenemase (IMI), NMDS

(NMC), Guiana extended-spectrum β-lactamase (GES) and *Klebsiella pneumoniae* carbapenemase (KPC) families. Class D carbapenemases consist of oxacillin-hydrolysing (OXA)-type β-lactamases. Class B carbapenemases include members of the imipenemase (IMP), Verona integron-mediated metallo-β-lactamase (VIM), New Delhi metallo-β-lactamase (NDM), São Paulo metallo-β-lactamase, German imipenemase, Seoul imipenemase, Dutch imipenemase, Adelaide imipenemase, *Serratia* metallo-β-lactamase, Tripoli metallo-β-lactamase, and Florence imipenemase. (35).

Ambler molecular class	Bush–Jacoby group	Preferred substrate	Inhibited	Representative enzyme
A (serine penicillinases)	2a	Penicillins	+	PC1 from *S. aureus*
	2b	Penicillins, narrow-spectrum cephalosporins	+	TEM-1, TEM-2, SHV-1
	2be	Penicillins, narrow- spectrum and extended-spectrum cephalosporins	+	SHV-2 to SHV-6, TEM-3 to TEM-26, CTX-Ms, BEL-1, VEB-1, PER-1
	2br	Penicillins	–	TEM-30, SHV-72, SHV-19
	2c	Penicillins, carbenicillin	+	PSE-1
	2e	Extended-spectrum cephalosporins	+	FEC-1, CepA
	2f	Penicillins, cephalosporins, carbapenems	+/–	KPC-2, SME-1, NMC-A
B (MBLs)	3	Most β-lactams including carbapenems	–	IMP-1, VIM-1. NDM-1, CcrA and BcII, CphA, L1
C (cephalosporinases)	1	Cephalosporins	–	AmpC, CMY-2, ACT-1
D (oxacillinases)	2	Penicillins, cloxacillin	+/–	OXA-1, OXA-10
	2de	Extended-spectrum cephalosporins	+/–	OXA-11, OXA-15
	2df	Carbapenems	+/–	OXA-23, OXA-48

Figure 1: Classification of β-lactamases. Image reproduced with permission of the rights holder(36)

1.1.5.3 Vancomycin-resistant enterococci (VRE)

Enterococcus faecium is an opportunistic Gram-positive organism that is associated with healthcare associated infections and has a low intrinsic susceptibility to a wide range of antimicrobials (37). It is also a gut commensal. There are six resistance phenotypes resulting in resistance to Vancomycin in *Enterococcus* (VanA, VanB, VanC, VanD, VanE, VanG), with vanA being the most prevalent worldwide. Resistance to Vancomycin results from a genetic change at the locus that encode peptidoglycan synthesis. At their terminal portion, D-Alanine-D-Alanine (D-Ala-D-Ala) switches to either D-Alanine-D-Lactate (D- Ala-D-Lac) (VanA, VanB and VanD) or D-Ala-D-Serine (VanC, VanE and VanG). This modification results in the prevention of action of vancomycin by reducing its binding affinity to peptidoglycan during synthesis (32). Treatment options for VRE infection are limited and antibiotics such as linezolid and tigecycline confer significant side effects.

1.1.6 Prevention of MDRO acquisition and transmission

Although WHO have recommended the research in drug development for CPE as a priority, new drug development is expensive, slow, and vulnerable to the eventual evolution of resistance to newer antibiotics(38). Only 27 drugs are currently in development to tackle pathogens considered critical by WHO(39). Current strategies rely on the prevention of spread of MDROs across patients and MDRO decolonisation of the intestine.

1.1.6.1 Infection Control

Infection control measures can help arrest the spread of the MDROs described. This involves a horizontal approach, implementing strict hand hygiene measures, care bundles and basic contact precautions to stop spread between healthcare workers(28). Surveillance of patients by detection of asymptomatic carriers by rectal screening for intestinal carriage of MDROs in patients in healthcare settings can help identify patients. Following identification of patients

colonised with MDROs, a vertical strategy of active isolation and implementing augmentation of contact precautions can stop further spread (40). This approach works less well for organisms such as NDM-1 than VRE or MRSA as elements are more transferable across species(28).

1.1.6.2 Antimicrobial Stewardship

Antibiotic misuse and overuse are recognised to proliferate the development of AMR (41) as well as increasing the risk of *Clostridioides difficile* infections and adverse drug reactions, and the impact on the gut microbiota (see page 48)(42). Reduction in use of broad-spectrum antibiotics can help decrease the selection pressure of intestinal colonisation of MDROs (43) An antimicrobial stewardship programme works to select the optimal dose, duration, and route of administration of antimicrobials. For such programmes to work requires a multidisciplinary team to work together to coordinate interventions such as utilising diagnostic tools like microbiology results to select the most appropriate antibiotic, which may not be possible to apply to every patient on antibiotics within a healthcare trust (44). Many programmes focus on narrowing the spectrum of activity of an antibiotic, but do not always consider the varying impact of antibiotics on disruption to the intestinal microbiota(28,45).

1.1.6.3 Intestinal decolonisation of MDROs

Spontaneous decolonisation rates vary greatly in the literature, in part due to the heterogeneity of screening tests, but it is agreed that most people spontaneously decolonise at some point (46). One meta-analysis reported 35% of patients were still colonised at 12 months (47), and prolonged carriage is associated with use of antimicrobial drugs(48). However, intestinal decolonisation does persist during hospital admissions when the MDROs are most at risk of translocation and invasive disease as well as spread to other vulnerable individuals (49). Efforts have been made to actively target pathobionts that colonise the intestine to reduce the incidence of invasive infection and transmission to others.

1.1.6.3.1 Selective digestive tract or oropharyngeal decontamination

Selective digestive tract decontamination (SDD) where topical antibiotics such as tobramycin and colistin are applied to the mouth and stomach, or selective oropharyngeal decontamination (SOD) where antibiotics are applied only in the mouth, both utilise poorly absorbed antimicrobials, sometimes in combination with systemic antimicrobials(50). These methods have mainly been used in intensive care settings and is not currently recommended due to insufficient available evidence and some literature reporting the contribution to selection pressure of MDROs (51).

1.1.6.3.2 Probiotics

Probiotics are selected commensal bacteria that are believed to influence intestinal carriage of MDROs by a combination of competitive adherence and improvement of colonisation resistance (see below) (52). In humans, there have been several studies looking at decolonisation of intestinal MDROs using probiotics. In an early trial in 2007, Manley *et al.,* demonstrated significant eradication of VRE colonisation in patients treated with *Lactobacillus rhamnosus* GG in a randomised controlled trial of 23 patients (53). This has not been replicated in further studies including the use of stain cocktails (54). In the use of probiotics (*Lactobacillus bulgaris* and *Lactobacillus rhamnosus*) for Gram-negative colonisation, decolonisation rates have been similar to spontaneous decolonisation rates (55–57). Recent reports have suggested clinically significant bacteraemia seen in Intensive Care patients and paediatric hematopoietic cell transplant patients following probiotic use (58,59).

1.1.6.3.3 Phage therapy

Bacteriophages or phages are viruses that infect and replicate within bacteria. They are recognised as a component of the gut microbiota and are an attractive option as they are species specific, therefore likely to have low toxicity within the microbiome (60). They exist in either a lytic (virulent) or lysogenic (non-virulent) life cycle, which is dependent on a change in the host environment or external cues such as antibiotics (61). Their use in intestinal decolonisation of MDROs has been explored however to date, human studies demonstrating

a partial response to MDRO decolonisation, have also involved the use of antibiotics, rather than phage therapy alone (62,63). Beyond the lack of robust data, disadvantages include the manufacturing challenges, phages are targeted to single specific organisms unlike antibiotics, which may limit its utility in both infection and colonisation(64). The greatest limiting factor however include the inevitable evolution of bacteria to become resistant to the phage (65).

1.2 Intestinal microbiota

The human gut microbiota is composed of 10^{14} bacteria. The intestinal bacterial community in healthy adults is approximately 90% dominated by *Firmicutes* and *Bacteroidetes* with smaller percentages of *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* also present. Each individual person demonstrates a unique microbiota profile however a conserved set of gut bacteria, the core microbiota, is shared, suggesting that this plays a role in maintaining health (66). Although the exact ideal composition has not been qualified, metrics such as higher alpha-diversity (Shannon Index) have been associated with healthier phenotypes(67).

1.2.1 The Resistome

The intestinal microbiome acts as a reservoir for antimicrobial resistant genes known as the resistome (68). Disruption by antibiotics increases the risk of colonisation with these MDROs by direct reduction of antibiotic-susceptible commensals(69). This loss of species diversity results in the consequent reduction in the role that these commensal bacteria play in mediating colonisation resistance and innate immune defences(70)

1.2.2 Colonisation Resistance and gut barrier function

The intestinal microbiome has an important function in the defence against infectious diseases. This defensive system includes a consortium of phylogenetically diverse commensal microbes, including bacteria and other components. Colonisation resistance is the term used to describes the way in which the microbiome operates both directly and indirectly to prevent colonization and invasive infection from pathogens, as well as to provide immune regulation (71). Examples of direct actions by members of the intestinal microbiota community include.

Examples of direct actions by members of the intestinal microbiota community include.

- Direct competition from commensal bacteria with pathogens for resources and niches
- Secretion of antimicrobial peptides (AMPs) which have bacteriostatic or bactericidal activity against pathogens.
- Production of inhibitory compounds (72)

Examples of these inhibitory compounds include short-chain fatty acids (SCFAs) and bile acids. SCFAs are saturated aliphatic organic acids that consist of one to six carbons(73). Examples include formate, acetate, propionate, butyrate and valerate. They are by-products of bacterial fermentation from non-digestible carbohydrates and amino acids and can induce production of AMPs (74) and inhibit growth and fitness of pathogens, both directly and via routes including intracellular acidification (75). Bile acids are cholesterol derivatives with a steroid ring component. The primary bile acids (cholic acid and chenodeoxycholic acids) are synthesized by cholesterol oxidation the liver and are secreted via the bile duct into the intestine and are where they are deconjugated by bile salt hydrolases secreted by commensal bacterial of the gut microbiota into the secondary bile acids, deoxycholic acid and lithocholic acid(76,77). Their impact on maintaining the gut barrier function and colonisation resistance is far reaching and includes regulating lipid absorption, antimicrobial properties, activating host nuclear receptors and cell signalling pathways and intestinal barrier regulation(78).

Indirect mechanisms of colonization resistance include.

- Microbiome-mediated regulation of the integrity of the gut barrier function to present penetration/ translocation of potential pathogens, such as mucins which are glycoproteins which act to protect the gut barrier against inflammation and colitis, (79)
- Modulation of the innate and adaptive immune cells to enhance mucosal immunity is an important role of microbially-secreted metabolites and microbial-associated molecular patterns (MAMPs)(80–82).
- Pattern repetition receptors such as toll-like receptors maintain intestinal homeostasis with their interaction with commensal bacteria(83).

- SCFAs including butyrate have a role in providing an energy source for intestinal epithelial cells as well as influencing T helper cell responses and promoting gut barrier integrity (84).
- In the presence of commensal bacteria, dendritic cells selectively induce immunoglobulin A (IgA) which also has an important immune function in prevention against invasive disease(85).

In addition to this, certain enteric pathogens possess the virulence factors that can trigger intestinal inflammation which disrupt gut barrier function thereby translocating across and resulting in invasive disease(86). An example of this is *Salmonella* spp. which enacts a variety of mechanisms to modify the conditions of the microbiome to create a more aerobic environment in which it can expand. Examples of how it does this is by depleting butyrate-producing *Clostridia* and enacting a neutrophil burst to oxidise thiosulfate to tetrathionate, which acts as a respiratory electron acceptor so the pathogen can to outcompete commensals(86,87). Pathogens such as *C. difficile* are recognized to decrease the level of major intestinal mucin, *muc2*(88).

1.2.3 Commensals recognised to play a role in MDRO colonisation and invasive infection.

Commensal bacteria which reside within the gut microbiota play a crucial role in the mobilisation of colonisation resistance. *Bacteroidetes, Firmicutes, Bifidobacterium sp., Coprococcus, Clostridium, Roseburia, Faecalibacterium* are all recognised to produce SCFAs and *Bifidobacterium, Bacteroides, Clostridium, Lactobacillus, Enterobacter* spp have a role in the modification of bile acids by deconjugation through the secretion of bile salt hydrolases (89). Members of the *Bacteroidetes* produce Type VI secretion system (T6SS), which is a protein translocation complex that attacks bacterial cell walls (90,91). Indigenous *E. coli* compete with pathogenic *E. coli* 0157 for the amino acid proline (which it can exploit to promote growth)(92). *E. coli* Nissle 1917 can compete with *Shigella* and limit its ability to

cause invasive disease within the gut wall(93). *Bifidobacterium longum* has a function in restoring mucin production (94)

Table *1* describes significant bacteria described in the literature that have demonstrated to limit MDRO colonisation or infection.

Table 1: Literature of commensal bacteria involved in limiting (non-*C. diff*) intestinal pathobiont colonisation and infection.

Reference	Commensal	Type of study	Summary
(95)	*Prevotella stercorea*	Human	Increased in liver transplant patients who were never colonised with an MDRO
	Bacteroides		
	Bifidobacterium		
	Faecalibacterium prausnitzii		
	Parabacteroides distasonis		
	Prevotella copri		
(96)	*Bacteroidales*	Human	Associated with absence of MDRO colonisation
(97)	*Akkermansia muciniphila*	Human	Levels significantly greater in subjects who did not acquire an MDRO
	Odoribacter laneus		
(98)	*Bacteroides*	Human	Decreased in critically ill patients colonised with CRE
	Barnesiella		

(99)	*Bifidobacterium bifidum*	Human	Significantly increased post-IMT in “responders”
(100)	*Bacteroides*	Human	Higher proportion in donor stool more likely to be associated with a response
	Barnesiella		
	Butyricimonas		
(101)	*Atopobiaceae*	Human	Higher abundance in nursing home residents never colonised with an MDRO versus colonised
	Dorea		
	Lachnospiraceae ND3007		
(102)	*Bacillales Family XI incertae sedis*	Human	Lower risk of colonization by MDRO, infection, and death
	Prevotella spp		
(103)	*Coprococcus*	Human	Significantly more abundant in the microbiota of individuals not colonised with ESBL-E

	Desulfovibrio		
	Oscillospira		
	Parabacteroides		
(104)	*Prevotella massiliensis*	Human	Higher abundance in healthy adults who were not colonised with an MDRO versus colonised
	Pseudomonadaceae		
(105)	*Bacteroides dorei*	Human	Associated with CPE negative stool samples
	Faecalibacterium prausnitzii		
	Bifidobacterium pseudocatenulatum		
	Bifidobacterium bifidum		
	Colinsiella aerofaciens		
	Eubacterium rectale		

	Streptococcus salivarium		
(106)	*Ruminococcaceae*	Human	Associated with a decreased risk of intestinal Gram-negative domination
(107)	*Blautia producta*	Human	Rectal colonization with VRE was inversely associated with B. producta
(108)	*Lactobacillus spp*	Mice	Patients who did not colonise with an MDRO were more likely to be colonised with this
(109)	*Blautia producta*	Mice	Prevented colonisation and had antimicrobial activity against *Listeria monocytogenes*
	Hungatella hathewayi		
	Thomasclavelia ramosa		
	Thomasclavelia saccharogumia		
(110)	*Blautia producta*	Mice	Cleared VRE colonisation
	Clostridium bolteae		
(111)	*Ruminococcus gnavus E1*	Mice	*Clostridium perfringens* was eliminated from the digestive tract

(112)	*Lactobacillus paracasei CNCM I-3689*	Mice	Significantly decreased VRE burden in the faeces
(113)	*Bacteroides thetaiotaomicron*	Mice	Repressed Shiga toxin 2 mRNA expression (virulence factor in *E. coli* O157:H7)
(114)	*Ruminococcus obeum*	Mice	Restricted *Vibrio cholerae* colonisation
(115)	*Lactobacillus spp*	Mice	Controlled *E. coli* multiplication in small intestine and stomach
(116)	*Blautia producta* BP_{SCSK}	Mice	Inhibited colonisation of VRE in intestine
(117)	*Lactobacillus HT121*	Mice	Administration reduced intestinal VRE burden
	Lactobacillus Y74		
(93)	*E. coli HS*	Mice	Prevented colonisation with *E. coli* O157:H7
	E. coli Nissle 1917		
(118)	*Bifidobacterium animalis subsp. lactis*	Mice	Reduced the faecal number of *Salmonella* in gnotobiotic mice
(119)	*Klebsiella oxytoca*	Mice	Inhibited intestinal Klebsiella pneumoniae colonisation

(120)	*Bacteroides thetaiotaomicron*	Mice	When administered to mice they were less likely be colonised with VRE
(121)	*Barnesiella*	Mice	Reconstitution with *Barnsiella* correlates with VRE elimination
(122)	*Bifidobacterium animalis*	In vitro	Antimicrobial activity against *Staphylococcus aureus, E. coli* and *Pseudomonas aeruginosa*
	Bifidobacterium bifidum		
	Lactobacillus casei		

1.2.4 Impact of antibiotics on the intestinal microbiota

Decreased gut microbiota variability from the action of antibiotics, particularly those active against anaerobes, can decrease colonsization resistance and enable colonisation of pathogenic MDROs (123). The impact of antibiotics depends on the spectrum of activity, dose, duration, pharmacodynamics, and pharmacogenetics of the agent(124). A seven-day course of clindamycin was noted to have resulted in significant disturbances to the intestinal microbiota that persisted over two years(125). Disruption to the microbiota by antibiotics reduces butyrate producing commensals(124). Butyrate serves as the main energy source for colonocytes, the ATP required to generate an osmotic gradient across the colonocyte comes from the oxidation of butyrate to CO_2. Depletion of butyrate can lead to increased oxygenation of the epithelium. This facilitates the expansion of aerobic bacteria such as *Salmonella* (87).

Besides the reduction of commensal bacteria that produce functional metabolites, antibiotics can also interfere with immune defences such as reduction of IL-17, INF-γ and Treg cells post exposure(126). They also act to thin the mucus barrier allowing increased susceptibility to bacterial invasion(127) Intestinal dominance of *Enterococcus* spp. has been noted to have a direct correlation with mortality in intensive care patients (128) and BSIs in haematology patients (129).

1.2.5 Vulnerable populations

Disruption to the intestinal microbiota is recognised to more adversely affect vulnerable populations who are more likely to have had previous exposure to hospitals, had previous antibiotic use and invasive procedures. The following are specific populations adversely affected by MDROs colonisation and risk of invasive infection.

1.2.5.1 Renal transplant patients

Although renal transplantation is the definitive treatment for patients with end-stage renal failure, the treatment course necessitates immunosuppressants and antibiotics which adversely impact the diversity of the gut microbiota (130). Underlying disease processes also

impact on diversity (131). Lee *et al.,* noted that intestinal microbial diversity was significantly altered post kidney transplant (132). Use of proton pump inhibitors, mycophenolate mofetil and a low glomerular filtration rate (a marker of reduced kidney function) were noted to be independent determinants of the lower diversity in transplant patients, who were noted to have a significantly higher abundance of *E. coli* in the intestine and lower abundance of *Bifidobacterium,* a commensal recognised to produce butyrate (133). Abundance of these intestinal pathogens is a risk factor for development of UTI and subsequent related UTIs (134). Recurrent urinary tract infections in renal transplant patients are more likely to be caused by MDROs and are associated with poor graft function of the transplanted kidney and poorer patient outcomes (135,136).

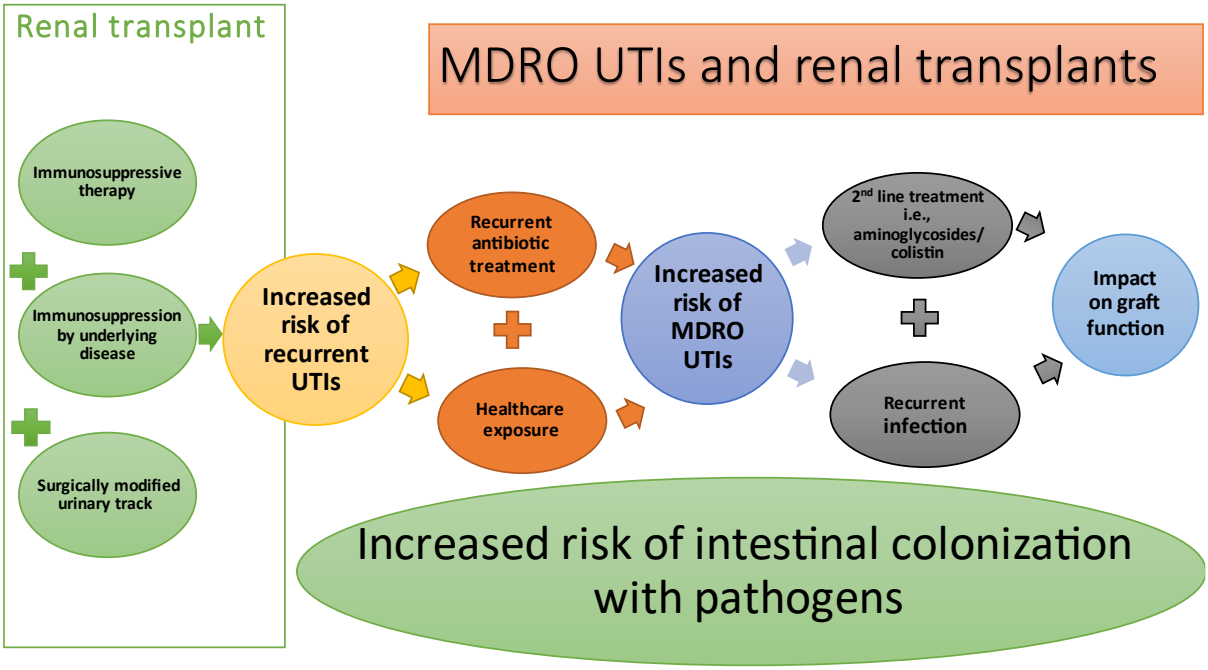

Figure 2: Schematic representation of the impact of recurrent UTIs on renal transplants.

1.2.5.2 Haematology patients

An allogeneic haematopoietic stem cell transplant (allo-HCT) is a well-established potentially curative treatment for many haematological, oncological, metabolic and immunology conditions (137). Conditioning treatments prior to the transplant induce a mucositis and a

neutropenia which increase the risk of translocations and resultant invasive infections arising from pathobionts colonising the intestine (138). Prophylactic antibiotic regimens prior to the transplant are recognised to lower intestinal microbial diversity (139) and intestinal domination of VRE is seen in patients treated with vancomycin, which precludes VRE BSIs post allo-HCT (129). A decrease in diversity is seen in patients post allo-HCT (140), and patients with higher diversity are associated with lower mortality (141). Biliński *et al* demonstrated that colonisation with MDROs resulted in lower survival rates post allo-HCT and increased with incidence of systemic infection (142). 30-day mortality rates for patients with haematological malignancies or post-allo-HCT range from 50% to 72.7% (24).

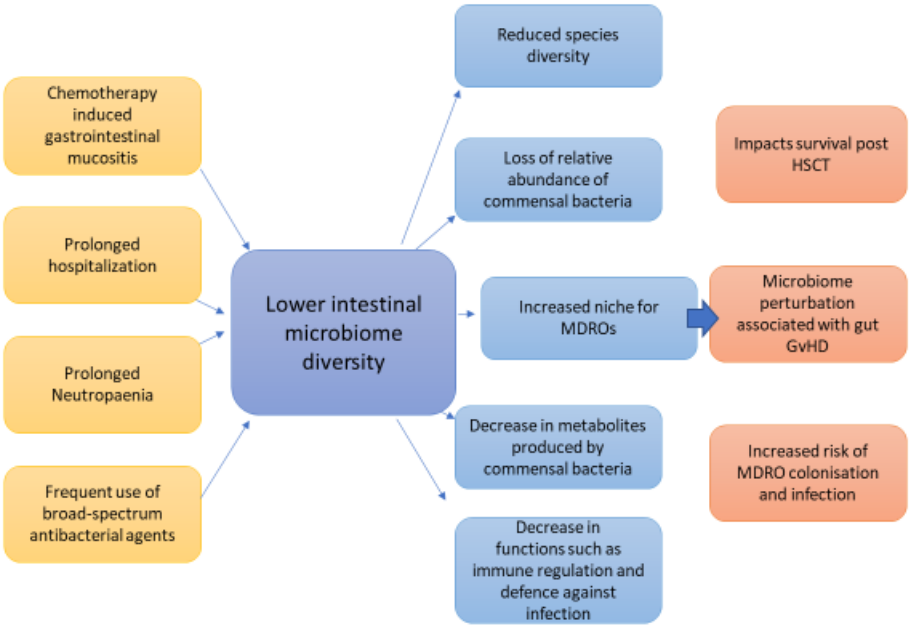

Figure 3 Impact on the intestinal microbiota in haematological disease.

1.2.6 Methods of evaluating gut microbiome functionality

To explore gut microbiome signatures, the mainstay of studies has explored looking at the taxonomic and functional profiling of the microbiome in collected faecal samples, the main

advantage being that it is non-invasive and can be collected at home. Until recently investigation of the gut microbiota was through culture-based techniques, however only a minority of commensal bacteria can be isolated by standard culture methods, but this has recently improved (143). There is also the added disadvantage of being labour-intensive and time-consuming considering the need for selective media and the correct, specific culture environments. The use of next-generation microbial sequencing technologies, developed over the last two decade as a high-throughput, comprehensive method has revolutionised the means of defining the composition and functionality of the intestinal microbiota.

1.2.6.1 Metataxonomics

Metataxonomics uses 16S rRNA gene amplification and sequencing of certain variable regions of the 16S rRNA gene present bacterial genomes to make community wide taxonomic classifications and demonstrate phylogenetic relationships between sequences(144,145). Alpha diversity is a metric used to describe the mean diversity of species in different sites or habitats within a local scale. Some commonly alpha diversity indices used include.

- Chao1: an estimator based on abundance of individual samples belonging to a certain class (richness).
- Shannon: an estimator for both species' richness and evenness. (Total number of bacterial taxa observed, $S_{obs)}$
- Inverse Simpson: a measure of diversity which considers the number of species present, as well as the relative abundance of each species.
- Faith PD: measurement of phylogenetic diversity, in particular the sum of branch lengths between the observed species on a phylogenetic tree.
- Rao's quadratic entropy: the proportion of the abundance of species present in a community and some measure of dissimilarity among them.

Beta diversity assesses inter-sample microbial community differences. To quantify the dissimilarities between samples, typically Bray-Curtis dissimilarity for compositional data, and UniFrac distances are used(146).

One issue with metataxonomics is that two organisms with the same 16S rRNA gene sequence might be classified as the same strain e.g., *E. coli* O157 and *E. coli* Nissle 1917, and provides limited information on function.

1.2.6.2 Metagenomics

Metagenomics uses shotgun sequencing of total DNA to assign taxonomy and microbial diversity classification allows us to catalogue the genetic makeup of the bacteria and can achieve strain level discrimination(147). It is also able to shed light on the viral ecology of the gut unlike metataxonomics.

1.2.6.3 Metabolomics and Metabonomics

Metabolomics is the quantitative descriptions of the low-molecular weight components (<1kDa) of endogenous metabolites (148). Metabonomics refers to the generation of a complex metabolite profiles when one or more tissue has contributed to the metabolite pool. This is conventionally performed using nuclear magnetic resonance (NMR) spectroscopy (for global metabolic profiling) and liquid chromatography-mass spectrometry to target a particular metabolite or a set of metabolites (i.e., liquid chromatography for bile acid profiling and gas chromatography for volatile compounds such as SCFAs)(149).

Other methods utilised are metatransciptomics where complementary DNA (cDNA) is synthesised and sequenced from transcribed microbial genes (mRNA), although this has less utility in intestinal tissue as there is much more host than microbial RNA(145) and most of the bacterial RNA is rRNA and not mRNA (145). Metaproteomics uses liquid-chromatography coupled to mass spectrometry for peptide identification to characterise the entire protein complement of a sample at a given point in time(149).

Utilising a “multi-omics” approach where metagenomics, metataxonomics and metabolomics are combined can give an oversight of the metabolic activity of microbial genomes including expressed proteins and produced metabolites and its impact on the host. (150)

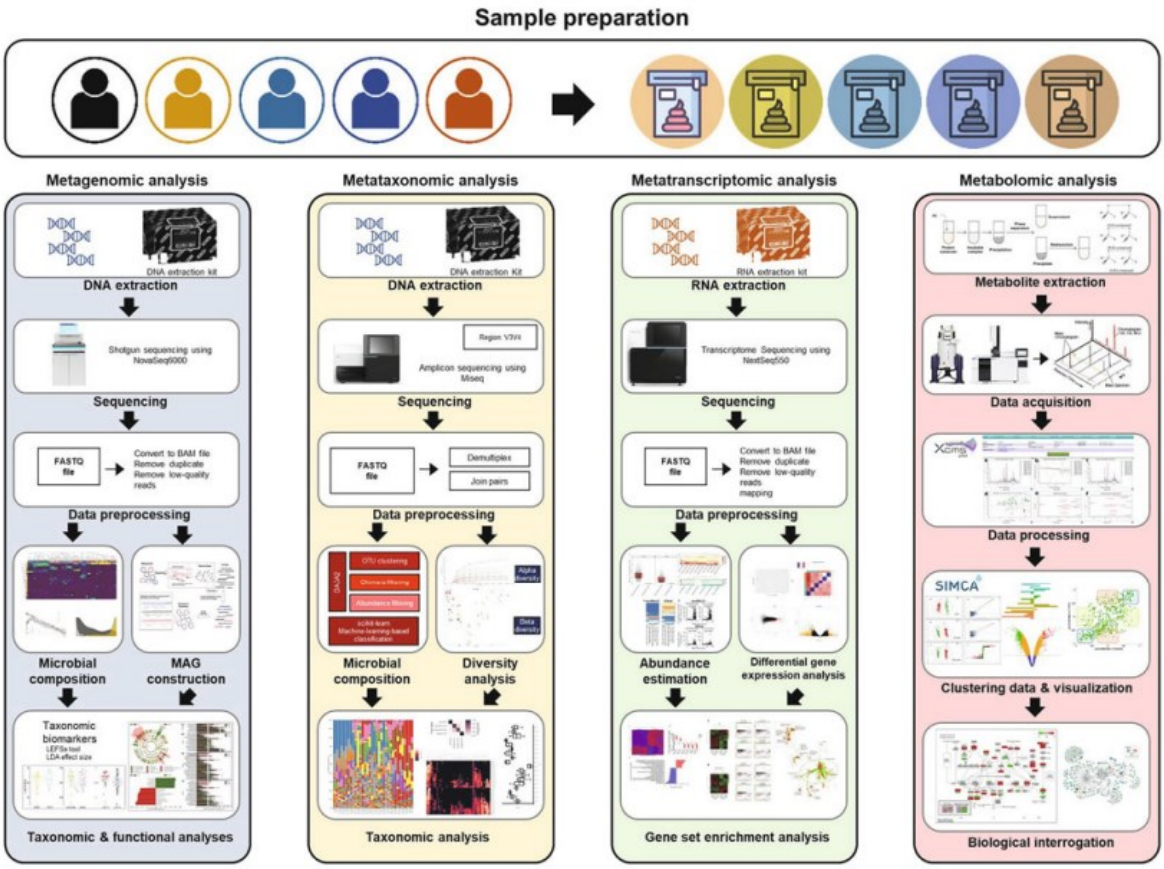

Figure 4: A schematic model for omics approaches to study the gut microbiome. Image reproduced with permission of the rights holder, Springer(144).

1.2.6.4 Immunological assessment of gut barrier function and permeability

The gastro-intestinal mucosa is a semi-permeable barrier with multiple properties. The term gut barrier function refers both to the permeability of the gut which allows solute and fluid exchange between the lumen and tissues (such as absorption of nutrients) and the ability of the mucosa and extracellular barrier components, e.g., mucus, to prevent this exchange(151). Gut barrier function is recognised to be disrupted by antibiotics and immunocompromised patients, and a disrupted gut barrier increases in gut permeability allowing the translocation of MDROs into the bloodstream (152). Intestinal fatty acid-binding protein-2 (FABP2) may be used as an estimation of gut permeability. Fatty acid-binding proteins are small cytosolic proteins which transport fatty acids and include several

isotypes which are expressed in different tissues, such as heart, liver, intestine, muscle, and adipocyte. Intestinal FABP (i-FABP or FABP-2) is uniquely located in mature small-intestinal enterocytes. When damage to the intestinal mucosa occur, the intestinal villi can facilitate its leakage into the circulation and may be detected in plasma or urine because of intestinal ischaemia(153). A pro-inflammatory gut environment may also lead to an increased intestinal permeability and susceptibility to invasive pathogens (154). Dynamic measurements of cytokines such as IL-1, TNF-α and IFN-γ in the stool and serum which are increased in pro-inflammatory states may reflect the relative permeability of the gut.

1.3 Intestinal Microbiota Transplantation (IMT)

1.3.1 Overview of IMT

Intestinal Microbiota Transplantation (IMT) (also known as faecal microbiota transplantation or IMT) is the transfer of screened healthy donor stool to a recipient's gastrointestinal tract. The aim is to restore an affected intestinal microbiome to its premorbid microbiome composition and function, as well as aiding recovery of host-microbiome interactions.

1.3.2 *Clostridioides difficile* studies

Clostridioides difficile is a spore forming anaerobic Gram-positive bacillus. It is recognised as a leading cause of antibiotic associated diarrhoea (155), and recurrent *C. difficile* infection (rCDI) carries a significantly higher mortality than a single occurrence due to progressive loss of microbial diversity(156,157). *C. difficile* spreads via the faeco-oral route, most likely as spores, and causes disease by producing two protein exotoxins (toxin A and toxin B), which are cytotoxic to colonic epithelial cells (158). Disease occurs when *C. difficile* spores germinate into their active vegetative state, and this is accelerated by antibiotic use creating a metabolic environment that encourages this transition (159). Two early randomised trials demonstrated that there was a significantly improved response of IMT versus vancomycin therapy (160,161), and further studies demonstrated a benefit of IMT over fidaxomicin (162). The first trial in fact was stopped early after an interim analysis revealed that 81% of patients in the IMT arm recovered compared with 31% in the arm that received vancomycin alone (160). Due to the

success of this application, many studies have investigated the mechanisms of IMT related to rCDI and although there are likely to be several contributory methods, including the restoration of short-chain fatty acids such as valerate directly preventing the growth of *C. difficile* (75) and bile salt hydrolase restitution which deconjugates primary bile acids and inhibits vegetative growth of *C. difficile* (163). Similar to studies looking at the risk of Gram-negative infection, colonisation with the taxa *Bacteroidetes*, *Lachnospiraceae*, and *Ruminococcaceae* were all found to be protective in patients post allo-HCT against developing *C. difficile infections* (164). Importantly a consistent finding in both CDI and non-CDI IMT studies is that high donor microbiota diversity as well as enrichment with commensals appear to be associated with IMT success (165).

1.3.3 Administration

Most of the evidence we currently have for IMT administration comes from trials looking at treatment for recurrent *C. difficile.* These have been formulated into guidelines which serves at a reference point for the use of IMT for other conditions (166).

1.3.3.1 Donor stool and screening

Unrelated donor which was frozen was found to be as efficacious as related fresh stool in early *C. difficile* studies(167). The advantage of use of an unrelated donor was that stool could be more easily stored in advance and the advantage of this is that it can prevent shortages, and one donor can supply several recipients, avoiding the need for repeated screening. (168,169). Frozen was found to have similar efficacy to fresh stool for the treatment of rCDI (170) and contained similarly viable microbial communities (171). Donors must be screened through taking a clinical history, and sampling of blood and stool samples to avoid the risk of transmitting diseases and adversely impacting the recipient's microbiota. Age and BMI of the patient is taking into consideration because of the compositional change seen in the intestinal microbiota of older and overweight individuals (172,173).

Screening of donor blood and stool of transmissible organisms varies according to the risk assessment of the immunocompetence of the patient and local prevalence. Table 2:

Recommended IMT donor screening from Joint British Society of Gastroenterology and Healthcare Infection Society guidelines details the recommended guidelines for screening donor for IMT(174).

Table 2: Recommended IMT donor screening from Joint British Society of Gastroenterology and Healthcare Infection Society guidelines

Recommended donor history/questionnaire	**Recommended blood screening for stool donors**	**Recommended stool screening for stool donors**
Receipt of antimicrobials within the past 3 months.	Hepatitis A IgM	*Clostridioides difficile* PCR
Known prior exposure to HIV and/or viral hepatitis and known previous or latent tuberculosis.	Hepatitis B (HBsAg and HBcAb)	*Campylobacter, Salmonella,* and *Shigella* by standard stool culture and/ or PCR
Risk factors for blood borne viruses, including high risk sexual behaviours, use of illicit drugs, any tattoo/ body piercing/ needlestick injury/ blood transfusion/ acupuncture, all within the previous 6 months.	Hepatitis C antibody	Shiga toxin-producing *Escherichia coli* by PCR
Receipt of a live attenuated virus within the past 6 months.	Hepatitis E IgM	Multidrug-resistant bacteria, at least CPE and ESBL
Underlying GI conditions/symptoms (e.g., history of IBD, irritable bowel syndrome (IBS), chronic diarrhoea, chronic constipation, coeliac disease, bowel resection or bariatric surgery), also including acute diarrhoea/GI symptoms within the past 2 weeks	HIV-1 and HIV-2 antibodies	Stool ova, cysts and parasite analysis, including for *Microsporidia*
Family history of any significant GI conditions (e.g., family history of IBD or colorectal cancer).	HTLV-1 and HTLV-2 antibodies	Faecal antigen for *Cryptosporidium* and *Giardia*
History of atopy (e.g., asthma, eosinophilic disorders).	*Treponema pallidum* antibodies	Acid fast stain for *Cyclospora* and *Isospora*
Any systemic autoimmune conditions.	Epstein–Barr virus IgM and IgG	*Helicobacter pylori* faecal antigen

Any metabolic conditions, including diabetes and obesity.	Cytomegalovirus IgM and IgG	Norovirus, rotavirus PCR
Any neurological or psychiatric conditions or known risk of prion disease.	*Strongyloides stercoralis* IgG	
History of chronic pain syndromes, including chronic fatigue syndrome and fibromyalgia.	*Entamoeba histolytica* serology	
History of any malignancy.	Full blood count with differential	
Taking antimicrobials, proton pump inhibitors, immunosuppression, chemotherapy in the last three month	Creatinine and electrolytes	
History of receiving growth hormone, insulin from cows or clotting factor concentrates.	Liver enzymes	
History of receiving an experimental medicine or vaccine within the past 6 months.	C-reactive protein	
History of travel to tropical countries within the past 6 months.		

1.3.3.2 Route of administration of IMT

Traditional routes of IMT administration were either via upper GI (i.e., nasoduodenal, nasogastric tube, or gastroscopy) or lower GI (i.e., colonoscopy, flexible sigmoidoscopy, or enema). Colonoscopy was shown to reduce the risk of rCDI by 90% compared to 80% with upper GI administration (175). Enemas are less preferred due to the need for repeated administration to reach the equivalent success rate to other methods (176), and is less frequently used for the use of IMT in indications other than rCDI due to the need to restore the entire intestinal tract versus the local effects of *C. diff within* the colon(177).

A more recent, attractive route is the administration of IMT via capsules. These may be capsulised frozen slurry, lyophilised or using a dehydrating matrix. The capsule format is clearly an attractive option as it negates the need for in-house procedures and the risks associated with endoscopy. Studies have shown non inferiority relative to colonoscopy (178,179).

1.3.3.3 Safety issues and tolerability

The main direct adverse effects from IMT are generally mild and self-limiting, and include flatulence, abdominal pain, increased stool frequency, vomiting and fever. More serious adverse effects are related to the endoscopic administration of the product such as aspiration or intestinal perforation (180).

There are however ongoing concerns about the risk of infection transmission via IMT, particularly when inadequate screening has occurred. In 2019 in the United States (U.S.), two patients suffered from ESBL–producing *Escherichia coli* bacteraemia transmitted from IMT donor stool, resulting in one fatality (181). In 2020 in the U.S., transmission of Shiga toxin–producing *Escherichia coli* (STEC) via IMT from a single donor to seven patients was reported, resulting in six serious adverse events and one non-serious adverse event (182).

Case reports have described both transmission of cytomegalovirus (CMV) in IMT for patients with ulcerative colitis and CMV reactivation post-IMT in solid organ transplant (SOT) patients (183,184), and concern exists regarding the theoretical oncogenic risk of transmission of Epstein-Barr Virus (EBV) in immunocompromised patient (185).

1.3.3.4 Terminology

Previously the term faecal microbiota transplantation (FMT) was more commonly used. The term “Intestinal Microbiota Transplantation” (IMT) is now felt to be more correct as the aim is to confer to confer an intestinal bacterial community structure more similar to that of the healthy donor rather than the faeces, viewed as a waste product of the human body(186). Patient uptake and acceptability of the procedure may be improved by changing the name from “faecal” to “intestinal” (187). Also it is biologically-accurate to describe the transplanted material as arising from the intestinal microbiota as the only component of the transplant recognised to engraft are the bacteria that are either mucosally-derived bacteria not attached to mucin, or sloughed off mucin to which mucosally-associated bacteria are attached and the origin of the bacteria may come from either the small or large intestine which gives “intestinal microbiota” versus “faecal microbiota” more accuracy related relating to the actual material transferred (188).

1.3.4 Use of IMT in MDRO colonized or infected patients.

The success of the improvement in rCDI with IMT has led research to expand to other intestinal pathobionts. The aim of IMT is to reverse the MDRO domination and arrest gut translocation to prevent invasive infection. Although mechanisms of colonisation resistance and gut barrier function have been explored the utility of restoring the gut microbiota in patients who are colonised with organisms known to or at risk of invasive disease is not fully understood.

1.3.4.1 IMT as a modality to decolonise the intestine from MDROs.

One modality that has been explored has been looking at eradication of carriage of MDROs, with one of the perceived benefits being that there may be a reduced risk of nosocomial spread, and potential for invasive infection.

Table 3: Literature to date on the use of IMT for MDRO decolonisation details the literature to date. As previously discussed, administration to mice of IMT containing the commensal bacteria *Barnesiella* was associated with intestinal clearance of VRE(121). Studies of IMT in the treatment of rCDI in humans have demonstrated a reduction in diversity and number of ARGs post-IMT(189–192) and a similar finding has also been described in patients being treated with IMT for liver cirrhosis(193).

Results from clinical studies looking at intestinal decolonization of MDROs following IMT have been highly variable, in part due to the heterogeneity of the study design, patient cohorts, and IMT administration protocols. High decolonisation rates were reported by Biliński *et al.,* of 75% in 20 patients(100) and Saïdani also reported similarly high rates of 80% in 10 patients at 14 days (with a decolonization rate of 10% in a comparator arm)(194). However, some studies reported rates similar to spontaneous decolonisation rates such as Davido *et al.,* who reported intestinal decolonisation in eight patients as of 37.5% after three months(195). The only reported randomized control trial (RCT) to date in this area demonstrated a non-significant decrease in rates of ESBL-E and CPE carriage in IMT-treated patients compared to

the control group; partly attributed to the low number of recruited patients recruited and early drop out by participants due to diarrhoea(196).

Table 3: Literature to date on the use of IMT for MDRO decolonisation

Ref	Author	Year	No. of patients	Delivery method	Indication for IMT	Outcome post IMT	Follow up period
(197)	**Aira**	2020	1	Colonoscopy	rCDI with recurrent UTIs (3 episodes in one year)	Reduction of intestinal *Enterobacteriaceae* from 74% to 0.07%	n/a
(198)	**Baron**	2019	1	nasogastric tube	CPE colonization with osteitis infection	Stool negative for CPE	12 months
(99)	**Bar-Yoseph**	2021	15	oral capsules	Colonization with MDRO	Stool negative for CPE 10/24 (41.7%) in control versus 9/15 (60%) in IMT group 8/12 (66.7%) negative in IMT group	1 month
(199)	**Battipaglia**	2019	10	enema or nasogastric tube	Allo-HCT colonized with CPE/VRE or ESBL	Stool negative for MDRO in 7/10 (70%) patients	4-40 months
(200)	**Biliński**	2016	1	nasoduodenal tube	Multiple myeloma plus autologous HCT colonized with CPE and ESBL-E	Stool culture negative for MDRO PCR positive for NDM	26 days

(100)	**Biliński**	2017	20	nasoduodenal tube	1) Haematological malignancy 2) Lung cancer 3) Renal transplant all colonized with CPE	Rectal swab negative for MDRO in 13/14 (93%)	6 months
(201)	**Crum-Cianflone**	2015	1	Colonoscopy	Sacral wound plus spinal epidural abscess with rCDI colonized with multiple MDROs	Reduction from 24 MDROs pre-IMT to 11 post-IMT detected on culture	15 weeks
(202)	**Davido**	2017	8	nasogastric tube	Colonization with MDRO only	Rectal swab negative for CRE 3/8 (37.5%)	3 months
(203)	**Davido**	2019	8	nasoduodenal tube	Chronic renal failure colonized with VRE	Rectal swab negative for CRE 7/8 (87.5%)	3 months
(204)	**Dias**	2018	2	n/a	rCDI colonized with CPE	Rectal swab negative for CPE in 2/2 (100%) patients	3 months
(205)	**Dinh**	2018	17	nasogastric tube	Colonisation with MDRO only	Rectal swab negative for CRE 4/8 (50%) Rectal swab negative for VRE 7/8 (87.5%)	3 months
(206)	**Eysenbach**	2016	15	n/a	rCDI colonised with VRE	Negative stool for VRE in 4/4 (100%) in IMT group versus 6/7 (86%) in control group	6 weeks
(207)	**Freedman**	2014	1	nasoduodenal tube	Haemophagocytic lymphohistiocytosis with CPE bacteraemia and osteomyelitis	Stool cultures negative for CPE	8 months

(208)	**García-Fernández**	2016	1	Colonoscopy	rCDI colonized with CPE	Stool cultures negative for CPE	6 months
(209)	**Grosen**	2019	1	nasojejunal tube	Renal transplant with recurrent ESBL UTIs (7 hospital admissions in 5 months)	Stool negative for ESBL *Klebsiella pneumoniae*	8 months
(196)	**Huttner**	2019	22	nasogastric tube	1) Colonization with CPE 2) Invasive ESBL infection	Stool negative for MDROs in 9/22 (41%) in treatment group versus 5/17 (29%) in control	48 days
(210)	**Innes**	2017	1	nasogastric tube	Acute lymphoblastic leukaemia undergoing allo-HCT colonized with CPE	Stool negative for CPE	12 months
(211)	**Jang**	2015	1	Enema and nasoduodenal tube	rCDI with spastic tetraplegia colonized with VRE	Stool positive for VRE	3 months
(189)	**Jouhten**	2016	n/a	Colonoscopy	rCDI	Reduction in diversity of antibiotic resistant genes, except vanB	2 months
(212)	**Lagier**	2015	1	nasogastric tube	Nursing home resident colonized with CPE	Stool negative for CPE	14 days
(213)	**Lahtinen**	2017	4	Colonoscopy	Recurrent ESBL *E. coli* UTIs	Stool cultures negative for ESBL-E	6 weeks

(190)	**Leung**	2018	8	Enema	rCDI	Reduction in 95 antimicrobial resistance genes Increase in 37 resistance genes	90 days
(214)	**Merli**	2020	5	nasogastric tube	Pre-allo-HCT 3/5 patients - carbapenem resistant Gram-negative bacteraemia	Stool negative for CPE	113 days
(191)	**Millan**	2016	20	Colonoscopy	rCDI	Reduced number and diversity of antibiotic resistant genes	1 year
(215)	**Ponte**	2017	1	nasoduodenal tube	rCDI colonized with CRE	3 stool samples negative for CRE	100 days
(194)	**Saïdani**	2019	10	nasogastric tube	rCDI colonized with CRE	Rectal swab negative for CPE/A in 8/10 (80%) IMT patients versus 2/10 (20%) in control group	14 days
(216)	**Singh**	2014	1	nasoduodenal tube	End-stage renal failure with recurrent ESBL *E. coli* transplant pyelonephritis	Perineal and throat swab positive for ESBL at 1 week, Negative at 2,4,12 months	12 months

(217)	**Singh**	2018	15	nasoduodenal tube	1) Renal transplant 2) Recurrent ESBL UTIs	Stool negative for ESBL in 3/15 (20%) after the first transplant 6/15 (40%) negative after the second transplant	4 weeks
(218)	**Sohn**	2016	3	Enema	rCDI colonized with VRE	No eradication of VRE in 3/3	21 weeks
(219)	**Stalenhoef**	2017	1	nasoduodenal tube	Peritoneal dialysis with recurrent Pseudomonas UTIs	5 negative stool culture for *Pseudomonas aeruginosa* Positive stool for ESBL *E. coli*	3 months
(220)	**Stripling**	2015	1	nasogastric tube	Renal and heart transplant with rCDI colonised with VRE	Decrease in abundance in stool of *Enterococcus* from 84% to 0.2% (7 weeks)	7 weeks
(221)	**Wei**	2015	5	nasojejunal tube	MRSA enteritis post colorectal surgery	Stool negative for MRSA	3 months

1.3.4.2 Prevention of invasive infection

Early studies looking at rCDI outcomes post IMT noted a reduction in BSIs(222) and this has also been noted in IMT studies of MDRO colonised patients where patients were noted to have had a reduction in MDRO and non-MDRO BSIs(99,199). This has also been noted in studies looking at the impact of IMT on UTIs.

Table 4: Literature review of the use of IMT to prevent clinical infection in MDRO colonised patients.

Reference	Author	Year	Outcome post FMT	Follow up period
Prevention of Clinical Infection in MDRO colonized patients				
(99)	Bar-Yoseph	2021	Death: 8/24 (33%) in control versus 0/15 in FMT group Clinical CPE infection: 9/24 (37.5%) in control versus 0/15 in FMT group	6 months
(199)	Battipaglia	2019	ESBL *E. coli bacteraemia* in 1 patient No MDR bacteraemia in 9/10 (90%) patients	90 days
(200)	Biliński	2016	No subsequent infections	26 days
(201)	Crum-Cianflone	2015	Reduction from five to one infective episode	15 weeks
(207)	Freedman	2014	No subsequent infections	1.5 years
(214)	Merli	2020	2/5 (40%) carbapenem resistant Gram-negative bacteraemia	113 days
(220)	Stripling	2015	No further episodes of VRE sepsis	7 weeks
(223)	Su	2021	No CPE bacteraemia	12 months

Table 5: Literature review of the use of IMT to prevent urinary tract infections in MDRO colonised patients.

Reference	Author	Year	Outcome post FMT	Follow up period
Recurrent Urinary Tract Infections (UTIs)				
(197)	Aira	2020	No further UTIs	12 months
(209)	Grosen	2019	One further ESBL UTI 6 days post FMT	12 months
(213)	Lahtinen	2017	1 episode of cystitis with fully sensitive organism	6 weeks
(216)	Singh	2014	No clinical infection	3 months

(219)	Stalenhoef	2017	No recurrent Pseudomonas infection One *E. coli UTI*	18 months
(224)	Biehl	2018	No further UTIs	9 months
(225)	Hocquart	2019	No further UTIs	8 months
(226)	Ramos-Martínez	2020	No further UTIs	10 months
(227)	Steed	2020	Reduction in number of infections Improved resistance profile of positive isolates	one year

(228)	Tariq	2017	0-4 UTIs No change in control group	one year
(229)	Wang	2018	No further UTIs	25 months

The mechanism of IMT aims to aid the recovery of host-microbiome interactions. It is likely that the benefits seen are due to the consortium of commensal microbes and the metabolites produced and their function in restoring host immunity, gut barrier function, improving colonisation resistance and decreasing pathobiont dominance.

1.4 Hypothesis and Objectives

1.4.1 Hypothesis

Invasive MDRO infection from intestinal pathogens carries significant morbidity and mortality, and it is recognised that disruption to the microbiota composition increases the likelihood of progression to invasive disease. Restoration of the microbiota by IMT may stop the progression to invasive disease. To date studies have been of small numbers and there has yet to be a study to take a multi-faceted approach to address this, by looking at the clinical outcomes as well as gene and metabolite expression pre- and post-IMT.

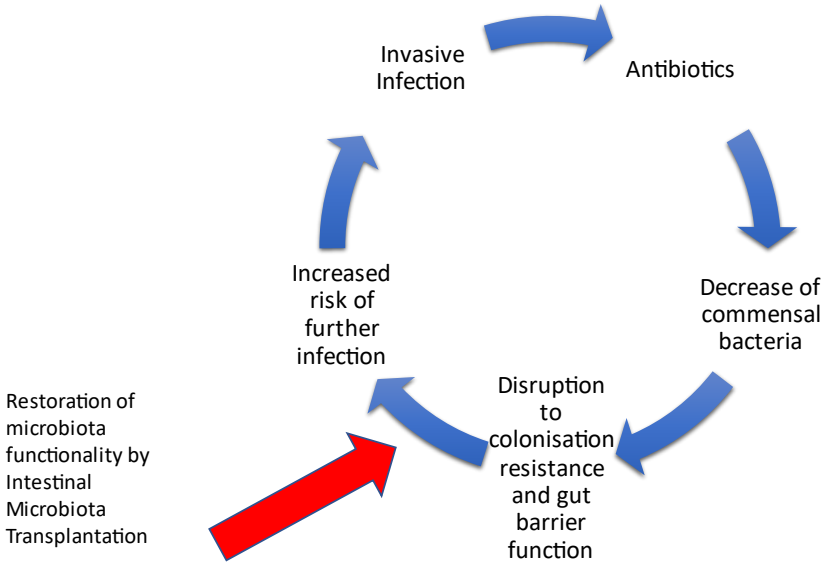

Figure 5: Aim of IMT to stop progression of intestinal pathobionts to invasive disease.

The aims of the project were to evaluate the relationship between colonisation of the intestinal microbiota with MDROs and the progression to invasive diseases and to

1.4.2 Objectives

- Evaluate the prevalence and impact of MDRO colonisation in a healthcare facility.

- Create a programme for administering IMT to appropriate patients colonised with MDROs.
- Assess the clinical impact of IMT on MDRO colonised patients and efficacy.
- To accurately map the mucosa-associated antimicrobial resistance genes in MDRO-colonised patients pre- and post-IMT
- To identify microbiome and or metabolome-based markers which may play a role in restoration of colonisation resistance and gut barrier in MDRO colonised patients.

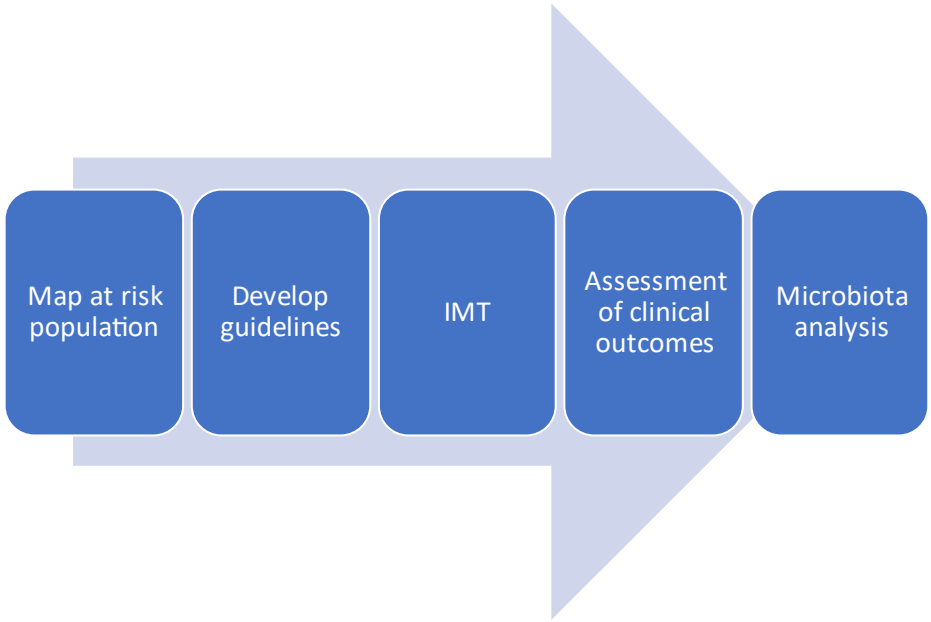

Figure 6: Flow chart of aims of the study to explore outcomes and mechanisms of IMT on MDRO colonised patients.

Chapter 2: Epidemiology of MDRO colonised and infected patients

2.1 Introduction

To first understand the impact that restoration of the intestinal microbiota would have on MDRO colonised patients, we sought to map out which organisms were most prevalent and which populations of patients who were most likely to benefit from this intervention. To do this we interrogated local data to identify the most common MDROs colonising the intestine to result in invasive disease, and secondly to identify and describe the cohorts of patients who are most at risk of developing invasive MDRO disease and adverse outcomes.

2.2 Aims

To map out the scale and impact of MDRO colonisation and infection within a hospital population and identify target populations who could benefit from IMT.

2.3 Methods

2.3.1 Clinical setting

This was a retrospective case-control study performed at Imperial College Healthcare NHS Trust. The study population were inpatients admitted between September 2013 – April 2017. Imperial College Healthcare NHS Trust (ICHNT) covers approximately 1500 patient beds across five hospital sites in West London with a centralised microbiology laboratory. Since 2015 ICHNT routinely performs rectal screening to identify rectal carriage of carbapenemase-producing genes in inpatients.

2.3.2 Microbiology sampling and antimicrobial susceptibility testing

Microbiology samples were processed at the clinical laboratory at Imperial College Healthcare NHS Trust. Screening and clinical isolates were cultured on either chromogenic media or MacConkey Agar with an ertapenem disc. Species identification was performed using Biotyper matrix-assisted laser desorption/ionization–time of flight mass spectrometry (Bruker Daltonics, Germany) according to the manufacturer's protocol. Testing of antimicrobial susceptibility was performed in accordance with European Committee on Antimicrobial

Susceptibility Testing guidelines (230). OXA-48, KPC, NDM, VIM, and IMP carbapenemase genes were identified by PCR (Xpert® Carba-R, Cepheid Inc, USA). Urine samples with more than 100 white cell count on microscopy were cultured on chromogenic media agar. Plates were incubated at 37°C and read at 16 hours, following which testing of antimicrobial susceptibility was performed in accordance with European Committee on Antimicrobial Susceptibility Testing guideline (230).

2.3.3 Definitions

ESBL Enterobacteriaceae resistant to cefalexin AND ceftazadime/ceftriaxone.

Recurrent Urinary Tract Infection (rUTI): 1) > three in a year (>two weeks apart) or 2) > two in six months (>one week apart) with the same organism/sensitivity profile.

Length of Stay: Number of days that the patient remained in hospital.

2.3.4 Analysis

2.3.4.1 Prevalence of CPE colonised patients in hospital inpatients

Patient electronic health records between 2008 and 2018 were reviewed for all patients who had clinical samples that were positive for CPE to identify baseline risk factors and identify at risk groups.

2.3.4.2 Impact of CPE bloodstream infections

All patients admitted between September 2013 – April 2017 with CPE BSI were matched with controls (Gram negative bacteraemia with no ESBL/AMPC/CPE mechanism identified) and the following characteristics were analysed.

- Demographics such as age, sex, underlying condition etc
- 30-day mortality
- Length of admission post detection of bacteraemia – measured in number of days.
- Renal function at day zero and day fourteen

- Patients who required renal replacement therapy such as dialysis were excluded from this analysis.

2.3.4.3 Clinical outcomes of CPE positive patients undergoing hematopoietic cell transplant.

The following parameters were assessed for Haematology patients who underwent an autologous or allograft hematopoietic cell transplant (HCT) and were colonised with CPE on rectal screening between September 2015 and December 2017.

- Length of stay (LoS) post HCT, including readmissions for sepsis
- Number of days of antibiotics therapy following detection of bacteraemia.

Controls were chosen were patients with a negative CPE screen on rectal screening but were matched for time and type of HCT in 3 controls to 1 case ratio.

2.3.4.4 Prevalence of MDRO urinary tract infections

All urine specimens which were culture positive for *Enterobacteriaceae* between June 2015 and January 2018 were collated. The dataset was analysed to identify recurrent UTIs and MDR isolates were matched with antibiotic sensitive culture positive urinary isolates in a 4:1 ratio. The patient electronic health record was interrogated to evaluate associations, origin of patient and resistance pattern.

2.3.4.5 Description of urology patients with MDRO positive urinary isolates

Retrospective analysis of culture positive urine isolates was obtained from 2015 to 2018 at a tertiary level service. Clinical profiling of patients under the urology service with recurrent ESBL producing isolates was performed.

2.4 Results

2.4.1.1 Prevalence of CPE colonised patients in hospital inpatients

Between 2008 and 2018, 1050 isolates from 792 individual patients were isolated as positive for CPE on PCR. 82% of these isolates were from rectal screening, with urine, wound swabs and bloodstream infections being the most common following isolates.

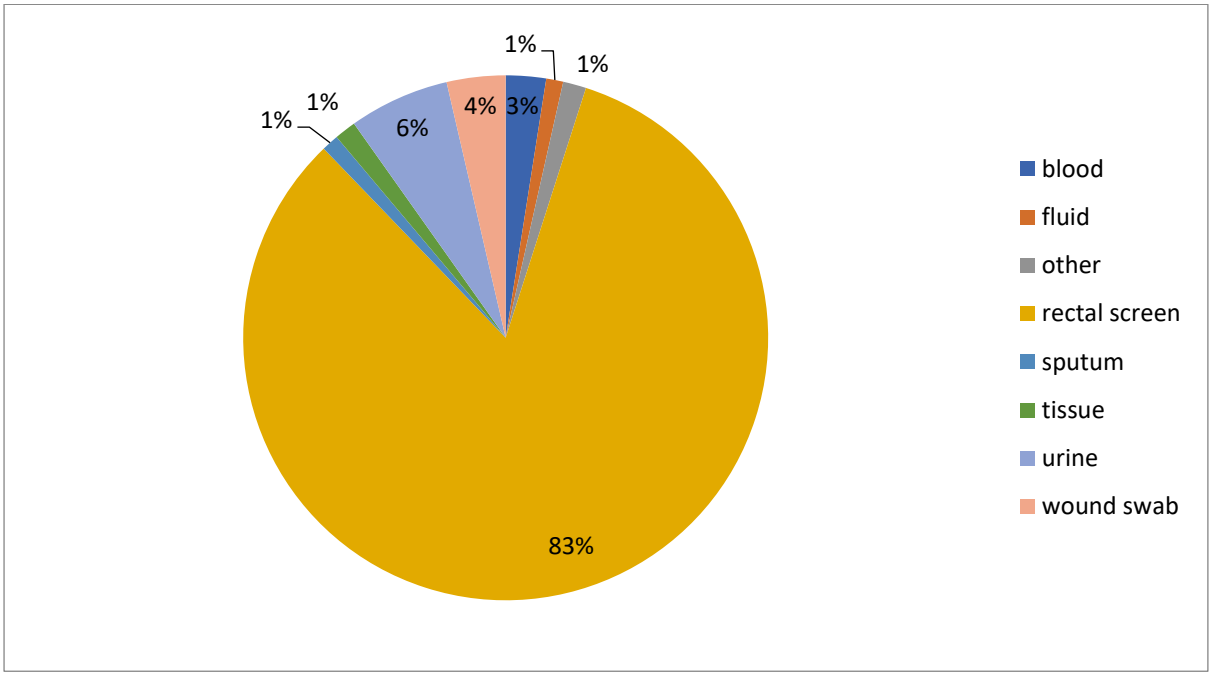

Figure 7: Pie chart displaying origin of CPE positive isolates identified on rectal screening of inpatients at Imperial College Healthcare NHS Trust between 2008 and 2018

The highest rates of CPE colonised or infected patients were under the renal (17% of all CPE positive patients, 17% of all CPE BSIs and 28% of all positive urine culture results), haematology (8% of colonised patients but 27% of CPE BSIs) and vascular (6.8% of CPE colonized patients, 7.7% of all CPE BSIs and 50% of deep tissue samples) services. 23% of vascular patients who were colonised with CPE were also under joint care with the renal services. The OXA-48 gene was the most prevalent CPE gene and was found on 39.8% of isolates.

2.4.1.2 Impact of CPE bloodstream infections

26 patients were identified between September 2013 to April 2017 with a CPE BSI. This represented 3.2% of all patients who were positive for CPE on rectal screening. Mortality (P=0.0194), length of hospital admission (P=0.0442) and deterioration in renal function (in non-dialysis patients) (P=0.0045) were all worse in the CPE bacteraemia group compared to control (Gram negative bacteraemia – no ESBL/AMPC/CPE mechanism identified).

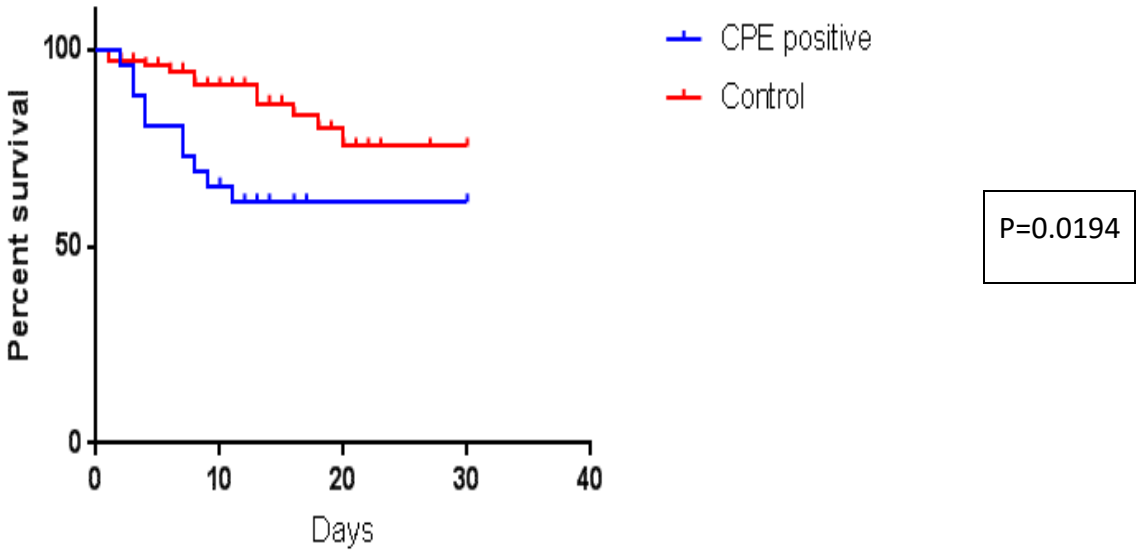

Figure 8: Kaplan-Meier 30-day mortality of patients with a CPE bacteraemia compared with those with a blood stream infection caused by a Gram-negative bacteraemia with no ESBL/AMPC/CPE mechanism identified.

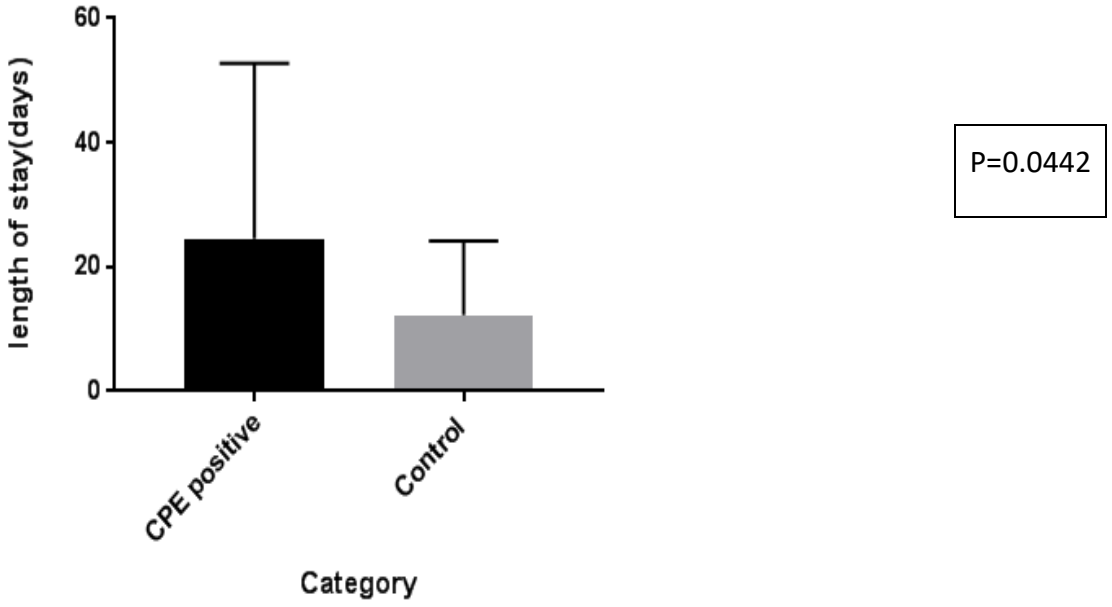

Figure 9: Comparison of length of hospital admission (days) post detection of bacteraemia between those with a CPE bacteraemia and those with a bacteraemia caused by a Gram-negative organism with no ESBL/AMPC/CPE mechanism identified.

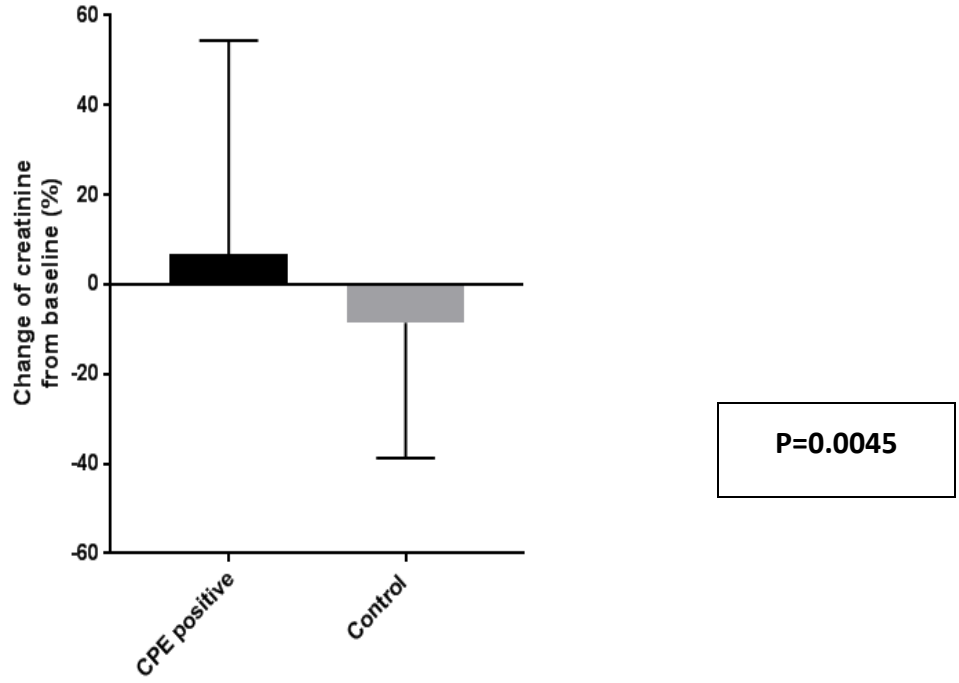

Figure 10: Comparison in the Percentage change in creatinine at day 14 between those with a CPE bacteraemia and those with a bacteraemia caused by a Gram-negative organism with no ESBL/AMPC/CPE mechanism identified, excluding those who underwent renal replacement therapy.

2.4.1.3 Clinical outcomes of CPE positive patients undergoing hematopoietic cell transplant (HCT).

The case sample had 20 patients with CPE detected on rectal screening, of which nine patients underwent an allograft and eleven underwent an autograft transplant. The control sample was made up of 59 HCT patients with negative rectal screening (allograft (n=27) and autograft (n=32)). All patients received antibiotic therapy post HCT.

The average length of stay for the case sample was significantly longer in the CPE colonised autograft group (41.7 vs. 23.6 days, case versus control, P=0.01), but not significant in the CPE colonised allograft group (75.1 vs. 58 days, P=0.12).(Figure 9)

Both CPE colonised autograft and allograft case samples had significantly longer duration of meropenem therapy, 24.8 vs. 14.4 days for allograft (P=0.03) and 9.4 vs. 5.5 days for autograft (P=0.03), cases versus control. Colistin therapy was longer in both case samples (P=0.03 in

autograft and P=0.006 in allograft). Tigecycline therapy was significantly longer in the autograft case versus control sample (P=0.006), with teicoplanin and piperacillin-tazobactam therapy significantly longer in the autograft case versus control sample, P=0.015 and P=0.03 respectively.

Table 6: Demographics of CPE colonised HCT patients compared with controls.

	ALLOGRAFT GROUP		AUTOGRAFT GROUP	
	CPE Colonized	Control group	CPE Colonized	Control group
Number	9	27	11	32
Age average	53.1	52.9	51.78	53.67
Sex M:F	04:06	16:11	08:03	21:12

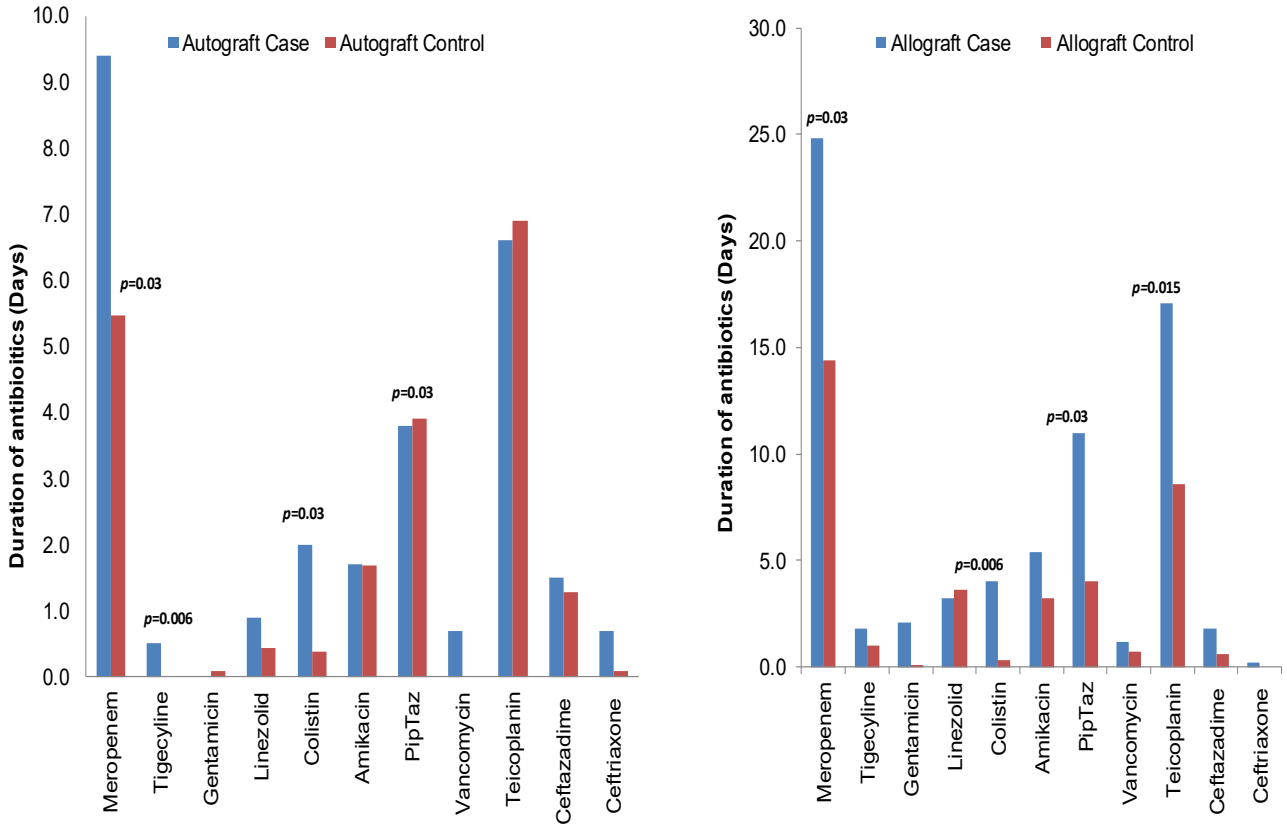

Figure 11: Comparison of duration of antibiotic length between CPE colonised HCT patients compared with HCT patients with negative rectal screening for CPE.

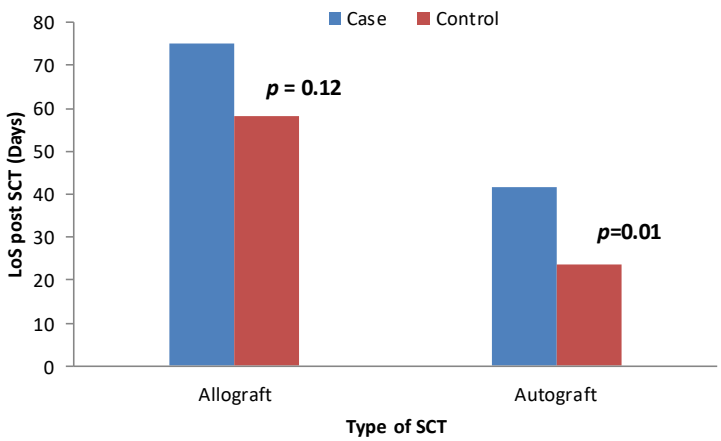

Figure 12: Comparison of length of stay (measured in days) between CPE colonised HCT patients compared with HCT patients with negative rectal screening for CPE.

2.4.1.4 Prevalence of MDRO urinary tract infections

A total of 1449 "recurrent UTI", patients were identified: 281 had ESBLs. Within the ESBL recurrent UTI group, patients were significantly older (P=<0.001), higher male proportion (P=<0.001), were more likely to have associated bacteraemia (P=0.001) and were more likely to have gut colonisation with carbapenemase-producing Enterobacteriaceae (CPE) compared to controls (P=<0.001). Of the renal patients, 75% had a kidney transplant and 81% of urology patients had a drainage tube insertion. There were higher resistance rates to non-beta-lactam antibiotics in the ESBL group (see Table 7).

Table 7: Recurrent ESBL UTIs versus recurrent non-ESBL UTIs

	Recurrent ESBL UTI Number (%)	Recurrent non-ESBL UTI Number (%)	p value (Chi-squared test)
Patients	281	1168	

Organism	***Klebsiella* spp (%)**	42 (14.9)	10 (0.8)	<0.001
	***E. coli* (%)**	199 (70.8)	811 (69.4)	0.665
Demographic	**Age (mean, SD)**	64.1, 19	58.7, 22	<0.001
	Male	137 (48.8)	328 (28)	<0.001
Associations	**Gram negative bacteraemia**	35 (12.4)	37 (3.1)	0.001
	Colonisation with CPE organism	21 (7.5)	31 (26.5)	<0.001
Speciality	**Renal**	84 (30.0)	196 (16.8)	<0.001
	Urology	47 (16.7)	104 (8.9)	<0.001
Antibiotic resistance	**Trimethoprim**	219 (77.9)	354 (30.3)	<0.001
	Ciprofloxacin	214 (76.2)	160 (13.7)	<0.001
	Nitrofurantoin	49 (17.4),	128 (11)	0.002
	Gentamicin	135 (48).	64 (5.5)	<0.001
	Amikacin	8 (2.8)	0 (0)	n/a

2.4.1.5 Description of patients under the Urology services with MDRO positive urinary isolates

Between June 2015-January 2018, 2059 patients under the Urology service had a positive urine culture, 128 (6%) were positive for ESBL. A total of 456 patients had more than one positive urine culture and of these, 62 (14%) had more than one ESBL UTI. This constituted 8% of all recurrent ESBL UTIs in the trust (806 totals).

We registered that 52/62 (84%), with recurrent ESBL UTIs, had an underlying urological diagnosis, while 10/62 (16%) were purely managed for recurrent UTIs. We found that 19/62 patients had more than one underlying diagnosis. While 7/62 (11%) patients underwent urological reconstructive surgery, and 40/62 (65%) had long term prosthetic devices (stents, nephrostomies, or catheters) *in situ*. Table 8 describes their outcome.

Table 8: Demographics of Urology patients with recurrent ESBL UTIs

Diagnosis		Number
Lower tract obstruction		23
Urinary tract calculi		13
Upper tract obstruction		7
Functional including reflux		5
Uro-Oncology		5
Post-operative complication		3
Urological reconstructive surgery	Ureteric reimplantation	4
	Ileocystoplasty	2

(n=7)	Ileal conduit	1
Drainage tubes (n=40)	Indwelling catheter	18
	Intermittent self-catheterisation	13
	Nephrostomy	10
	Stent	9

2.5 Discussion

Our program at Imperial College NHS Trust is carried out in a 1700 bed hospital trust in West London where administration of IMT for treatment of rCDI was already well-established as a clinical service.

As a Central London trust, the population that the hospital trust serves is one that is highly diverse in terms of the varied ethnic population, frequent travel outside of the UK is recognised as a risk factor for MDRO colonisation(231). There are also specialist tertiary centres within the trust, including renal and haematology, who by default of requiring prolonged hospitalisation and frequent antibiotic courses, are also at increased risk of MDRO colonisation(18). MDRO transmission within the Trust (in particular, outbreaks of carbapenemase-producing Enterobacterales (CPE) carriage) has been an increasing issue in high-risk populations.

2.5.1 Prevalence of CPE colonisation and infection

A retrospective cohort analysis of the laboratory information management system was performed to compare concurrent MDRO positive stool/rectal screens and invasive isolates. The highest rate of CPE colonised patients who became infected with a CPE were in the renal and haematology groups, strikingly within the haematology group there were a stark

difference between having relatively low rates of CPE colonisation (8% of colonised patients but 27% of CPE BSIs). The reasons for these likely include the disturbance to the commensal bacteria within the intestinal microbiome from immunosuppression, antibiotics, and prolonged hospitalisation, leading to these patients being more vulnerable when exposed to these organisms in hospital settings to intestinal colonisation. By identifying cohorts who are more likely to progress to invasive disease, we can focus on which groups would benefit most from IMT as a method to prevent invasive disease.

The results from this study also demonstrate the need to prevent invasive CPE bacteraemia. CPE bacteraemia patients were noted to have worse outcomes than invasive bloodstream infections that did not have a resistance to all beta lactams. The reasons for this are likely multifactorial. Firstly, patients who are colonised with CPE who develop an invasive infection are likely colonised due to risk factors that disrupt the intestinal microbiota precluding CPE colonisation such as prolonged hospitalisation and immunosuppression (see 1.1.4) therefore have lower reserves in the defence against infection. Secondly the treatment for a CPE bacteraemia is often delayed whilst the laboratory sensitivities are awaited, and there is often cross resistance to several other drugs, and the last line of therapy such as tigecycline or colistin are often not as effective and more toxic (particularly in terms of renal function) contributing to worse outcomes.

As there was a such as marked difference in haematology patients who were colonised with CPE and developed invasive disease, the impact of CPE colonisation on hematopoietic cell transplant patients was explored as this is one of the most immunosuppressive regimens utilised and therefore the highest increase in the risk of invasive disease. Colonisation alone was seen to have a negative impact on the length of stay and on the use of antibiotics recognised to have higher toxicity. Often this cohort of patients develop fevers which are treated empirically with no guiding microbiology, so one possibility for these outcomes is the fact that their colonising organisms are causing culture negative invasive disease, resulting in worse outcomes. Clinicians will often pre-emptively use more toxic agents such

as tigecycline and colistin based on the rectal colonisation which could contribute to the increased use of these agents seen.

2.5.2 Prevalence of ESBL recurrent urinary tract infections

ESBL recurrent UTIs were noted in this study to be more prevalent in older male patients and were more likely to be associated with Gram negative bacteraemias and were more likely to be associated with patients who had underlying conditions associated with the genitourinary tract. This observation demonstrates once again that MDRO infections have a greater burden on the healthcare system and are more likely to affect more vulnerable populations.

An important finding which demonstrates the impact that rUTIs have, was the fact that there was cross resistance with non-beta lactam antibiotics. This emphasises the fact that intravenous (IV) therapy is more likely to be necessitated, adding increase cost and risk of further line associated infections versus oral medication. A further analysis of patients under the Urology service demonstrated that many of these patients (65%) had long term prosthetic devices, which may suggest that resistance develops in those patients who may have a prosthesis where infection seeds and is trickier to eradicate because of biofilm formation.

2.5.3 Study limitations.

This study was a retrospective study which relied on clinical laboratory results. Based on previous local epidemiological data, the decision had been made to screen every hospital admission for MRSA and CPE, but not ESBL. Screening for rectal colonisation is only of benefit if the healthcare team would act on the result. ESBL colonisation rates are now so prevalent, that a hospital would not have the capacity to isolate all patients colonised with these organisms. Only patients admitted to intensive care were screened for VRE. This meant that we were only able to compare the rates of CPE colonisation which were a relatively small number. The number of CPE bacteraemia was only 26 patients, therefore excluding those who underwent dialysis decreased the numbers further. Data was not

available for patients in the ESBL UTI study to look at treatment courses as many of these patients will have received treatment in the community.

2.5.4 Future directions

The purpose of this analysis was to set a framework in preparation for administering IMT for MDRO colonised patients and identify suitable recipients. From the data produced from this portion of the study, we decided to define MDRO-colonised patients who are most likely to benefit from IMT broadly fall into the following two categories; (1) patients at risk of MDRO invasive infection due to altered immune status and (2) those with recurrent MDRO-mediated invasive disease and considered at risk of further disease. Due to geographical differences and differences in service provision, these findings may not be globally applicable, but it was clear that patients with haematological disorders were at risk of invasive MDRO diseases, and therefore likely to fall into category 1 and patients with recurrent UTIs were likely to fall into category 2, in particular those who were immunosuppressed i.e. by a renal transplant. The value of the data generated extends beyond this project and gives a valuable insight into the prevalence and risk factors of MDRO colonisation and the relevance of colonisation in at risk populations.

Chapter 3: Development and adaptation of IMT Programme to target MDRO colonised patients.

3.1 Introduction

IMT is well established as a method for treating rCDI(232). However, with rCDI patients the outcome measured is resolution of clinical infection and patients are treated when they are acutely unwell. Studies looking at IMT for MDRO colonised patients to date were highly heterogenous, and there was no information in the literature regarding the most effective method of delivering IMT to this cohort of patients. To start safely and effectively carrying out IMT for MDRO patients it was necessary to conduct a literature review of the most relevant studies that have carried out IMT and construct a framework to follow.

3.2 Aims

To establish a guideline to deliver IMT to patients colonised with MDROs to deliver IMT safely and effectively with a suitable measurable outcome.

3.3 Methods

Abstracts cited on PubMed were searched for up to July 2014. The terms 'Faecal Microbiota Transplantation, 'Intestinal Microbiota transplantation', 'multidrug-resistant', 'antimicrobial resistance' were used as search terms with these terms applied together in different logical combinations. English language abstracts only were the only restriction set. Once identified, the abstracts were read to decide if the study contained material relevant to the review, and if so, the full text article was retrieved and reviewed. Bibliographies were manually cross-referenced to identify potentially relevant articles that may have been missed by the initial search. Once the review of the literature was performed (see

Table 3: Literature to date on the use of IMT for MDRO decolonisation) and cross referenced against current standard of practice for the treatment of rCDI using IMT(174,233). Any salient features were presented to a specialist panel (comprising of Microbiology doctors, Infectious Diseases doctors, Gastroenterology doctors, Pharmacists, and Clinician Scientists).

A multi-disciplinary team discussion was carried out to confirm among specialists the most effective options. The areas targeted to address were donor screening, choice of recipient, timing of delivery and measurable outcomes. Regional guidelines were constructed to inform clinicians on how IMT would be carried out for MDRO colonised patients based on findings.

3.4 Results

3.4.1 Screening of donor stool

Donor stool was prepared in-house (see Appendix 3: Preparation and administration of IMT). Guidance for donor assessment and laboratory screening are based on the published current UK and international guidance on IMT delivery for recurrent CDI(168,233,234). Due to the choice of recipients, who were more likely to be immunocompromised from their underlying process, the decision was made to additionally test for the following microorganisms in addition to the screening laid out in Table 2: Recommended IMT donor screening from Joint British Society of Gastroenterology and Healthcare Infection Society guidelines.

- Cytomegalovirus and Epstein-Barr Virus serology:

Due to case reports having describing both transmission of cytomegalovirus (CMV) in IMT for patients with ulcerative colitis and CMV reactivation post-IMT in solid organ transplant (SOT) patients (184,235), and concerns regarding the theoretical oncogenic risk of transmission of Epstein-Barr Virus (EBV) in immunocompromised patients (185), the decision to test for serology for CMV (IgM and IgG) and EBV (IgM and IgG VCA and EBNA) is required of donors before donation. All donors are required to have negative CMV IgG status regardless of immune status of recipient, EBV IgG status should be screened to match that of the recipient.

- Transmissible MDROs in stool:

In 2019 in the United States (U.S.), two patients suffered from ESBL–producing Escherichia coli bacteraemia transmitted from IMT donor stool, resulting in one fatality (236). In 2020 in

the U.S., transmission of Shiga toxin–producing *Escherichia coli* (STEC) via IMT from a single donor to seven patients was reported, resulting in six serious adverse events and one non-serious adverse event (237). To minimize the risk of transmission, all donor stool was screened via culture and STEC and CPE are screened via real-time Polymerase Chain Reaction (RT-PCR) at each donation. A risk assessment for MDRO acquisition was taken at each donation, regarding foreign travel, hospitalisation, or recent use of antibiotics(238).

- SARS-CoV-2:

Prolonged shedding of the SARS-CoV-2 virus in stool after exposure has been described(239). To avoid potential transmission, we tested donors using a nasopharyngeal swab and donor stool be sent for RT-PCR in an ISO Accredited lab to detect SARS-CoV-2 (240).

3.4.2 Choice of MDRO colonised recipient

Based on the results from Chapter 2: Epidemiology of MDRO colonised and infected patients. The following inclusion category was selected.

- ≥18 years of age.
- Confirmed colonisation with MDRO (CPE, ESBL or VRE) and likely to have an adverse outcome should they develop invasive disease OR
- Recurrent infection with MDRO organism which they are known to be colonised with.

Based on previous findings, the two cohorts selected for active recruitment were haematology-oncology patients who were about to undergo an HCT and renal transplant patients who were known to have received multiple courses of antibiotics for recurrent MDRO UTIs. Other MDRO colonised patients were considered on a case-by-case decision.

Each patient referred for IMT would be discussed in a multidisciplinary meeting to decide appropriateness of the referral. Patients referred for the purpose of “decolonisation” alone were not considered.

Exclusion criteria for IMT for MDRO colonised patients was decided on the following criteria.

- < 18 years of age.
- Decompensated cirrhosis.
- Life-threatening food allergies, e.g., nuts.
- Pregnancy or currently breastfeeding.

Relative contraindications:

- Active ulceration/ bleeding of the upper GI tract.
- Altered anatomy of the GI tract that may prevent nasogastric tube insertion, e.g., gastrectomy.
- HIV infection with a CD4 count of < 240.

3.4.3 Delivery and timing of IMT

Delivery for IMT for MDRO colonised patients is described in Appendix 3: Preparation and administration of IMT and based on guidelines for rCDI(166)

However, timing was considered an important distinction from other uses for IMT. For IMT for patients who were due to undergo further immunosuppression (e.g., myeloablative chemotherapy followed by HCT), it was decided to be ideally planned for around 2–6 weeks before the immunosuppressive event is scheduled to occur. This was to reach a balance between allowing sufficient time for bacterial engraftment from the IMT to occur prior to any potential future intervention (such as HCT, plus empirical antibiotics given following any febrile neutropenic event) that may impact engraftment, but equally to ensure at least partial recovery from the neutropenia related to preceding chemotherapy.

In contrast to other uses of IMT, prevention of invasive disease was a key outcome measure. Failure of IMT has been described with antibiotic usage after IMT for CDI patients (241). It was advised in the guidelines to take care to facilitate engraftment of IMT by ensuring that it is performed when the patient is infection free, and therefore not likely to require antibiotics post IMT. Patients who are treated with IMT for recurrent MDRO UTIs are

required to have finished treatment for previous infections and have a negative urine culture prior to IMT.

3.4.4 Selective digestive decontamination with antibiotics

Selective digestive decontamination with antibiotics to our patients prior to IMT administration was decided against. Huttner et al utilised this approach in their randomised controlled trial looking at IMT to eradicate carriage of multidrug-resistant Enterobacteriaceae(196). However, in their study, they noted that antibiotics were responsible for most of the side effects in the study (mainly diarrhoea) and noted the theoretical concern of drive to further resistance and therefore may be counterproductive. The focus of our study was on patients at risk of invasive disease and as an approach to disease prevention and not intestinal decolonisation (i.e utilising antibiotics therapy as an approach to eradicate the pathobionts was not necessary. It was recognised that in HCT patients that decreased diversity is predictive of mortality(242) and MDRO colonisation is recognized to be driven by antibiotic administration which lowers intestinal bacterial diversity(243), although external factors such as early neutropenic fever requiring antibiotics following HCT cannot be avoided even when infectious aetiology is not the primary cause. Phage therapy for similar reasons was not considered as the aim of the IMT was not to target a specific pathobiont, and the use of phage therapy in this context is not yet widely reported in other studies, although could be considered for future studies as an adjunctive therapy in disruption of biofilm formation.

3.4.5 Measurable outcomes following IMT.

The following steps were recommended following IMT.

- Any potential adverse events should be followed up and recorded for at least 8 weeks after IMT.
- Education of patients on antimicrobial stewardship and requested that their clinician liaise with the IMT team if antibiotics are required.

- Avoidance of antibiotics to which the colonising MDRO is resistant, which would drive the selection pressure of the organism within the intestinal microbiome.
    - When unavoidable, antibiotics with least impact upon the transplanted intestinal microbiota (i.e., omitting anaerobic cover where possible) should be taken into consideration, for as short duration as possible (45,244).
- The patients who received an IMT were to undergo a 6-month follow-up to monitor the following.
    - Clinical response
    - Microbiological evidence of invasive disease
    - Mortality and morbidity
    - Relative length of inpatient admission (including intensive care admission), re-admission
    - Antibiotics usage.
- Patients should be questioned about health-related quality of life indicators.
- Follow-up should take place within the secondary care service they usually attend in a multidisciplinary setting.

It was decided that failure of IMT would not be considered when MDROs were detectable on rectal or stool screening after IMT (i.e., absolute intestinal MDRO decolonisation), as prevention of invasive disease from colonising MDROs is the primary goal. Repeated IMT for rCDI is recognized to increase the success rate cumulatively(245). Repeat IMT using different donor stool was decided to be offered to patients who saw a reduction in their MDRO mediated invasive infection, but experienced recurrence requiring further antibiotic courses (as the underlying contributor to recurrent infection such as anatomical defects and immunosuppressant therapy remains).

3.5 Discussion

The research carried out in this project would be a novel therapy on immunosuppressed patients, therefore a literature review and construction of clinical guidelines was an essential part of this research project to ensure patient safety. The key areas identified and

modified included donor stool, and additional tests were added to endure reduction of risk of transmission of disease. The guidelines generated were used to carry out IMT in MDRO colonised patients in a safe and consistent manner.

As IMT is still a new modality, particularly in the use in MDRO colonised patients, there was limited research published to help create guidelines. The main guidance to date has been for the use of IMT for rCDI. Not only is the patient cohort different, our selection of MDRO colonised patients were all on some form of immunosuppression, but also IMT for rCDI is enacted when the patient is unwell, whereas IMT for MDRO colonised patients should be carried out as a method of preventing them from becoming unwell, a pre-habilitation versus a rehabilitation. Previous studies looking at IMT in MDRO colonised patients had not specifically advised on such idiosyncrasies of this modality. Only one randomised control trial to date had been carried out looking at MDRO colonised IMT patients. These recommendations therefore were based on anecdotal evidence and expert opinion on hypothetical situations. Data also does not exist on the long-term effects of IMT. As more studies are published, and as emerging and evolving infections present as public health issues, these recommendations will require regular review and adaptations.

Chapter 4: Clinical outcomes of MDRO colonised patients undergoing IMT.

4.1 Introduction

The need to specifically try to prevent MDRO invasive infection in vulnerable cohorts comes from the fact that MDRO infection results in worse morbidity and mortality than infections that respond to first line therapy. The relationship between MDRO colonisation and increased risk of invasive infection and worse clinical outcomes is well recognised in vulnerable cohorts (246,247). rCDI patients who were given IMT were noted to have a lower rate of BSIs 90 days post IMT than patients treated with antibiotics (222). The benefit of IMT to patients in terms of direct clinical outcomes has been noted in some IMT studies for MDRO colonised patients, although most studies looking at IMT in MDRO colonised patients

have focused on IMT as a “decolonisation” method. The purpose of MDRO decolonisation is a tool to reduce the spread of infection in community (51) and healthcare settings as the risk of infection is noted to be increased with colonisation(248), however the actual prevention of invasive infection would be a more valuable modality to reduce morbidity and mortality from MDRO disease.

4.2 Aims

The aim of this part of the study was to evaluate the impact of IMT on the clinical outcomes of patient colonised with MDROs and whether there was prevention in invasive infection.

4.3 Methods

4.3.1 Clinical Setting

This study was an observational pre/post study of patients with MDRO colonisation or infection who received IMT to prevent disease occurrence and or recurrence. It was performed between 2015 and 2019 in a London group of 5 hospital sites with approximately 1700 inpatient beds, when IMT had started to be put into practice at Imperial College Healthcare NHS Trust.

4.3.2 Ethics

The study was approved by a UK Research Ethics Committee (REC reference: 19/LO/0112). All patients, including patients used as controls, provided informed consent authorizing the use of their personal information for research purposes.

4.3.3 Definitions

Multidrug-resistant organism was defined as vancomycin-resistant enterococci, carbapenem-producing Enterobacteriaceae, or extended-spectrum β-lactamase (ESBL)–producing Enterobacteriaceae.

4.3.4 Patient selection

The study was not randomised. Patients were selected from two distinct groups, either at risk of invasive infection, or having experienced recurrent infection and considered at risk of further infection, as different endpoints were evaluated for both groups.

Group 1/ Haematology patients:

Patients with intestinal colonisation (diagnosed on stool or rectal screening) with an MDRO and considered at risk of invasive MDRO disease. These were patients with an underlying haematological condition where further planned immunosuppression was planned (i.e., allogeneic hematopoietic cell transplantation [HCT]). IMT would aim to take place at least 2 weeks before further immunosuppression. The groups were further subdivided based on whether they received an HCT or not.

Group 2/ Recurrent UTIs

Patients with recurrent MDRO-mediated invasive disease and considered at risk of further disease. This included patients with recurrent MDRO urinary tract infections (rUTIs)—in particular, renal transplantation patients where recurrent infection was adversely impacting graft function. Patients were selected if they had received treatment for either two infections in six months or three infections in one year requiring antibiotic therapy.

In both groups, IMT was scheduled when patients were not receiving antibiotic therapy and considered infection free. The aim of IMT in both groups was to prevent invasive MDRO infections. (See Chapter 3: Development and adaptation of IMT Programme to target MDRO colonised patients.)

4.3.5 Donor Selection and IMT Administration

IMT was administered via nasogastric tube using pre frozen donor stool (see full details in Appendix 3: Preparation and administration of IMT).

4.3.6 Comparator Groups

Table 9: List of all cohorts of patients

Group Name	Description
Group 1/Haematology	**All** patients who received an IMT with an underlying haematological condition where further planned immunosuppression
Group 1a/Haematology (no IMT)	Comparator arm of patients with an underlying haematological condition who did **not** receive IMT and **were** MDRO colonised
Group 1/HCT	Haematology patients who received an IMT who underwent an **HCT** following IMT (subgroup of Group 1/Haematology)
Group 1b/HCT (no IMT)	Comparator arm of patients who received an **HCT** and **did not** receive IMT and **were** MDRO colonised
Group 1c/HCT (no IMT and no MDRO)	Comparator arm of patients who received an HCT and did **not** receive IMT and **were not** MDRO colonised
Group 2/Recurrent UTIs	**All** patients who received an IMT with recurrent MDRO urinary tract infections
Group 2a/Recurrent UTIs (no IMT)	Comparator arm of patients who had more than 4 MDRO episodes of UTI per year from 2015-2019 and **did not** receive an IMT

A comparator arm analysis was also performed. Comparator patients had clinical profiles similar to both IMT groups, were treated over the same time period, and had previous infection/colonisation with MDROs but were not considered for IMT (lead clinician or

patient choice). Analysis was performed for the first and second 6 months from the first identified MDRO.

4.3.6.1 Comparator Group 1a/Haematology

For Group 1a/ haematology patients, comparator patients were selected from a list of patients who were colonised on rectal screening or who had an MDRO bacteraemia between 2015-2019 and had not received IMT; age and sex matching to the IMT group was performed as much as possible. 20 patients were identified in this group. Medical notes were examined for: number of BSI (total and MDRO); days of carbapenem therapy (where available); and length of stay. The notes were examined for a 12-month period from date of first MDRO organism identification and split into 0-6 and 6-12 months from date of MDRO, for analysis purposes.

4.3.6.2 Comparator Group 1b/HCT

A further second comparator group was analysed to compare the differences in baseline characteristics of patients who underwent both IMT and HCT (Group1/HCT) and MDRO colonised patients who underwent an HCT only (Group 1b/HCT comparator group)

4.3.6.3 Matched pair analysis of Haematology patients.

For Group1/HCT patients two case-control cohort studies were also conducted (in addition to the direct comparison of these groups) to minimize the effects of known covariates on survival. Specifically, using a 2:1 matched pair analysis to account for the small sample size, outcomes of both the Group1/HCT (Underwent IMT and were MDRO colonised) and Group 1b/HCT (No IMT but MDRO colonised) to their respective control cohorts who were not MDRO-colonised (Group 1c/HCT). These were matched for disease type, disease stage, transplant intensity, donor type (matched sibling, matched unrelated, and haploidentical), and age was performed.

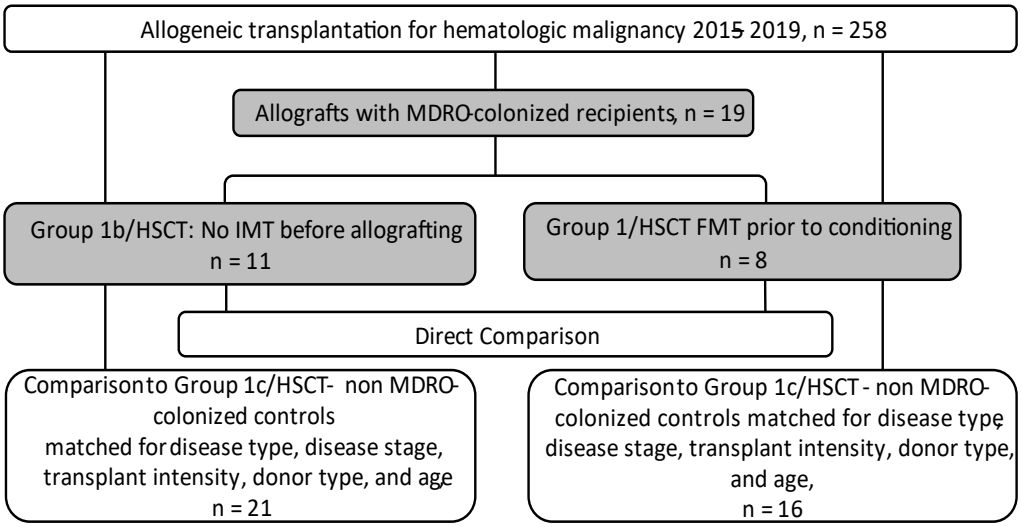

Figure 13: CONSORT diagram of case-cohort analysis of Group1/HCT patients pre and post IMT.

Adapted from Innes, Mullish, Ghani et al (2021) Fecal Microbiota Transplant Mitigates Adverse Outcomes Seen in Patients Colonized With Multidrug-Resistant Organisms Undergoing Allogeneic Hematopoietic Cell Transplantation. Front. Cell. Infect. Microbiol CC BY 4.0 (http://creativecommons.org/licenses/by/4.0)

4.3.6.4 Comparator Group 2a/Recurrent UTIs

For the Group 2a/ recurrent UTI comparator arm, 20 patients were selected from a list of patients who had more than 4 MDRO episodes of UTI per year from 2015-2019. Age and sex matching to the IMT group was performed as much as possible. This cohort of patients who were under the care of renal or urology services and who had not undergone urological surgery or other interventions in the 12 months from the date of first MDRO UTI. The medical notes were examined for: the number of MDRO urinary tract infections; number of BSI (total and MDRO); days of carbapenem therapy (where available); and length of stay. Again, data were split into 0-6 and 6-12 months from date of MDRO, for analysis purposes.

4.3.7 Outcome Metrics and Statistics

Patients were observed for at least 6 months post-IMT and monitored for MDRO carriage, invasive infection (bloodstream infection [BSI] or UTI), number and days of intravenous and oral antibiotic courses, antibiotic susceptibility of invasive/colonising isolates, and inpatient bed days. Days of antibiotic therapy, infection episodes, and length of stay in the 6 months pre- and post-IMT were recorded from clinical notes/electronic prescription charts. Multidrug-resistant organism decolonisation was assessed by serial rectal swab or stool sample analysis for at least 6 months post-IMT via opportunistic screening at clinic appointments.

4.3.8 Statistical methods

Statistical analysis was performed using GraphPad Prism version 9.3. Wilcoxon signed pairs rank test was used to assess statistical significance, for nonparametric data between the two time periods for each patient. Probabilities of survival were calculated using the Kaplan–Meier method, with the log-rank test utilized for comparison of groups; probabilities of NRM were calculated using the cumulative incidence procedure, with disease progression being the competing risk. Gray's test was used to compare groups. Days of fever were normalized for days of hospital admission, and compared by unpaired, non-parametric testing (Mann–Whitney U-test). Patient and transplant characteristics were compared using Fisher's exact test and the Mann-Whitney U-test as appropriate. P-values <0.05 were taken as statistically significant.

4.4 Results

4.4.1 Baseline characteristics of Group 1 and Group 2 IMT recipients:

4.4.1.1 Colonising MDRO of all IMT recipients (Group 1/Haematology and Group 2/Recurrent UTIs)

Table 10: All IMT patients' underlying condition and associated MDRO.

Patient Demographics		**Underlying diagnosis**	**Colonising/invasive organism**
Group 1/Haematology	33M	Acute myeloid leukaemia	*E. coli* ESBL (meropenem resistant)
	68F	Acute myeloid leukaemia	*E. coli* NDM
	68M	Acute myeloid leukaemia	*E. coli* GES5
	59M	Chronic myeloid leukaemia	*C. freundii* OXA-48
	63M	Mycosis fungoides	*K. pneumoniae* OXA-48
	54M	Diffuse large B cell lymphoma	*C. freundii* OXA-48 Vancomycin resistant *enterococci*

	17F	Sickle cell disease and gut GVHD	Vancomycin resistant *enterococci*
	70M	Acute myeloid leukaemia	Vancomycin resistant *enterococci*
	63M	Acute lymphoblastic leukaemia	*K. oxytoca* GES5
	55M	Chronic myeloid leukaemia	*E. coli* IMP-1
	59M	Acute myeloid leukaemia	*K. pneumoniae* OXA-48
Group 2/Recurrent UTIs	89F	Recurrent UTI/ CDI	*E. coli* ESBL *C. difficile*
	90F	Recurrent UTI/CDI	*E. coli* ESBL *C. difficile*
	80F	Recurrent UTI/CDI	*E. coli* ESBL *C. difficile*
	90F	Recurrent UTI/CDI	*E. coli* ESBL

			C. difficile
	59F	Recurrent UTI/Renal transplant	*K. pneumoniae* ESBL
	62M	Recurrent UTI/Renal transplant	*E. coli* ESBL
	60F	Recurrent UTI/Renal transplant	*E. coli* ESBL
	51F	Recurrent UTI/Renal transplant	*K. pneumoniae* ESBL
	78M	Recurrent UTI/Renal transplant	*E. coli* ESBL *C. freundii* OXA-48

4.4.1.2 Group 1/ Haematology Group Baseline Characteristics

Of 11 patients with an underlying haematologic disorder, six had an MDRO BSI pre-IMT, and nine patients had prolonged admissions (range 6-20 weeks) complicated by septic episodes. Patients were colonised with CPE (8), VRE (3) or ESBL (2) as detected on rectal screening. None of these patients received antibiotic therapy between the IMT and hematopoietic cell infusion. In one patient, IMT was delayed until three days after the hematopoietic cell infusion due to pre-transplant infection requiring antibiotic therapy.

4.4.1.3 Group 2/ Recurrent UTIs Baseline Characteristics

Nine patients underwent IMT for recurrent ESBL UTIs (seven patients with *Escherichia coli* and two *Klebsiella pneumoniae*). This included four patients with rCDI co-infection and five

patients with renal transplantation. The renal transplant patients had each required inpatient stay of >2 months duration within the preceding year with infection-related graft dysfunction; three patients had received multiple prolonged courses of intravenous antibiotics administered via the outpatient parenteral antibiotic team.

4.4.1.3.1 Comparator arm Baseline Characteristics

Twenty patients were included for Group 1a/ haematology and 20 for Group 2a/Recurrent UTI analysis. 12 of the 20 comparator Group 1a/ haematology patients had further chemotherapy, including allogeneic (4) or autologous (1) hematopoietic cell transplantation during the 12 months of analysis after their first isolated MDRO. Eight patients were diagnosed with MDRO colonisation or infection during their admission for hematopoietic cell transplantation. Seven of the 20 comparator Group 1a/ haematology patients died within the 12-month study period from date of first MDRO, one of whom received an 11-month course of outpatient parenteral tigecycline prior to death. Of the 20 comparator Group 2a/ recurrent UTI patients, all were under active follow-up with either renal or urology services and included 18 patients with previous renal transplantation. There were no significant differences seen over time in any of the clinical domains analysed in either of the comparator groups examined.

4.4.1.3.2 Comparison of Group 1b/HCT comparator group baseline characteristics.

A table was constructed to compare the differences in baseline characteristics of patients who underwent both IMT and HCT (Group1/HCT) and MDRO colonised patients who underwent an HCT only (Group 1b/HCT comparator group). The Karnofsky Performance Status (KPS) is a widely used method to assess the functional status of a patient. The European Group for Blood and Marrow Transplantation (EBMT) risk score provides a tool to assess instantly chances and risks of (HCT) for an individual patient pre-transplant.

Characteristic	Group1/HCT (n = 8)	Group 1b/HCT comparator group (n = 11)	*P*

Patient age (years) (Median, range)	61.9 (33-70)	59.8 (31-66)	0.32
Diagnosis to HCT (years) (Median, range)	0.8 (0.3-9.6)	0.5 (0.3-13.3)	0.89
Disease			
CML	2 (25%)	2 (18%)	
AML/MDS	3 (38%)	7 (64%)	0.68
ALL	2 (25%)	1 (9%)	
T-cell lymphoma	1 (13%)	1 (9%)	
Disease Risk Index (EBMT)			
Low	4 (50%)	7 (64%)	
Intermediate	4 (50%)	2 (18%)	0.89
High	0	2 (18%)	
Karnofsky score at HCT.			
<=80%	3 (38%)	2 (18%)	
90%	3 (38%)	3 (27%)	0.27
100%	2 (25%)	6 (55%)	
Donor type			
Matched sibling	3 (38%)	4 (36%)	
Matched unrelated	4 (50%)	4 (36%)	0.71
Haploidentical	1 (12%)	3 (27%)	

Conditioning			
Reduced intensity	7 (88%)	7 (64%)	0.34
Myeloablative	1 (13%)	4 (36%)	
Patient - Donor sex match			
Female into male	1 (13%)	2 (18%)	1.00
Other	7 (88%)	9 (82%)	
CMV donor/recipient			
negative to negative	2 (25%)	2 (18%)	
positive to negative.	0	1 (9%)	0.62
negative to positive	3 (38%)	2 (18%)	
positive to positive	3 (38%)	6 (55%)	
HCT - comorbidity index			
0	1 (13%)	3 (27%)	
1 or 2	4 (50%)	4 (36%)	0.45
>=3	3 (37%)	4 (36%)	
Year of HCT			
<2018	3 (38%)	7 (64%)	0.37
>2017	5 (62%)	4 (36%)	

Table 11: Comparison of MDRO colonised patients who underwent an IMT followed by an HCT with MDRO colonised patients who underwent HCT only.

4.4.1.3.3 Comparison of baseline characteristics for the matched pair group analysis of HCT patients.

Table 12: Demographics and further clinical details of MDRO-colonised HCT recipients and controls who did not receive an IMT.

Characteristic	Group 1b/HCT comparator group (n = 11)	Group 1c/HCT (n = 21)	*P*
Patient age (years) (Median, range)	59.8 (31-66)	51.4 (31-73)	0.24
Diagnosis to HCT (years) (Median, range)	0.5 (0.3-13.3)	0.7 (0.2-21)	0.82
Disease			
CML	2 (18%)	5 (24%)	
AML/MDS	7 (64%)	12 (57%)	0.91
ALL	1 (9%)	2 (10%)	
T-cell lymphoma	1 (9%)	2 (10%)	

Disease Risk Index (EBMT)			
Low	7 (64%)	10 (47%)	
Intermediate	2 (18%)	9 (43%)	0.62
High	2 (18%)	2 (9%)	
Karnofsky score at HCT.			
<=80%	2 (18%)	2 (10%)	
90%	3 (27%)	7 (35%)	0.89
100%	6 (55%)	11 (55%)	
Donor type			
Matched sibling	4 (36%)	9 (43%)	0.86
Matched unrelated	4 (36%)	8 (38%)	
Haploidentical	3 (27%)	4 (19%)	
Conditioning			

Reduced intensity **Myeloablative**	7 (64%) 4 (36%)	13 (62%) 8 (38%)	0.92
Patient - Donor sex match **Female into male** **Other**	 2 (18%) 9 (82%)	 3 (14%) 18 (86%)	 0.77
CMV donor/recipient **negative to negative** **positive to negative.** **negative to positive** **positive to positive**	 2 (18%) 1 (9%) 2 (18%) 6 (55%)	 4 (19%) 4 (19%) 3 (14%) 10 (48%)	 0.89
HCT - comorbidity index **0** **1 or 2**	 3 (27%) 4 (36%)	 6 (29%) 10 (48%)	 0.73

>=3	4 (36%)	5 (24%)	
Year of HCT			
<2018	7 (64%)	15 (71%)	0.39
>2017	4 (36%)	6 (29%)	

Table 13. Demographics and further clinical details of IMT MDRO-colonised HCT recipients and controls

Characteristic	**Group1/HCT (n = 8)**	**Group 1c/HCT (n = 16)**	***P***
Patient age (years) (Median, range)	61.9 (33-70)	59.2 (31-73)	0.65
Diagnosis to HCT (years) (Median, range)	0.8 (0.3-9.6)	0.6 (0.3-13)	0.82
Disease			
CML	2 (25%)	4 (25%)	
AML/MDS	3 (38%)	6 (38%)	1.00
ALL	2 (25%)	4 (25%)	
T-cell lymphoma	1 (13%)	2 (13%)	
Disease Risk Index (EBMT)			
Low	4 (50%)	8 (50%)	1.00
Intermediate	4 (50%)	8 (50%)	
High	0	0	
Karnofsky score at HCT.			
<=80%	3 (38%)	6 (40%)	

90%	3 (38%)	1 (7%)	0.15
100%	2 (25%)	8 (53%)	
Donor type			
Matched sibling	3 (38%)	3 (38%)	
Matched unrelated	4 (50%)	4 (50%)	1.00
Haploidentical	1 (12%)	1 (12%)	
Conditioning			
Reduced intensity	7 (88%)	12 (75%)	0.63
Myeloablative	1 (13%)	4 (25%)	
Patient - Donor sex match			
Female into male	1 (13%)	2 (13%)	0.96
Other	7 (88%)	13 (87%)	
CMV donor/recipient			
negative to negative	2 (25%)	2 (13%)	
positive to negative.	0	1 (7%)	0.81
negative to positive	3 (38%)	6 (40%)	
positive to positive	3 (38%)	6 (40%)	
HCT - comorbidity index			
0	1 (13%)	3 (19%)	
1 or 2	4 (50%)	6 (38%)	0.83
>=3	3 (37%)	7 (44%)	
Year of HCT			
<2018	3 (38%)	10 (63%)	0.39
>2017	5 (62%)	6 (37%)	

4.4.2 Clinical outcomes for Group 1/Haematology and Group 2/Recurrent UTIs (Both IMT groups)

All patients tolerated IMT well with no serious adverse events. Mild adverse effects included self-limiting constipation, bloating, and diarrhoea

4.4.2.1 Group1/Haematology Clinical Outcomes

Table 14: Baseline characteristics, clinical outcomes six months pre and post IMT for patients with an underlying haematological disorder

Background				Pre IMT						Post IMT						
Age /Sex	**date of IMT**	**Underlying diagnosis**	**Colonising Organism**	**Recent inpatient admission prior to IMT**	**Days of admission**	**Invasive MDRO infection pre IMT**	**No of MDRO BSIs**	**No of BSIs total**	**Days of Meropenem**	**Inpatient admission post IMT**	**Days of admission**	**Invasive MDRO infection post IMT**	**Days of Meropenem**	**No. of MDRO BSIs**	**No of BSIs total**	**?Intestinal decolonisation**
33 M	04/10/2018	Acute myeloid leukaemia	ESBL *E coli* meropenem resistant	15 /52 during pre-stem cell transplant (HCT) chemotherapy	105	ESBL meropenem resistant *Klebsiella pneumonia* 6 /52 of Meropenem and Colistin	0	2	42	Six-week admission post Allo-HCT	42	*VRE* bacteraemia 2 /52 Linezolid only	10	1	1	No

68 F	20/03/2017	Acute myeloid leukaemia	*E coli* NDM	16 /52 during pre HCT chemotherapy	112	Recurrent *E. coli* NDM bacteraemia. 2 courses of Meropenem and Colistin	0	0	17	Deceased 6 /52 post IMT (not related to sepsis)	42	No	D18	0	0	No
68 M	30/08/2018	Acute myeloid leukaemia	*E coli* GES 5	3 /52 during chemotherapy	21	None	0	0	0	None	0	No	0	0	0	Yes
59 M	12/05/2017	Chronic myeloid leukaemia	*Citrobacter freundii* OXA-48	10 /52 during pre HCT chemotherapy	70	*VRE* bacteraemia 2 /52 of Colistin and Daptomycin	1	2	19	4 /52 post Allo-HCT	28	No	14	0	1	No
63 M	07/02/2018	Mycosis fungoides	*Klebsiella* pneumoniae OXA-48	6 /52 during pre HCT chemotherapy	42	2 /52 of Meropenem and Colistin	0	0	15	3 /52 post Allo-HCT	21	No	13	0	0	No

54 M	10/12/2018	Diffuse large B cell lymphoma	*Citrobacter freundii* OXA48 VRE	20 /52 during chemotherapy	140	VRE bacteraemia Required Intensive Care support	2	3	61	4 /52 post radiotherapy	28	No	2	0	1	No
17 F	02/04/2019	Sickle Cell disease and Gut GvHD	Vancomycin resistant *Enterococci*	14 /52	98	Eight courses of antibiotics for non neutropaenic fevers		5	43	2 /52	14	No	0	0	0	yes
70 M	18/07/2018	Acute myeloid leukaemia	Vancomycin resistant *Enterococci*	8 /52 during pre HCT chemotherapy	56	VRE bacteraemia 2 /52 Meropenem and Linezolid	2	2	14	4 /52 post Allo-HCT	28	No	0	0	2	Yes
63 M	11/03/2016	Acute lymphoid leukaemia	*Klebsiella oxytoca* GES 5	12 /52 during pre HCT chemotherapy	84	Recurrent courses of Meropenem	0	0	n/a	4 /52 post Allo-HCT	28	No	n/a	0	1	Yes

55 M	26/06/2019	Chronic myeloid leukaemia	*E. coli* IMP-1	9 /52 during pre-HCT chemotherapy	63	29 days of Meropenem (2 /52 at high dose)	0	0	29	4 /52 post allo-HCT	28	No	6	0	1	VRE (not isolated pre-IMT)
59 M	08/08/2019	Acute myeloid leukaemia	*Klebsiella* pneumoniae OXA-48	4 /52	28	*Klebsiella* pneumoniae OXA-48 bacteraemia 2 /52 of Colistin 11 days of high dose Meropenem 9 days of Ceftazadime-Avibactam	1	2	11	4 /52 post allo-HCT	28	No	0	0	1	Yes

4.4.2.2 Group2/Recurrent UTI Clinical Outcome

Table 15: Baseline characteristics, clinical outcomes six months pre and post IMT for patients who experienced recurrent UTIs

Background				Pre-IMT							Post-IMT							
Age /Sex	**Date of IMT**	**Underlying diagnosis**	**Colonising Organism**	**Recent inpatient admission prior to IMT**	**Days of admission**	**Invasive MDRO infection pre IMT**	**No of MDRO BSIs**	**No of BSIs total**	**No of MDRO UTIs over 6 months**	**Days of Meropenem**	**Inpatient admission post IMT**	**Days of admission**	**Invasive MDRO infection post IMT**	**Days of Meropenem**	**No. of MDRO BSIs**	**No of BSIs total**	**No of MDRO UTIs over 6 months**	**?Intestinal decolonisation**
89 F	25/08/2017	Recurrent UTI/ *C.diff*	ESBL *E coli*	n/a*	n/a	Recurrent ESBL UTI	0	0	2	n/a	n/a*	0	No	0	0	0	0	Yes
90 F	20/04/2017	Recurrent UTI/ *C.diff*	ESBL *E. coli*	n/a*	n/a	Recurrent ESBL UTI	0	0	2	n/a	n/a*	0	No	0	0	0	0	Yes
80 F	20/05/2019	Recurrent UTI/ *C.diff*	ESBL *E. coli*	n/a*	0	2x ESBL *E. coli* bacteraemia)	2	2	2	n/a	n/a*	0	No	0	0	0	2	n/a

90 F	19/09/ 2019	Recurrent UTI/ *C.diff*	ESBL *Ecoli*	n/a*	n/a	Recurrent ESBL UTI	0	0	2	n/a	n/a*	0	No	0	0	0	0	n/a
59 F	16/08/ 2018	Renal transplant /Recurrent UTI	ESBL *Klebsiella pneumonia*	16/52 inpatient 6/52 OPAT	112	2 x ESBL *Klebsiella pneumonia* bacteraemia	2	2	4	87	2/52 IP 6/52 OPAT	14	ESBL *E. coli* bacteraemia 6/52 Ertapenem	45	1	1	3	No
62 M	08/03/ 2019	Renal transplant /Recurrent UTI	ESBL E. coli	10 /52	70	10 /12 ESBL *E. coli* UTIs Meropenem	1	2	6	51	None related to sepsis (dialysis dependent post septicaemia)	0	No	0	0	0	0	Yes

60 F	21/03/ 2019	Renal transplant /Recurrent UTI	ESBL *E. coli*	4 /52 as inpatient 16 /52 on outpatient parental therapy	28	5 months of carbapenem therapy for recurrent ESBL UTI over one year	1	2	5	92	2 /52	14	No	28	1	1	1	No
51 F	24/06/ 2019	Renal transplant /Recurrent UTI	ESBL *Klebsiella* pneumoniae	13.5 /52	94.5	12 courses of treatment for ESBL UTI over a year	0	0	6	n/a	16 /52	112	Yes	n/a	0	0	5	No
78 M	25/09/ 2018	Renal transplant /Recurrent UTI	ESBL E coli *Citrobacter* OXA-48	4 /52 inpatient 8/12 OPAT over one year	42	10 ESBL *E. coli* UTIs over one year	0	0	6	163	2 /52	14	No further ESBL detected in urine. following second FMT	0	0	0	1	No

4.4.2.3 Bloodstream infections

Across both IMT groups, there was a significant reduction in the number of BSIs 6 months after IMT. This was the case for both MDRO BSIs (P = 0.047) and all BSIs (P = 0.03) (n = 20). The reduction in BSIs was not seen in the comparator group (BSI across all patients, P = .24; n = 40; MDRO BSI for group 1 comparator arm only, P = 0.28; n = 20).

4.4.2.4 Length of stay

There was a significant reduction in inpatient length of stay post-IMT in both IMT groups (pre-IMT median = 70 ± 35 days, post-IMT median = 28 ± 26 days; P = 0.0002; n = 16). This was also not seen in comparator patients (P = 0.16, n = 40). One patient was an overseas patient (outlier in Figure 14 C) so had a prolonged admission due to visa issues.

4.4.2.5 Antibiotic usage

Patients had significantly reduced carbapenem use post-IMT (pre-IMT median = 36 ± 44 days, post-IMT median = 4 ± 13 days; P = .0005; n = 14) which was again not seen in comparator patients (P = .61; n = 32).

4.4.2.6 MDRO decolonisation

Seven of 17 (41%) patients were no longer colonised with MDROs on rectal screening following IMT (follow-up range: 6 weeks–24 months).

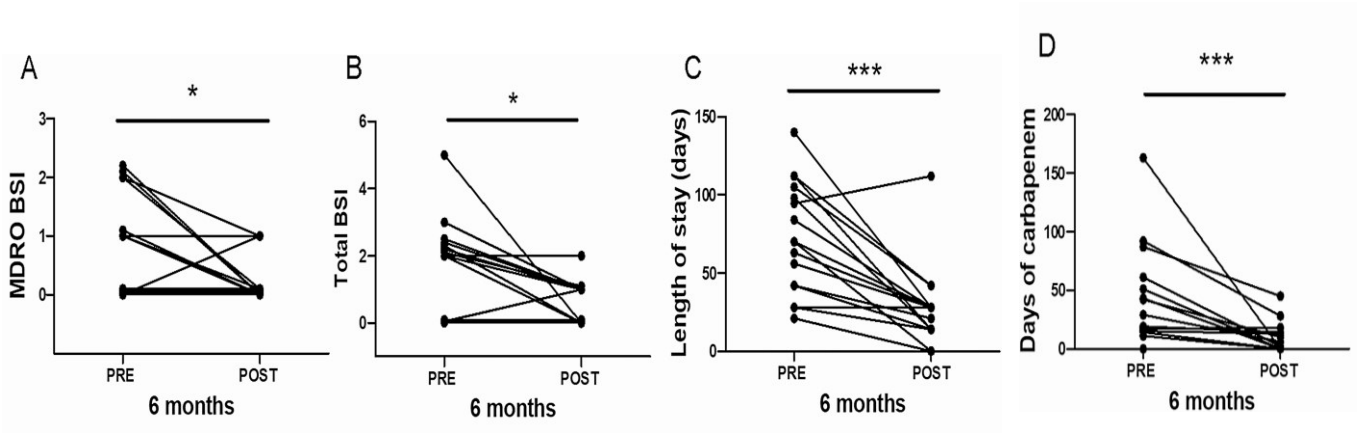

Figure 14: Ladder plots demonstrating clinical outcomes for patients pre and post IMT.

Clinical outcomes. A*, Number of MDRO BSIs 6 months pre- and post-IMT (*P *= 0.047; n = 20).* B*, Number of all BSIs 6 months pre- and post-IMT (*P *= 0.03; n = 20).* C*, Length of inpatient stay (days) 6 months pre- and post-IMT (pre-IMT = 70 ± 35 days [median ± SD], post-IMT = 28 ± 26 days; ***P *= 0.0002; n = 16; incomplete data available for 4 patients).* D*, Number of days of carbapenem use 6 months pre- and post-IMT (pre-IMT = 36 ± 44 days [median ± SD], post-IMT = 4 ± 13 days; ***P *= 0.0005; n = 14; incomplete data available for 6 patients).* E*, Number of MDRO UTIs 6 months pre- and post-IMT in group 2 (pre-IMT median = 4 ± 2 episodes, post-IMT median = 1 ± 2 episodes; **P *= 0.008; n = 9).*

4.4.3 Clinical outcomes for IMT patients versus comparator arms

There was no significance difference seen in the first 6 months since first MDRO isolate (0-6) compared to the second 6 months (6-12) for:

- The number of MDRO BSI in comparator Group 1a/ haematology patients (P = 0.28, n=20).
- The total number of BSI, comparator Group 1a/ haematology and Group 2/ Recurrent UTI groups (P = 0.24 n=40)
- The length of stay, comparator Group 1a/ haematology and Group 2/ Recurrent UTI groups (P = 0.16, n=40).
- The number of days of carbapenem therapy, comparator Group 1a/ haematology and Group 2/ Recurrent UTI groups (P = 0.16, n=32; full data unavailable for 8 patients).
- The number of positive MDRO urine samples in comparator Group 2/ Recurrent UTI patients (P = 0.18, n=20).

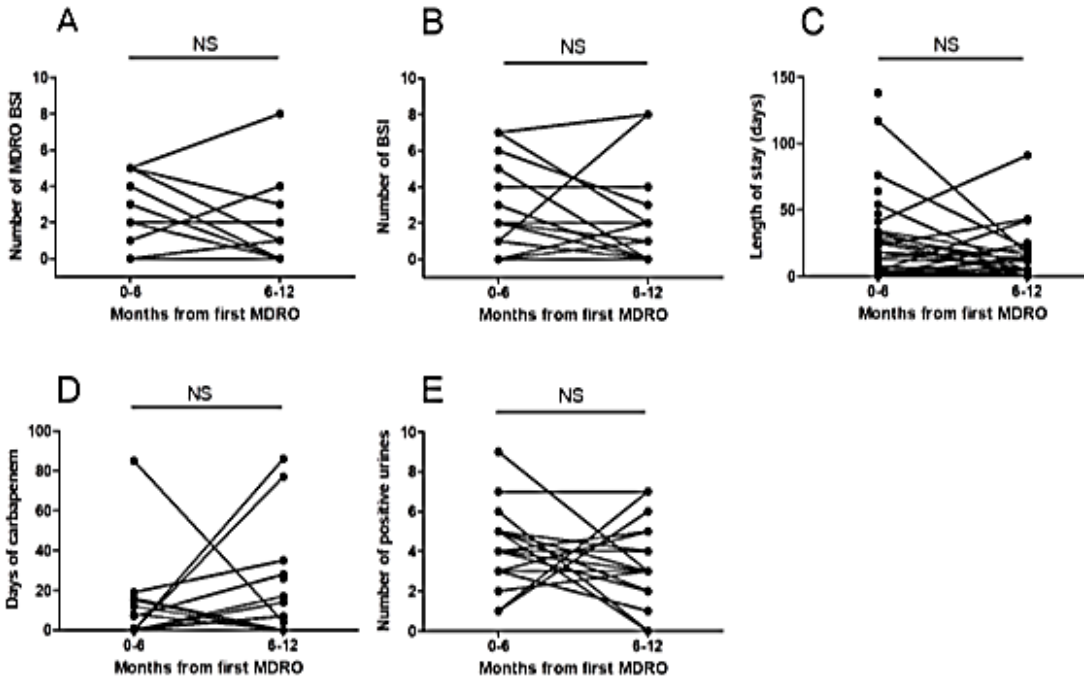

Figure 15: MDRO colonised comparator group (no IMT): outcomes compared between the first and second 6 months from the first identified MDRO.

A) the number of MDRO BSI in comparator Group 1/ haematology patients (P = 0.28, n=20); B) total number of BSI, comparator Group 1/ haematology and Group 2/ recurrent UTI groups (P = 0.24 n=40); C) length of stay, comparator Group 1/ haematology and Group 2/ recurrent UTI groups (P = 0.16, n=40); D) days of carbapenem therapy, comparator Group 1/ haematology and Group 2/ recurrent UTI groups (P = 0.16, n=32; full data unavailable for 8 patients); E) the number of positive MDRO urine samples in comparator Group 2/ recurrent UTI patients (P = 0.18, n=20). NS: non-significant.

4.4.4 Clinical outcomes for Group 1/Haematology patients versus comparator groups

4.4.4.1 Clinical outcomes for Group 1/Haematology patients compared with Group 1a/Haematology comparator group.

Post-IMT, 8 patients underwent an allogeneic HCT. All Group 1/ patients had shorter inpatient stays (P = 0.002) and fewer days on carbapenems compared with the preceding 6 months (P = 0.002). This reduction was not seen in Group 1a/ comparator patients (P = 0.48; n = 20). One patient undergoing HCT post-IMT developed an MDRO BSI caused by a different organism from their previous colonising organism. This BSI was treated with a

shorter course of antibiotics relative to their pre-IMT infections (42 days pre-IMT, 10 days post-IMT).

4.4.4.2 Clinical outcomes for Group1/HCT compared with Group 1b/HCT comparator group.

The probability of survival of Group1/HCT was 70% at 12 months, compared to 36% in MDRO-patients who did not (P=0.044). Fewer Group1/HCT needed an admission to the intensive care unit for inotropic support or respiratory failure (0% versus 46%, P=0.045) than Group 1b/HCT comparator group. Group1/HCT patients had fewer days of fever when normalized for the number of admission days (i.e., number of days with fever divided by total number of admission days) (0.11 versus 0.29 days, P=0.027) than Group 1b comparator group.

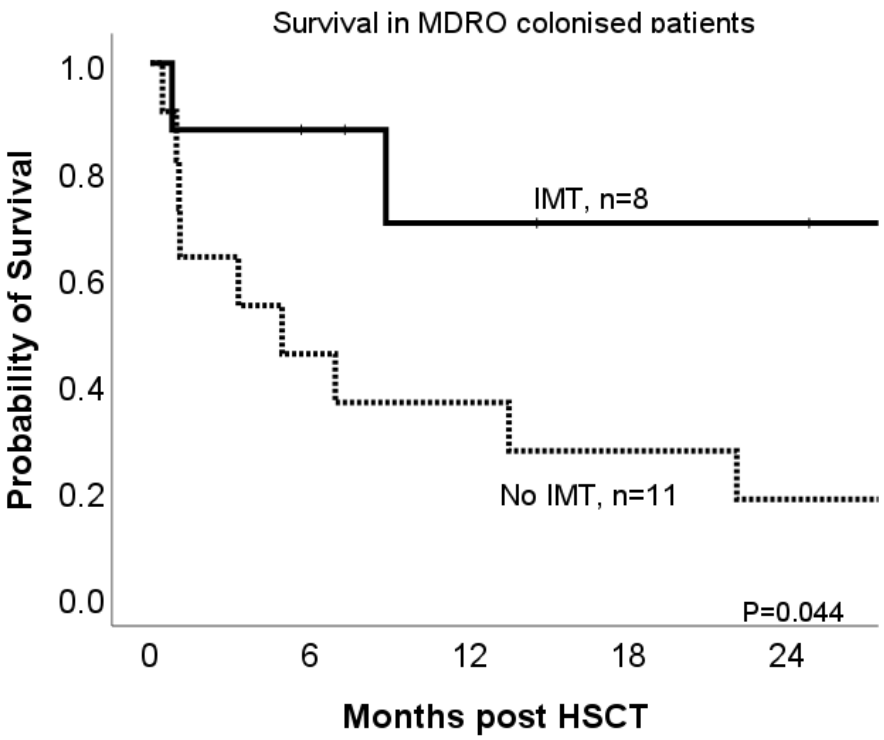

Figure 16: Kaplan-Meier curve of overall survival in MDRO-colonised HCT patients who underwent IMT (n=8) and those who did not (n=11). .

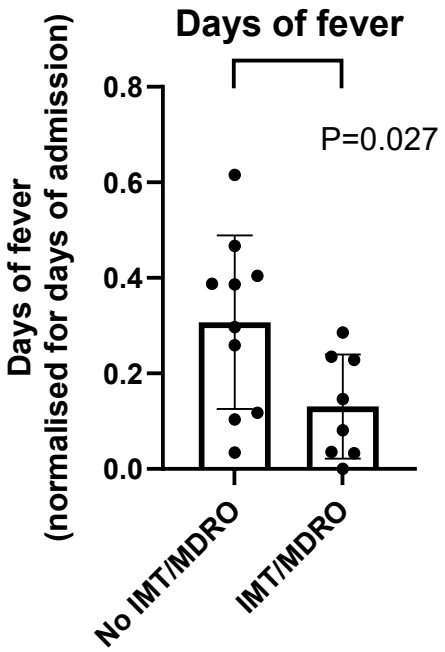

Figure 17: Comparison of days of fever in MDRO colonised HCT patients who underwent IMT (IMT/MDRO) vs those who did not (No IMT/MDRO).

Causes of death were classified as clinical infection in one Group1/HCT, and in five MDRO-patients in the Group 1b/HCT comparator group. One additional patient in the Group 1b/HCT comparator group died of infection on a background of graft-versus-host disease, and one of veno-occlusive disease/sinusoidal obstruction syndrome. One patient in the Group1/HCT and two in the Group 1b/HCT comparator group died of relapse of their malignancy. The one MDRO-patient who died of clinical infection in the Group1/HCT had no positive blood cultures or other positive bacteriology findings during the two weeks prior to death. Of the five MDRO-patients who died of clinical infection in the Group 1b/HCT comparator group, three died with MDRO bloodstream infection, and one with MDRO pneumonia. One died with from a *Candida albicans* BSI (with no bacterial growth), and one MDRO-patient's blood cultures were sterile during the two weeks prior to death.

Within this subgroup, two of eight (25%) Group1/HCT achieved MDRO decolonisation; (in one patient, a new MDRO (different from the original isolate) became detectable). Two of eleven (11%) Group 1b/HCT comparator spontaneously decolonised.

4.4.4.3 Matched Pair Analysis Studies of Group1/HCT patients

Patients who were not given an IMT but were colonised with an MDRO had significantly lower survival than their paired cohort not colonised with MDRO (36.4% versus 61.9% respectively, P=0.012) at 12 months. In contrast, there was no statistical difference in survival between the patients who were MDRO colonised and received an IMT, Group1/HCT, and their paired cohort not colonised with MDRO, Group 1c/HCT (70% versus 43.4%, P=0.14). Similarly, NRM at 12 months was higher in patients who were not given an IMT but were colonised with an MDRO (60.2%) than in their matched controls (16.7%, P=0.009), but there was no significant difference in NRM between MDRO colonised patients given an IMT group (Group1/HCT) (12.5%) and their matched controls (Group 1c/HCT) (31.2%, P=0.24).

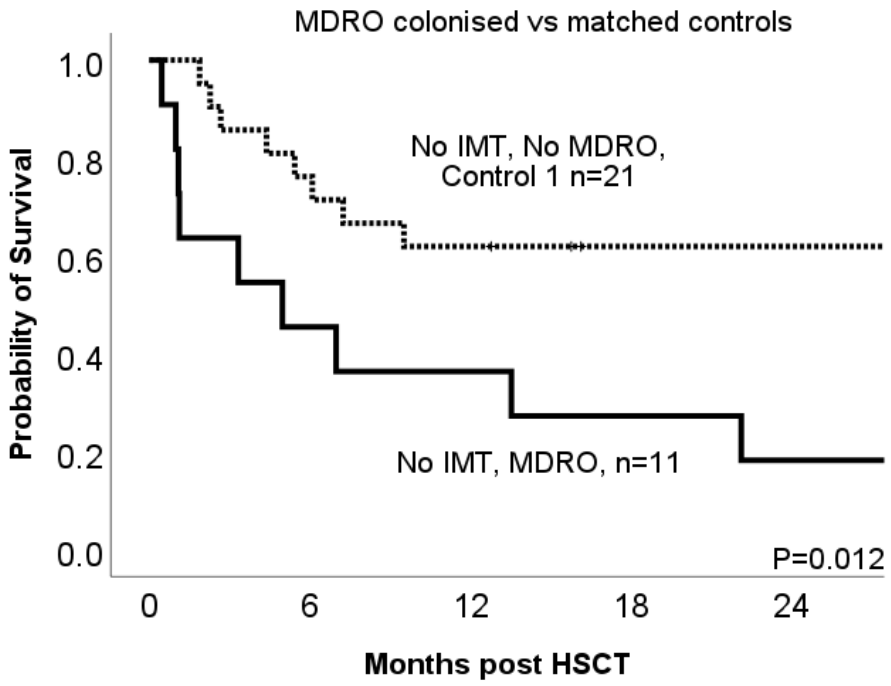

Figure 18: Kaplan-Meier curve of overall survival in patients colonised with MDROs in comparison to matched controls who were not colonised.

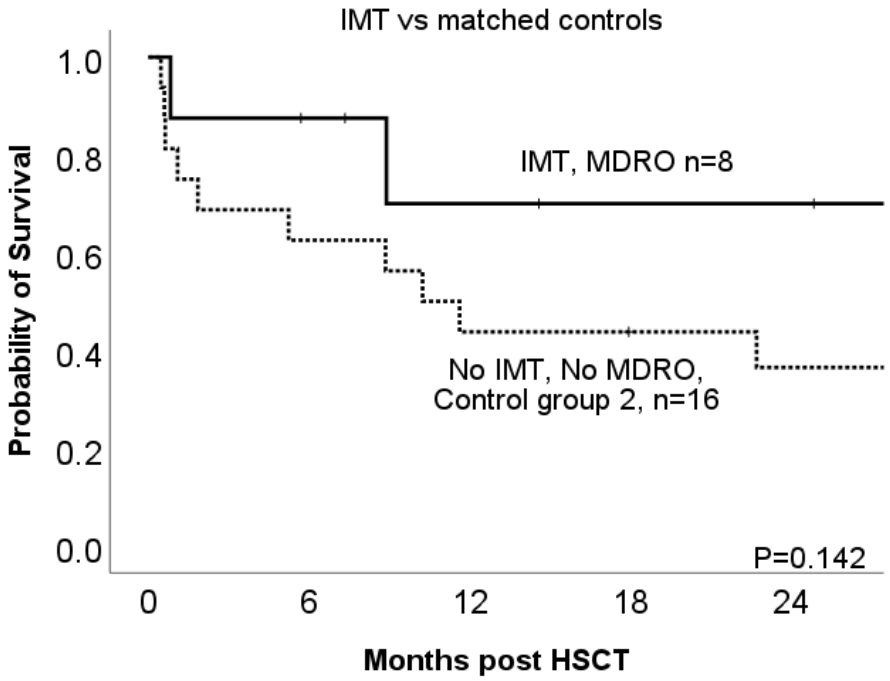

Figure 19: Kaplan-Meier curve of overall survival in patients colonised with MDROs who underwent IMT in comparison to matched controls who were not MDRO colonised.

4.4.5 Group 2/ Recurrent UTI patient

There was a significant reduction in frequency of MDRO UTIs post-IMT (see Figure 20) (pre-IMT median = 4 ± 2 episodes, post-IMT median = 1 ± 2 episodes; P = 0.008; n = 9. This was not seen in the Group 2 comparator arm (P = 0.18; n = 20). Only 1 patient with recurrent CDI/UTI coinfection developed a further ESBL UTI 6 months post-IMT. Three renal transplant patients had a marked reduction in days of antibiotics and both inpatient and outpatient attendances post-IMT. Of note, two patients required inpatient antibiotic therapy immediately post-IMT for ESBL-driven infection (for both patients, urine collected at the time of IMT was culture-positive for ESBL organisms); this may have impacted the efficacy of the IMT. One patient underwent a second IMT after a 6-month interval, resulting in no further MDRO UTI during the study period.

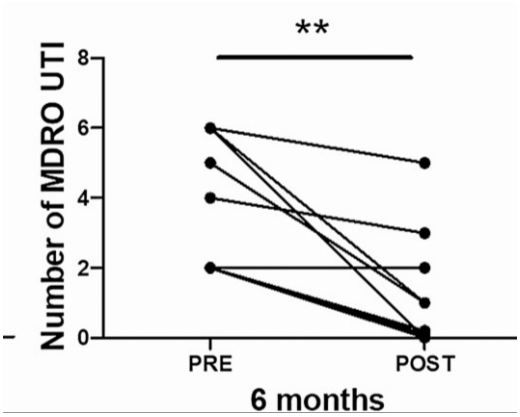

Figure 20: Number of MDRO UTIS 6 months pre- and post-IMT in Group 2 patients

Number of MDRO UTIs 6 months pre- and post-IMT in group 2 (pre-IMT median = 4 ± 2 episodes, post-IMT median = 1 ± 2 episodes; ***P* = 0.008; n = 9)

4.5 Discussion

4.5.1 Main findings

The major novel finding of the study was the significant post-IMT reductions in inpatient bed days, bacteraemias and antibiotic use in both the haematology and recurrent UTI cohort despite modest rates of intestinal decolonisation. The low rate of decolonisation is in line with the existing literature and did not necessarily correlate with whether the incidence of clinical infection was reduced.

Particularly notable was the reduction in BSI in haematology patients, where no patients developed bacteraemia with their pre-IMT colonising bacteria, despite ongoing systemically active chemotherapy and/or immunosuppression, including allogeneic HCT. This observation starkly contrasts with the group 1a comparator arm (Comparator arm of patients with an underlying haematological condition who did not receive IMT and were MDRO colonised), where there was no reduction in BSI over time, and a marked number of deaths, findings that require further analysis. In patients with recurrent UTI, there was difficulty establishing an infection-free window in which to perform IMT for some patients. Nevertheless, there was a significant reduction in antibiotic use and use of oral antibiotics rather than intravenous. In 1 case, a second IMT was performed, with improved effectiveness.

In the closer look at the haematology patients who underwent an HCT and the matched-pair study, IMT mitigated the worse outcomes seen in MDRO-colonised post-HCT patients, including survival, their survival was similar to those not colonised with an MDRO unlike the cohort of patients colonised with an MDRO who did not undergo IMT who had worse survival. Part of this appears likely attributable to IMT-related impact on infective complications after HCT, although the benefits of IMT may be more broad-reaching then just the prevention of invasive MDRO disease. For instance, one interesting observation was that whilst most patients in both MDRO groups developed fevers that were treated with antimicrobials as per institutional protocol, there was a clear reduction in the number of days of fever in patients who had received IMT; one interpretation could be that these patients were more responsive to conventional anti-infective treatment. This observation is further supported by the lower requirement for intensive care support in the IMT group, and the lower rate of death from sepsis. Importantly, IMT was also well tolerated in this cohort of immunosuppressed patients, with no serious adverse events, confirming the applicability of its use in immunosuppressed patients.

The matched cohort analysis of those colonised with MDRO, also reflects previous findings from other groups that MDRO colonisation is associated with a poor outcome in the setting of HCT(249). The results from this study also suggest that although the findings that were accrued in patients with detectable MDRO in the intestinal microbiome, these benefits may be more broadly applicable. Due to their previous exposure to broad spectrum antibiotics, chemotherapy, and/or the underlying disease process itself, prospective HCT recipients are recognized to have a relative decrease in diversity of commensal bacteria which are recognized to play a role in immune recovery. IMT may provide comparable clinical benefits even in HCT patients who are not colonised with MDROs.

4.5.2 Limitations

The sample size was non-randomised with certain discrepancies between clinical characteristics. One limitation of this study was the fact that as Imperial College Healthcare NHS Trust was a tertiary centre for both haematology and renal care, therefore follow up for many patients including assessment of colonisation status could only be performed opportunistically at their preassigned follow up clinical appointments. Monitoring of

antibiotic use pre and post IMT was restricted as there was no access to antibiotics supplied in the community by General Practitioners (GPs). Infection was not fully prevented in the renal group, and factors such as structural abnormalities in renal transplant patients may have been contributed. However, delayed onset of efficacy of IMT seems to be apparent and requires further work to be fully understood. Pre-IMT antibiotic choices varied widely between patients, therefore may have had an impact on the pre-IMT microbiota composition. The broader impact of FMT on use of World Health Organization “Restrict” and “Watch” group antibiotics merits exploration in future studies.

Chapter 5: Analysis of the metataxonomic and metagenomic profile of the gut microbiota pre and post Intestinal Microbiota Transplantation.

5.1 Introduction

Chapter Four demonstrates a clinical benefit to patients who received IMT in both prevention of invasive disease and decreasing the frequency of infections in patients who had previously experienced MDRO invasive disease from bacteria that commonly colonise the intestinal microbiota. The reasons for the effect of this are not clear, although it is accepted that IMT restores the gut microbiota of critically altered gut ecosystem back to a premorbid state, the underlying mechanisms for this are not clear. To explore this, we took a holistic view of the microbiome, considering both the commensal and pathogenic bacteria residing within the gut via metagenomics, and utilising metataxonomics to evaluate the overall diversity of these bacteria.

5.2 Sample collection.

In order to be able to extrapolate the findings to a more heterogeneous population, we liaised with groups in Poland and Israel who were also involved in IMT in MDRO colonised patients and were able to receive patient samples to increase the samples size for the mechanistic studies.

5.2.1.1 Initial data set (United Kingdom)

The initial data set was collected in the UK at Imperial College from MDRO colonised participants pre and post IMT and donors. The sample set was as follows.

- Ten patients with stool samples pre and post IMT.
- Five donor stool samples
- Seven unmatched MDRO colonised stool samples (grouped with the pre-IMT cohort)
    - These were patients who were assessed for an IMT but did not end up receiving one.

Samples were taken prior to IMT and collected for at least 6 months (range 15- 266 days) post-IMT via opportunistic screening at clinic appointments. Samples were collected using a

FECOTAINER® collection device. Within two hours of sample donation, faecal samples were separated into twelve equal aliquots and frozen at -80°C in an Ultra-Low temperature freezer until processing for analysis consistently with standard protocols.

5.2.1.2 Israeli data set

Further samples were sent from Haifa, Israel from a prospective, interventional, cohort study at a single tertiary centre (Rambam Health Care Campus [RHCC]) preformed between February 2018 and April 2019 (99). The sample set consisted of

- 13 IMT recipient stool samples were all colonised with CPE. The samples were collected before IMT and at 14 days post IMT.
- Six donor stool samples
- Six unmatched CPE colonised stool samples (grouped with the pre-IMT cohort)

Faeces were delivered from Israel to London, the transfer took less than 24 hours and samples were kept in dry ice. All samples were kept at -80 °C until sample preparation for further analysis was performed.

5.2.1.3 Polish data set

These were samples taken from primarily Haematology patients from Warsaw, Poland. The sample set consisted of thirteen patients colonised with an MDRO with stool samples pre and post IMT at several time points (7,14 or 28 days).

These samples were delivered from Poland to London, the transfer took less than 24 hours and samples were kept in dry ice. All samples were kept at -80 °C until sample preparation for further analysis was performed.

5.2.1.4 Clinical and demographic characteristics of entire sample cohort

Table 16: Characteristics of entire patient cohort

Type of patient	Country of origin	Colonising MDR bacteria	Associated MDRO gene	Underlying condition	Sex	Age	No of post-IMT	No of days post IMT

								samples
IMT	Israel	*Klebsiella pneumoniae*	OXA-48	No data	M	27	1	14
IMT	Israel	*Enterobacter cloacae & Citrobacter freundii*	KPC	No data	M	70	1	14
IMT	Israel	*Klebsiella pneumoniae*	KPC	No data	M	75	1	14
IMT	Israel	*Klebsiella pneumoniae*	KPC	No data	F	60	1	14
IMT	Israel	*Enterobacter hormechai*	KPC	No data	M	49	1	14
IMT	Israel	*Klebsiella pneumoniae*	OXA-48	No data	M	48	1	14
IMT	Israel	*Klebsiella pneumoniae*	OXA-48	No data	M	68	1	14
IMT	Israel	*Klebsiella oxytoca*	KPC	No data	F	21	1	28
IMT	Israel	*Serratia marcescens*	KPC	No data	F	81	1	14
IMT	Israel	*Serratia marcescens*	KPC	Haematology	M	59	1	14
IMT	Israel	*Klebsiella pneumoniae*	OXA-48	No data	M	23	1	14
IMT	Israel	*Escherichia coli*	NDM	No data	M	50	1	14
IMT	Israel	*Klebsiella pneumoniae*	KPC	No data	M	65	1	14
Donor	Israeli	N/A	N/A	N/A	No data	No data	N/A	N/A
Donor	Israeli	N/A	N/A	N/A	No data	No data	N/A	N/A
Donor	Israeli	N/A	N/A	N/A	No data	No data	N/A	N/A
Donor	Israeli	N/A	N/A	N/A	No data	No data	N/A	N/A
Donor	Israeli	N/A	N/A	N/A	No data	No data	N/A	N/A
Donor	Israeli	N/A	N/A	N/A	No data	No data	N/A	N/A

MDRO colonised No IMT	Israeli	No data	No data	No data	No data	No data	N/A	N/A
MDRO colonised No IMT	Israeli	No data	No data	No data	No data	No data	N/A	N/A
MDRO colonised No IMT	Israeli	No data	No data	No data	No data	No data	N/A	N/A
MDRO colonised No IMT	Israeli	No data	No data	No data	No data	No data	N/A	N/A
MDRO colonised No IMT	Israeli	No data	No data	No data	No data	No data	N/A	N/A
MDRO colonised No IMT	Israeli	No data	No data	No data	No data	No data	N/A	N/A
IMT	British	*E coli*	ESBL	Renal	M	78	4	28,86, 247,431
IMT	British	*Klebsiella pneumoniae*	ESBL	Renal	F	51	1	14
IMT	British	*E coli*	IMP-1	Haematology	M	55	2	40,159
IMT	British	*Klebsiella pneumoniae*	OXA-48	Haematology	M	59	1	116
IMT	British	*Enterococci faecium*	VRE	Haematology	M	70	2	41,159
IMT	British	*E coli*	GES 5	Haematology	M	68	2	41, 197
IMT	British	*E coli*	ESBL	Renal	F	59	3	15, 56, 165
IMT	British	*E coli*	meropenem resistant	Haematology	M	33	2	84, 182
IMT	British	*Klebsiella pneumoniae*	OXA-48	Haematology	M	54	1	131
IMT	British	E coli	ESBL	Renal	F	61	2	266, 466
Donor	British	N/A	N/A	N/A	M	28	N/A	N/A
Donor	British	N/A	N/A	N/A	M	40	N/A	N/A
Donor	British	N/A	N/A	N/A	F	29	N/A	N/A

Donor	British	N/A	N/A	N/A	F	24	N/A	N/A
Donor	British	N/A	N/A	N/A	F	28	N/A	N/A
MDRO colonised No IMT	British	*E coli*	ESBL	Renal	M	63	N/A	N/A
MDRO colonised No IMT	British	*Enterococci faecium*	VRE	Haematology	F	16	N/A	N/A
MDRO colonised No IMT	British	*E coli*	OXA48 NDM	Haematology	M	68	N/A	N/A
MDRO colonised No IMT	British	*E coli*	ESBL	Renal	M	39	N/A	N/A
MDRO colonised No IMT	British	*E coli*	ESBL	Renal	F	70	N/A	N/A
MDRO colonised No IMT	British	*Klebsiella pneumoniae*	ESBL	Renal	F	42	N/A	N/A
MDRO colonised No IMT	British	*E coli Klebsiella pneumoniae*	ESBL NDM	Renal	F	73	N/A	N/A
IMT	Polish	*Klebsiella pneumoniae*	NDM	Haematology	M	63	3	7, 14, 30
IMT	Polish	*Klebsiella pneumoniae*	ESBL/MBL	Haematology	M	43	3	7, 14, 31
IMT	Polish	*Klebsiella pneumoniae*	ESBL	Haematology	F	61	1	7
IMT	Polish	*Proteus mirabilis/Enterococcus faecium*	ESBL/VRE	Haematology	F	49	2	7, 14
IMT	Polish	*Klebsiella pneumoniae*	ESBL/MBL		M	88	2	7, 14
IMT	Polish	*Enterococcus faecium/Klebsiella pneumoniae/Citrobacter freundii*	VRE/ESBL/MBL	Haematology	M	38	2	7, 14
IMT	Polish	*Klebsiella pneumoniae / E. coli*	ESBL/MBL	Haematology	M	36	1	7
IMT	Polish	*Klebsiella pneumoniae*	NDM	Haematology	M	38	2	7, 14
IMT	Polish	*Klebsiella pneumoniae*	MBL	Haematology	F	53	1	7

IMT	Polish	*Klebsiella pneumoniae*	NDM		F	86	1	14
IMT	Polish	*Klebsiella pneumoniae*	MBL	Haematology	M	68	3	7, 14, 30
IMT	Polish	*Klebsiella pneumoniae / Enterococcus faecium*	MBL/ VRE	Haematology	M	58	3	7, 14, 30
IMT	Polish	*Klebsiella pneumoniae*	NDM	Haematology	M	46	1	30
Donor	Polish	N/A	N/A	N/A	No data	No data	N/A	N/A
Donor	Polish	N/A	N/A	N/A	No data	No data	N/A	N/A
Donor	Polish	N/A	N/A	N/A	No data	No data	N/A	N/A
Donor	Polish	N/A	N/A	N/A	No data	No data	N/A	N/A
Donor	Polish	N/A	N/A	N/A	No data	No data	N/A	N/A
Donor	Polish	N/A	N/A	N/A	No data	No data	N/A	N/A
Donor	Polish	N/A	N/A	N/A	No data	No data	N/A	N/A
Donor	Polish	N/A	N/A	N/A	No data	No data	N/A	N/A
Donor	Polish	N/A	N/A	N/A	No data	No data	N/A	N/A
Donor	Polish	N/A	N/A	N/A	No data	No data	N/A	N/A
Donor	Polish	N/A	N/A	N/A	No data	No data	N/A	N/A
Donor	Polish	N/A	N/A	N/A	No data	No data	N/A	N/A

Donor	Polish	N/A	N/A	N/A	No data	No data	N/A	N/A

5.2.2 Metataxonomics Methods

Metataxonomics uses the 16S RNA gene to assign taxonomy and bacterial diversity characterization. Metataxonomic analysis was performed on stool samples from the initial data set (UK sample) to enable identification of specific gut bacterial taxa changes associated with IMT.

5.2.2.1 Microbial DNA extraction and quantification

Stool was thawed at room temperature on ice and an aliquot of. ≈ 250 mg was used for microbial genomic DNA extraction using the PowerLyzer PowerSoil DNA Isolation Kit following the manufacturer's instructions with minor adjustments.

The faecal water (FW) was added to a 2 ml microcentrifuge tube containing 0.1 mm glass beads. Sixty µL of C1 and 750 µL of bead solution were added and then vortexed. Briefly, solution C1 is a cell lysis solution including sodium dodecyl sulphate which lyses cell membrane fatty acids and lipids. Bead beating was carried out using the Bullet Blender Storm instrument for 3 min, while tubes were therefore centrifuged at 13,000 x g for 1 min. A 400-500 µL volume of the supernatant was transferred to a sterile tube where 250 µL of solution C2 was added. Tubes were vortexed, incubated 78 for 5 min at 4˚C and centrifuged for 13,000 x g for 1 min. This step leads to the physical separation of the liquid phase (containing the DNA) from the solid phase (pellet). Six hundred µL of supernatant was transferred to a sterile collection tube and 200 µL of solution C3 was added. At this stage, tubes were vortexed again, incubated for 5 min at 4˚C and centrifuged for 1 minute at 13,000 x g. Adding the C3 causes the additional cellular debris to be broken down and proteins to precipitate. Similarly, a volume of 750 µL supernatant was transferred to a sterile tube and added to a 1.2 mL of C4 solution. Six hundred and fifty µL of the supernatant mix was transferred onto a spin filter and centrifuged at 10,000 x g for 1 minute

with the remaining flow through discarded. This process was repeated for several passages until all the supernatants had fully been filtered, so that DNA was bound to the membrane. To clean the DNA bound to the filter, 500 µL of solution C5 was added to the spin filter and centrifuged for 1 min at 10,000 x g. The flow through was discarded and the spin filter centrifuged again for 1 min at 10,000 x g to remove all remaining ethanol. The spin filter was transferred to a sterile collection tube and 100 µL of solution C6 was added to elute the DNA, followed by centrifuging for 1 min at 10,000 x g. The so-obtained DNA was aliquoted into Eppendorf-tube and stored at -80 ˚C, while the spin filter was discarded.

Eluted DNA concentration was quantified using the Qubit dsDNA Broad Range Assay Kit and. afterwards DNA was divided into 20 µL aliquots and stored at – 80°C.

5.2.2.2 16S rRNA gene sequencing

Amplicon sequencing libraries were prepared using Illumina's 16S Metagenomic Sequencing Library Preparation Protocol(250). This protocol was adjusted as per previously published 16S rRNA gene sequencing standard operation procedure (SOP) from the Marchesi Lab (75). Firstly, microbial DNA was amplified using the V1-V2 hypervariable region of the 16S rRNA gene using a mixture of 28F forward primers 28F-YM, 28F-Borrellia, 28F-Chloroflex and 28F-Bifdo at a 4:1:1:1 ratio and 388R reverse primer (Table 17). Additionally, SequalPrep Normalization Plate Kits were used to clean and normalise the Illumina Index PCR product according to the manufactures protocol. Finally, amplicon libraries were quantified with the NEBNext Library Quant PCR Kit for Illumina. Sequencing libraries were loaded onto an Illumina MiSeq platform using the Illumina MiSeq Reagent kit v3 and paired end 300bp chemistry. Amplicon libraries also included 1 in-house mock community, 2 Zymo gut microbiome controls, 10 library preparation negative controls and, 2 extraction kit negative controls.

Table 17:: Primers used for 16S rRNA gene sequencing on the Illumina MiSeq.

The forward primer mix was composed of four different forward primers, mixed at a final ratio of 4: 1: 1: 1 (28F-YM: 28F-Borrellia: 28FChloroflex: 28F-Bifdo). Bases in bold are the MiSeq adapter sequences.

Primer name	Primer sequence
28F-YM (Forward primer)	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GAGTTTGATYMTGGCTCAG
28F-*Borrellia* (Forward primer)	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GAGTTTGATCCTGGCTTAG
28F-Chloroflex (Forward primer)	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GAATTTGATCTTGGTTCAG
28F-Bifido (Forward primer)	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GGGTTCGATTCTGGCTCAG
388R (Reverse primer)	**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TGCTGCCTCCCGTAGGAGT

5.2.2.2.1 16S rRNA gene sequencing data processing

Data analysis was initially performed in Mothur package (v1.35.1) (http://www.mothur.org/wiki/MiSeq_SOP#OTU-based_analysis) following the MiSeq standard operating protocol pipeline(251,252). Sequence alignments were performed using the Silva bacterial database (www.arb-silva.de/), and the RDP database reference sequence files were used for sequence classification using the Wang method (253). Operational taxonomic unit (OTU) taxonomies (from phylum to genus level) were established using the RDP MultiClassifier Script. Data was resampled and normalised to the lowest read count in Mothur (11604 reads per sample), which resulted in >99.5% coverage within each sample. Where possible, species were identified from OTU data using a standard nucleotide BLAST

of the 16S rRNA gene sequences (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=MicrobialGenomes) with strict criteria (query cover 100% and ≥97% identity, with no other candidate species above ≥97% identity) (254). Genus-level annotation was made where query cover was 100% and ≥94% identity. Non-metric multidimensional scaling (NMDS) plots and permutational multivariate ANOVA (PERMANOVA) p-values were generated using the UniFrac weighted distance matrix generated from Mothur and analysed using the Vegan library within the R statistical package. Extended error bar plots were generated at different taxonomic levels using the Statistical Analysis of Metagenomic Profiles (STAMP) software package, applying White's non-parametric t-test with Benjamini-Hochberg false discovery rate (FDR) correction (255).

α-diversity was used as a metric to evaluate the mean diversity of species each cohort within a local scale. The indices used for this study were.

- Chao1: an estimator based on abundance of individual samples belonging to a certain class (richness).
- Shannon: an estimator for both species' richness and evenness. (Total number of bacterial taxa observed, $S_{obs)}$
- Inverse Simpson: a measure of diversity which considers the number of species present, as well as the relative abundance of each species.
- Faith PD: measurement of phylogenetic diversity, in particular the sum of branch lengths between the observed species on a phylogenetic tree.
- Rao's quadratic entropy: the proportion of the abundance of species present in a community and some measure of dissimilarity among them.

To calculate phylogenetic diversity measurement, (FaithPD). BTools package was used. Rao's quadratic entropy was calculated using picante package (256).

β-diversity of samples was also measured to assess differences in microbial community differences in the three cohorts (Donor, Pre-IMT and Post IMT). To quantify the dissimilarities between samples, Bray-Curtis dissimilarity for UniFrac distances were used

using a non-metric multi-dimensional scaling (NMDS) model (146). A permutation test for homogeneity of multivariate dispersions was performed with the function permutest function (vegan package). A permutational multivariate analysis of variance (PERMANOVA) was used to compare the difference in the observed community composition across groups of samples and between difference distance metrics, using the adonis function in vegan package. The number of permutations was set at 1000 in every test, The homogeneity condition of the data was assessed with PERMDISP2 (mia package).

Statistical tests were performed using GraphPad Prism version 9.3. A p-value of 0.05 and a q-value of 0.05 was considered significant. Changes in microbial composition were also assessed down to the OTU level. Differences in means of relative proportions >1% were measured between donor and pre-IMT samples, and between pre-IMT and post-IMT samples, using White's non-parametric test and Benjamini- 73 Hochberg FDR. From these data, OTUs were analysed that were enriched in donors in comparison to pre-IMT samples, and those enriched post-IMT in comparison to pre-IMT samples.

5.2.3 Metagenomics Methods

Microbial DNA extraction and quantification for metagenomics Genomic DNA library preparation and metagenomic sequencing were performed at the Wellcome Sanger Institute [Cambridge, UK]. Genomic DNA was extracted and quantified (see 5.2.2.1). from the study stool samples. Following the Institute guidelines samples were aliquoted into an ABgene 0.2 ml full skirted 96-well plate. The plated volume was 50 µL to ensure the recommended amount of DNA for optimal quality control, library preparation, and sequencing pipeline of 200 ng.

Shotgun metagenomic sequencing Library preparation and sequencing were performed by the Core Sequencing facility at Wellcome Sanger Institute. DNA samples, including 3 extraction kits and 2 internal negative controls, were set for library preparation using TruSeq DNA PCR-Free from Illumina. A 150-bp paired-end read length library was produced with a total fragment length of 450-bp. The library was sequenced in an Illumina HiSeq 4000, 32 samples were multiplexed per flow cell.

Relative abundances of metagenome sequence reads were determined using a Kraken 2 v2.1.2 and Bayesian Reestimation of Abundance with KrakEN software (Bracken) database based on 4543 genomes present in the Unified Human Gastrointestinal Genome database. ARG markers present in the metagenome data were quantified by mapping sequences against the Comprehensive Antibiotic Resistance Database (CARD, v.3.0.3) using the Resistance Gene Identifier software. Statistical analysis was performed using GraphPad Prism 9.3.

5.3 Results

5.3.1.1 Metataxonomic gut ecosystem indices results

5.3.1.1.1 Alpha diversity in all samples

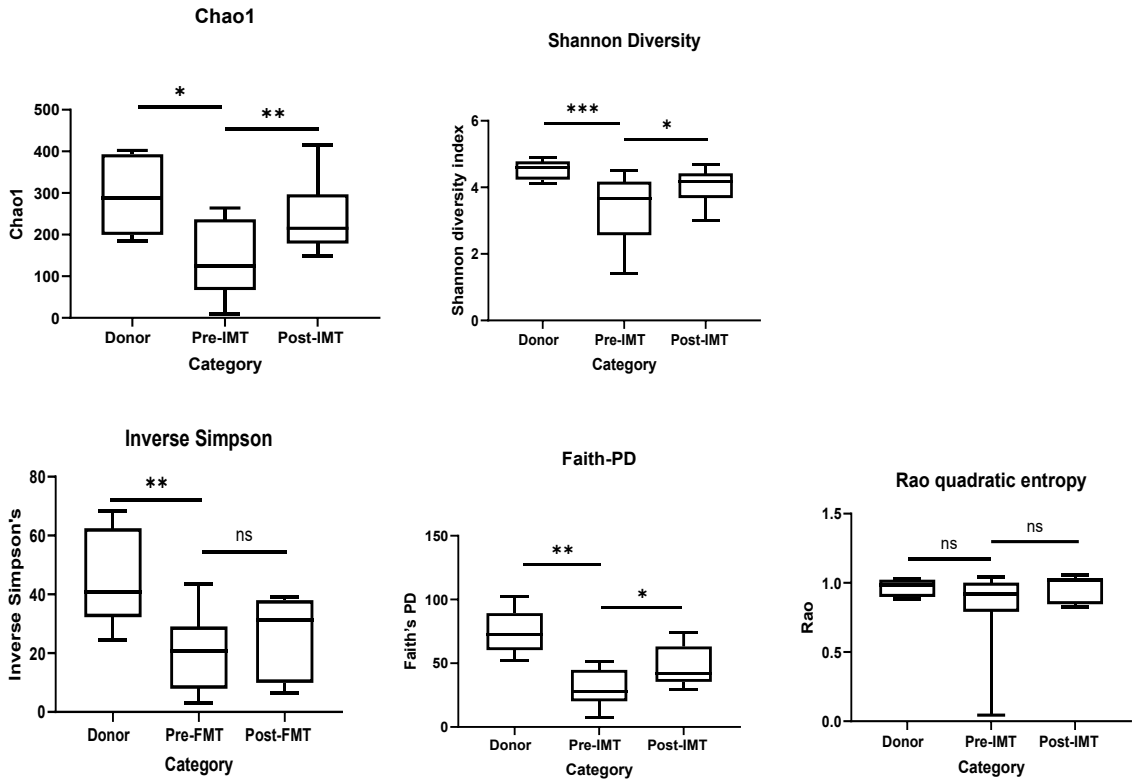

Figure 21: Box plots comparing the selected alpha diversity indices in donor, pre-IMT and post IMT stool. Welch's t-test, 2 sided. Donor: n=5; pre-IMT: n=16; post-IMT: n=9.

Metataxonomic analysis was performed on stool samples from donors (n=5), and patients pre- (n=16) and post IMT (n=9). The increase in number of pre-IMT samples was due to samples being donated from potential IMT patients who ultimately did not receive their IMT. Commonly used alpha diversity indices was compared. Donors had significantly increased richness in Chao1 (P=0.002), Shannon (P=0.0006), Inverse Simpson (P=0.002), and Faith PD (P=0.0034) metrics then pre-IMT patients who were colonised with MDROs. Post IMT there was a significant increase in the Chao1 (P=0.0091), Shannon (P=0.04), and Faith PD (P=0.03) metrics. There was no change between donors and pre-IMT or pre versus post in the Rao quadratic entropy metric, and no significant difference in Inverse Simpson between pre and post IMT patients. (Figure 21)

5.3.1.2 Alpha diversity in matched paired samples

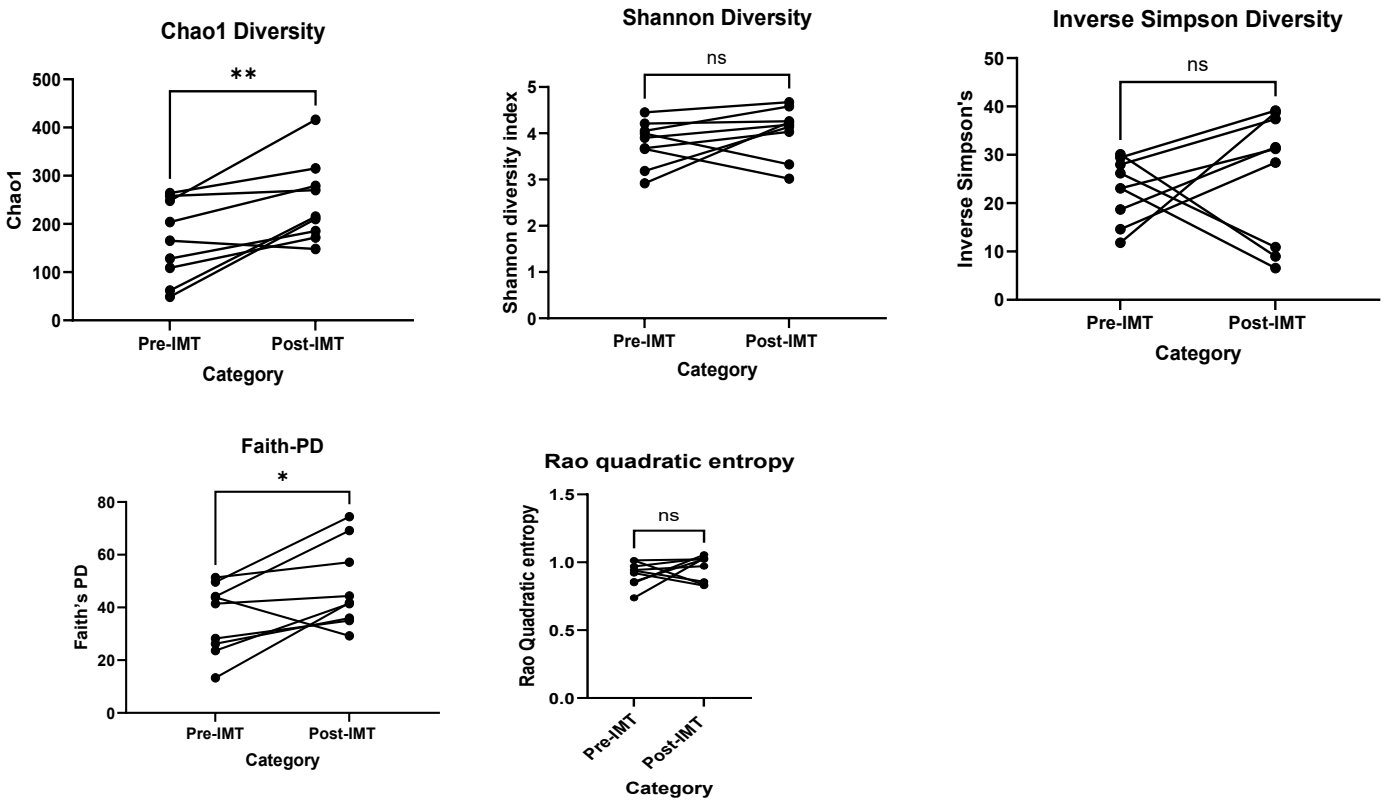

Figure 22: Ladder plots comparing the effect of IMT on selected alpha diversity metric indices.

Paired t-test, 2 sided-FMT: n=9; post-FMT: n=9

Alpha diversity metrics in paired samples of patient stool pre-and post-IMT was compared using a paired t-test. There was significant increase in in Chao1 diversity (P=0.0067) and Faith PD (P=0.03) diversity measures. (Figure 22)

5.3.1.3 Beta Diversity

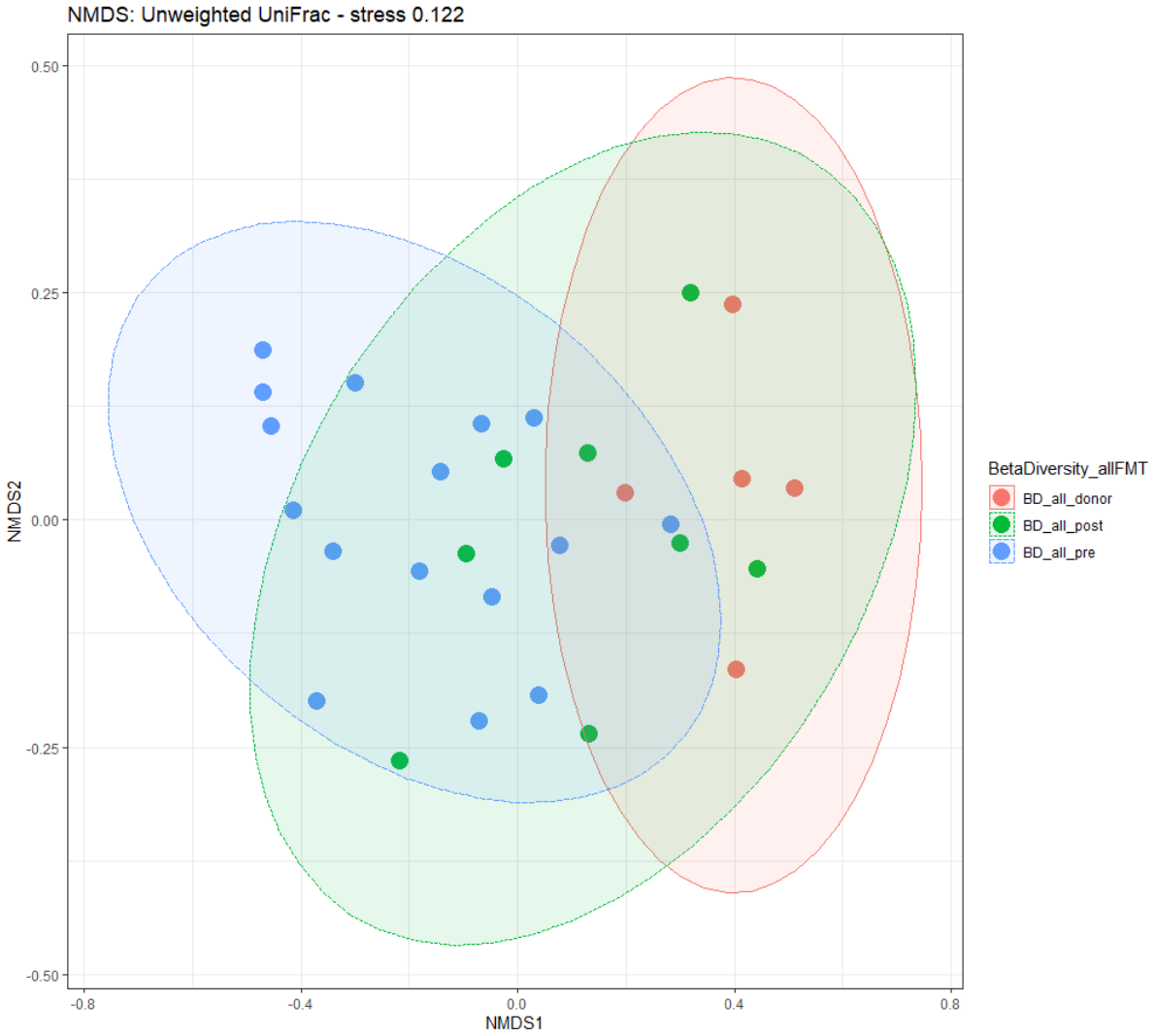

Figure 23: Beta diversity across donors, pre and post IMT

To compare the community diversity and phylogenetic relatedness between donors and patients pre and post IMT, beta diversity was analysed using weighted Unifrac distances between samples (Figure 23). Plotting these distances using a non-metric multi-dimensional scaling (NMDS) model demonstrated that IMT did not appear to cause a uniform uni-directional shift in phylogenetic relatedness. Although the permutational multivariate analysis of variance (PERMANOVA) was significant between donors and both pre-IMT (P=0.016) and post-IMT (P=0.03), there was not a significant change pre and post IMT.

5.3.1.4 Comparison of relative abundances of the stool metataxonomic profiles between donors, pre-IMT and post-IMT samples

16S rRNA gene sequencing data derived from the initial human dataset was analysed at multiple taxonomic levels. STAMP analysis did not demonstrate any statistical difference between the groups across family, genera, or phyla.

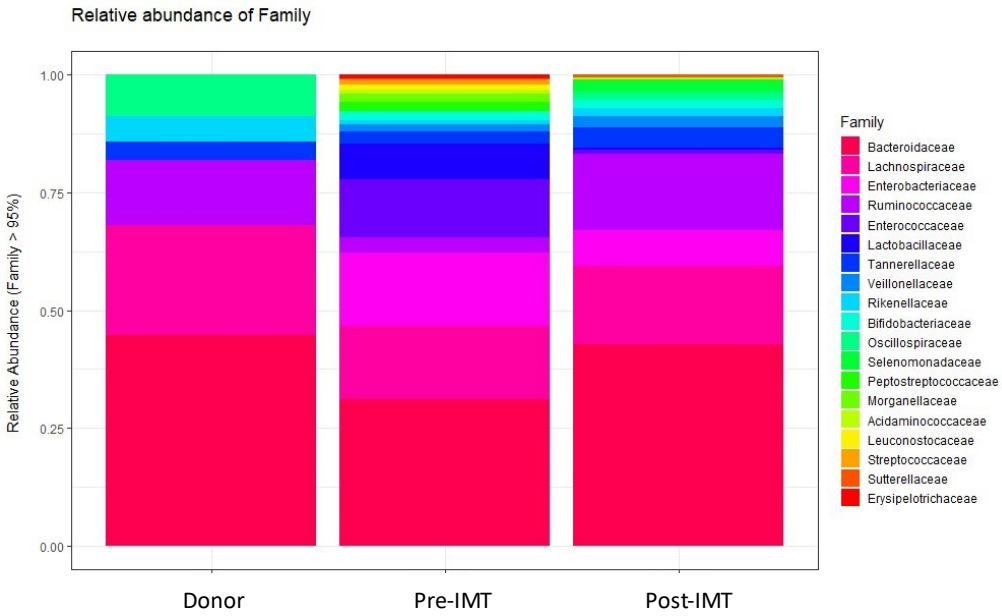

Figure 24: Bar chart showing the metataxonomic relative abundance by family comparing donors with pre- and post-IMT.

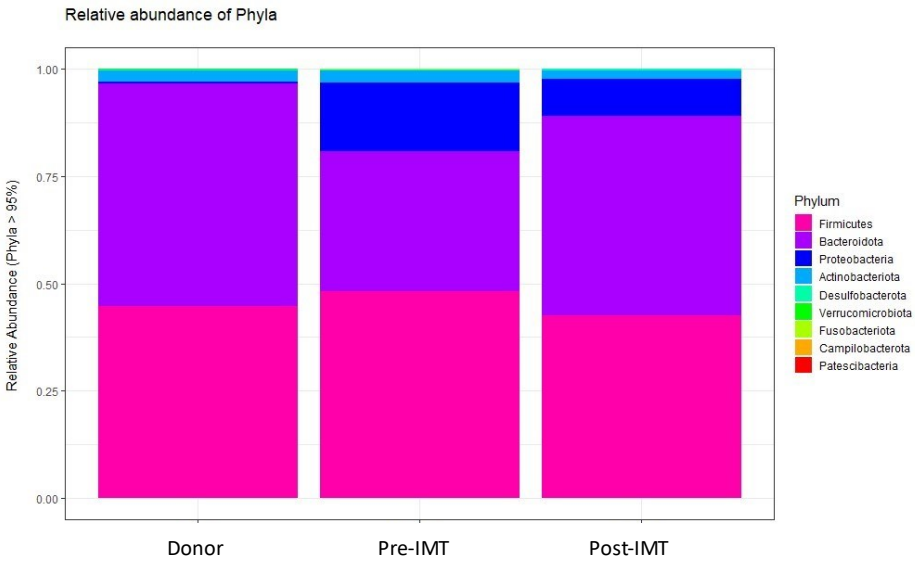

Figure 25: Bar chart showing the metataxonomic relative abundance by phyla comparing donors with pre- and post-IMT.

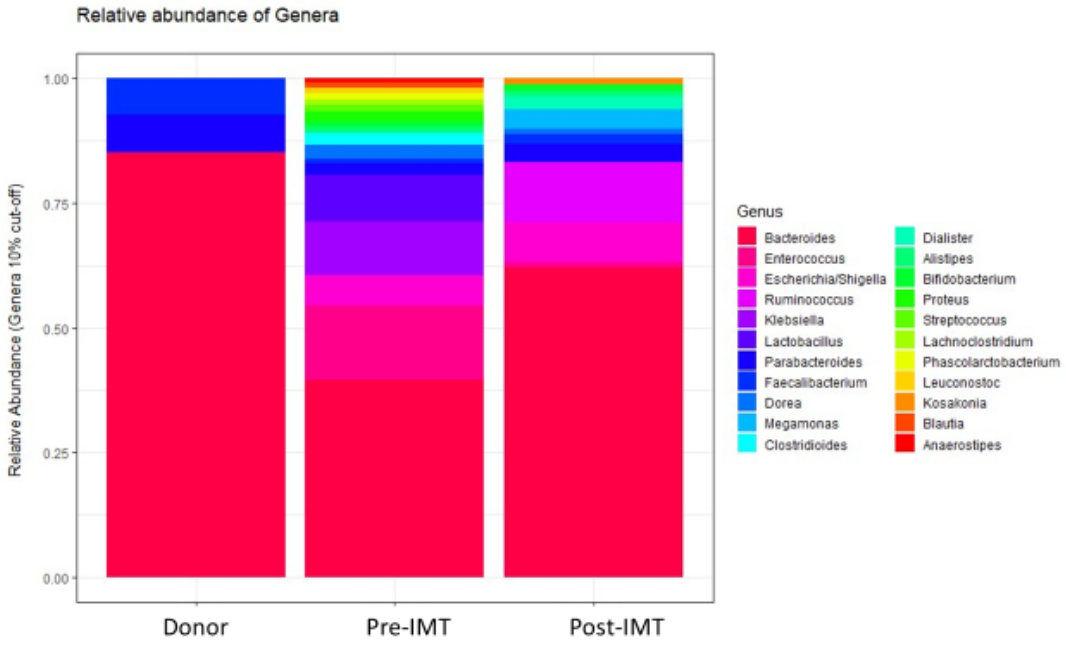

Figure 26: Bar chart showing the metataxonomic relative abundance by genera comparing donors with pre- and post-IMT.

5.3.2 Metagenomics Results

5.3.2.1 Metagenomic analysis of commensal bacteria

Table 18: Differences in commensal bacteria recognised to play a role in colonisation resistance pre and post IMT.

Commensal bacteria with reported effect on colonisation resistance					
Bacteria	**Paired t-test**		**Bacteria**	**Paired t-test**	
Akkermansia muciniphila	P=0.2500		*Desulfovibrio spp*	P=0.0926	
Bacillus thuringiensis	P=0.3624		*Erysipelatoclostridium ramosum*	P=0.026	↓
Bacteroides fragilis	P=0.5429		*Erysipelatoclostridium saccharogumia*	P=0.8907	
Bacteroides thetaiotaomicron	P= 0.3624		*Faecalibacterium prausnitzii*	P=0.1827	
Barnesiella spp	P=0.8614		*Lactobacillus spp*	P=0.3613	
Bifidobacterium bifidum	P=0.1694		*Lactobacillus crispatus*	P=0.3782	
Blautia hansenii	P=0.6012		*Lactobacillus paracasei*	P=0.698	
Blautia producta	P=0.0180	↑	*Lactococcus lactis*	P=0.0949	
Clostridium bolteae	P=0.0120	↓	*Odoribacter laneus*	P=0.0949	

Coprococcus spp	P=0.0247	↓	*Parabacteroides spp*	P=0.5919	
Coprococcus catus	P=0.0013	↑	*Pseudoflavonifractor capillosus*	P=0.1718	
Coprococcus eucatus	P=0.3118		*Roseburia hominis*	P=0.2853	

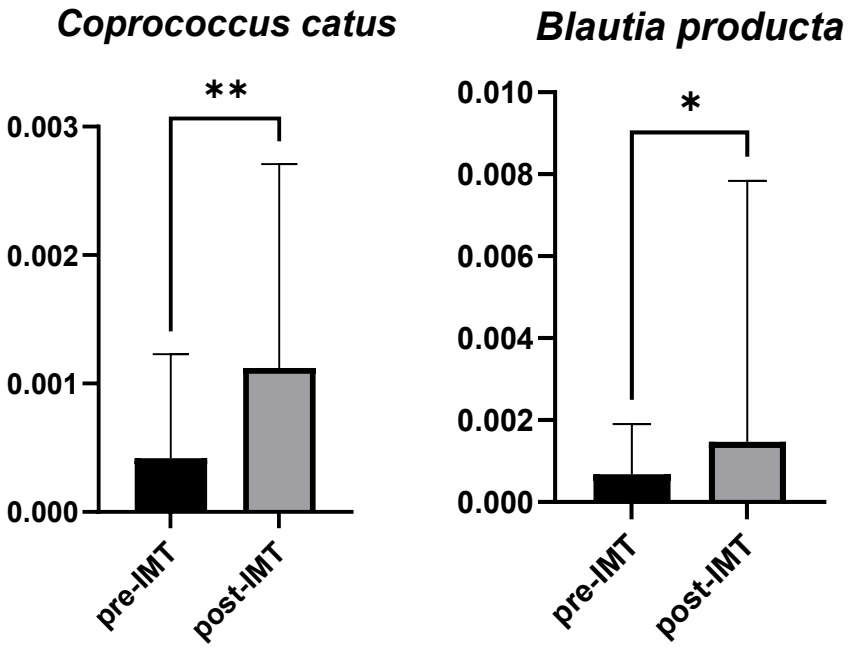

Figure 27 Difference in level of the commensal bacteria, Coprococcus catus and Blautia producta between pre and post IMT

Twenty-three commensal bacteria recognised to have play a role in colonisation resistance were analysed (Table 18). *Blautia producta* (P=0.018) and *Coprococcus catus* (P=0.0013) both significantly increased post-IMT (Figure 27).

5.3.2.2 Metagenomic analysis of pathobionts

Table 19: Paired t-test of known intestinal pathobionts pre and post IMT.

Pathobionts		
Bacteria	**Paired t-test**	
Acinetobacter baumannii	*p=0.25*	
Citrobacter freundii	*p= 0.6753*	
Enterobacter cloacae	*p= 0.6753*	
Enterococcus faecium	*p=0.0461*	↓
Escherichia coli	*p=0.5813*	
Klebsiella pneumoniae	*p=0.6623*	
Morganella morganii	*p=0.6564*	
Pseudomonas aeruginosa	*p=0.5000*	
Serratia marcescens	*p=0.0575*	

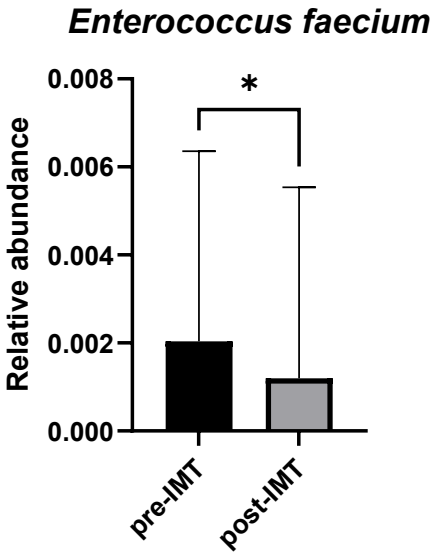

Figure 28: Effect of IMT on the level of Enterococcus faecium in the stool.

The relative abundance of known pathobionts that colonise the intestinal microbiome were compared in pre- and post-IMT donor stool. There was a significant relative decrease in

Enterococcus faecium (P=0.0461) post-IMT (Figure 28), but no difference in any of the *Enterobacterales* or non-fermentative Gram-negative bacilli (Table 19).

5.3.2.3 Metagenomic analysis of Antimicrobial resistance genes.

Table 20: Paired t-test of resistance genes expressed by Enterococcus *spp. pre and post IMT.*

ANTIMICROBIAL RESISTANCE GENES		
Glycopeptide resistance genes	**Paired t-test**	
vanY; glycopeptide resistance gene cluster	p=0.0051	↓
vanS; glycopeptide resistance gene cluster		
vanH; glycopeptide resistance gene cluster		
glycopeptide resistance gene cluster; van ligase		
glycopeptide resistance gene cluster; vanR		
vanZ; glycopeptide resistance gene cluster		
vanX; glycopeptide resistance gene cluster		
glycopeptide resistance gene cluster; vanT		
glycopeptide resistance gene cluster; vanXY		
vanW; glycopeptide resistance gene cluster		
glycopeptide resistance gene cluster; vanV		

Table 21: Paired t-test of Beta-lactamase resistance genes expressed pre and post IMT.

ANTIMICROBIAL RESISTANCE GENES		
Beta-lactamase genes		**Paired t-test**
CfxA beta-lactamase	SCO beta-lactamase	p=0.7158
ampC-type beta-lactamase	CBP beta-lactamase	
OXA beta-lactamase	OXY beta-lactamase	
TEM beta-lactamase	VIM beta-lactamase	
CTX-M beta-lactamase	CARB beta-lactamase	
SHV beta-lactamase	ACC beta-lactamase	
CblA beta-lactamase	DES beta-lactamase	
cepA beta-lactamase	MIR beta-lactamase	
CMY beta-lactamase	OKP beta-lactamase	
DHA beta-lactamase	YRC Beta-lactamase	
SRT beta-lactamase	BIL Beta-lactamase	
KPC beta-lactamase	BUT beta-lactamase	
ACI beta-lactamase	Sed beta-lactamase	
CcrA beta-lactamase	LEN beta-lactamase	
blaZ beta-lactamase	ADC beta-lactamase without carbapenemase activity	

NDM beta-lactamase	HERA beta-lactamase	
ACT beta-lactamase	CMH beta-lactamase	
LAP beta-lactamase	PDC beta-lactamase	
PER beta-lactamase	blaF family beta-lactamase	
	CphA beta-lactamase	

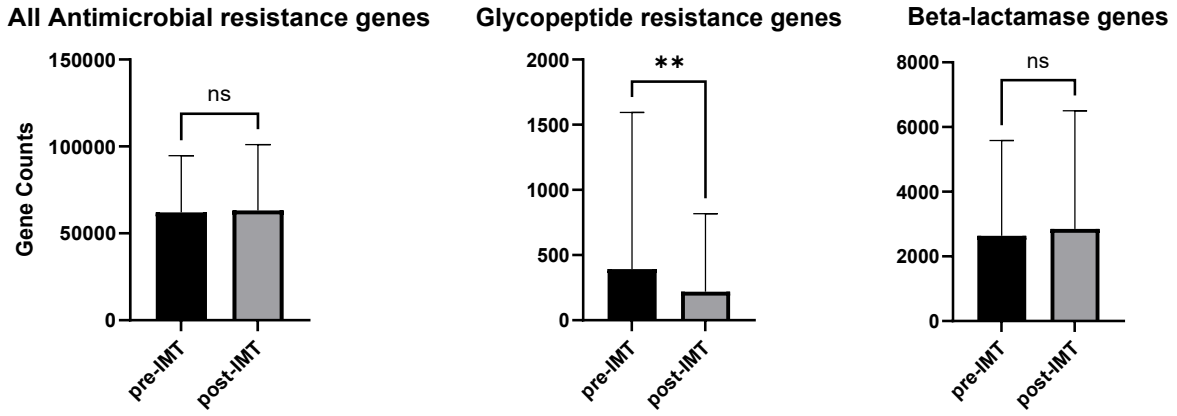

Figure 29: Comparison of shotgun metagenomic analysis of antimicrobial resistance genes in paired stool samples pre- and post-IMT

Relative abundances of ARG that cause transferrable resistance in *enterococci* and *Enterobacterales* were compared. There was a significant decrease in glycopeptide resistance genes (P=0.051); however, there was no difference in total ARGs overall or beta-lactamase genes.

5.4 Discussion

5.4.1 Metataxonomics

Consistent with previous studies looking at rCDI, IMT resulted in restoration of several measures of ecological diversity and richness of the microbiota back to a level comparable to that of healthy donor(178). Low alpha diversity has previously been reported as associated with increased risk of invasive disease in vulnerable populations, so could contribute to the effects seen in Chapter 4 in terms of reduction of invasive disease(69). Rao quadratic entropy diversity was not seen to have been significantly different in pre and post IMT, and interestingly this is closely related to beta diversity which was also not seen to be

significantly different. The reasons for this could be that this is not the correct measure for this cohort of patients, i.e., too broad, or could be related to sample size. Measures of "richness" such as Chao1 are likely to be more truly representative of the benefits of IMT versus the diversity in different microbial communities that Beta diversity (and Rao quadratic entropy) measure. Although Shannon and Inverse Simpson did not demonstrate significance, there appeared to be a distinct cohort of patients who appeared to have a positive change or no change. Further larger studies are needed to elucidate whether this could be due to patients who have and have not engrafted the IMT by this point. Further studies should likely use Chao1 as their baseline diversity indices as this appears to be the most sensitive in this context.

When comparing pre and post IMT patients, patients who did not have an IMT were excluded which meant the numbers were much smaller (n=9), so could explain the fact that there was a general trend in change post-IMT, but significance was only seen in Chao1 and Faith-PD diversity metrics.

5.4.2 Metagenomics

Metagenomics allowed for detailed analysis at a species level. Commensal bacteria that were recognised to have a positive impact in MDRO colonised patients (see Table *1*) were compared and *Blautia producta* and *Coprococcus catus* were seen to significantly increase post-IMT. In an ICU cohort, rectal colonization with VRE was inversely associated with *B. producta* (107). *Blautia producta* has been described in the literature in enabling inhibition of VRE colonisation in mouse studies(110). In another mouse model, Kim *et al*., identified a lantibiotic secreted by *Blautia producta,* the high abundance of which was associated with reduced density of *E. faecium.* (116). In other mouse studies, *B. producta* was seen to prevent colonisation and had antimicrobial activity against *Listeria monocytogenes.* The mechanisms by which *Coprococcus catus* might operate has not been explored in MDRO colonisation or infection. However, it is recognised to produce short chain fatty acids

(butyrate from fructose and propionate from lactate (via the acrylate pathway)(257), therefore may provide a wide-reaching protective role in production of SCFA, such as inducing production of AMPs (74) and inhibiting growth and fitness of pathogens, both directly and via routes including intracellular acidification (75).

In our cohort, IMT conferred a relative reduction of *Enterococcus faecium* and associated ARGs. Dominance of *Enterococcus* species has been described and is associated with low bacterial diversity, which is linked to poorer outcomes (69). Previous IMT studies which used decolonisation as their end point, noted that VRE was more readily decolonised than CPE. One reason for this could be that in mouse studies, *K. pneumoniae* was seen to be more effective than VRE at invading the mucus layer and translocating to mesenteric lymph nodes (258), which may be reflected by the fact it was more difficult to eradicate. The ability of Gram-negative organisms to form biofilms within the intestine could also be a potential factor in its persistence(259).

This lack of significant decrease in the Gram-negative bacteria and corresponding beta-lactamase genes reinforces the clinical data which did not reveal any change in rectal colonisation (see Table 3). Chapter 4 demonstrated an improvement in several clinical outcomes post-IMT. When looking at the diversity, the increase in alpha diversity was more similar to donors and increase in commensal bacterial recognised to have an immunomodulatory effect further strengthens the case that the microbiome may play a role in development of infection, and manipulation of its composition may be a key in preventing infection in at risk groups.

5.4.3 Limitations

A limitation of this study was the heterogeneity of the sample set. In the UK sample set, stool samples could only be collected opportunistically at their preassigned follow up clinical appointments, therefore there was a discrepancy in the post-IMT follow up dates. In the Polish sample set, samples were collected as early as seven days post procedure. Time to engraftment is variable in IMT, although by eight weeks around 71% of patients stabilise(260). The patient cohorts were also very different, the Israeli population were

mostly well outpatients who were noted to be colonised with an MDRO in the community, whereas the Polish patients (mostly haematology patients) and the UK patients (a mix of renal and haematology patients) likely had a different baseline microbiota composition.

6 Chapter 6 Analysis of the functionality of the gut microbiota via metabolomic studies and immunological assays

6.1 Introduction

The experiments in this chapter were to investigate the hypothesis that IMT provides restoration of gut barrier function. The samples used were from the same cohort as Chapter 5 but included serum samples donated from thirteen Polish recipients of IMT. Metabolomics allowed us to evaluate the dynamics of SCFA production pre and post IMT which are recognised to have a beneficial role in colonisation resistance. We also looked at the serum cytokine response which may reflect the function of the gut barrier. Multi-panel ELISA testing was performed on serum sample from eight patients for selected cytokines and proteins linked to gut barrier function and assess whether IMT was linked to restoration of gut barrier function by proxy of decreased markers of an inflammatory response such as selected cytokines and FABP-2,

6.2 Aims

The aim of this part of the study was to perform metataxonomic and metagenomic analysis on stool samples pre and post IMT to gain insights into the dynamic composition of the intestinal microbiota and analyse whether there are changes in the genes or bacteria which reflects the clinical benefit seen.

6.3 Ethics

The study was approved by a UK Research Ethics Committee (REC reference: 19/LO/0112). All patients, including patients used as controls, provided informed consent authorizing the use of their personal information for research purposes.

6.4 Methods

6.4.1 Sample collection.

See Table 16 for clinical and demographic characteristics of entire sample cohort.

6.4.1.1 Initial data set (United Kingdom)

The initial data set was collected in the UK at Imperial College from MDRO colonised participants pre and post IMT and donors. The sample set was as follows.

- Ten patients with stool samples pre and post IMT.
- Five donor stool samples
- Seven unmatched MDRO colonised stool samples (grouped with the pre-IMT cohort)

Samples were taken prior to IMT then collected for at least 6 months (range 15- 266 days) post-IMT via opportunistic screening at clinic appointments. Samples were collected using a FECOTAINER® collection device. Within two hours of sample donation, faecal samples were separated into twelve equal aliquots and frozen at -80°C in an Ultra-Low temperature freezer until processing for analysis consistently with standard protocols.

6.4.1.2 Israeli data set

Further samples were sent from Haifa, Israel from a prospective, interventional, cohort study at a single tertiary centre (Rambam Health Care Campus [RHCC]) preformed between February 2018 and April 2019 (99). The sample set consisted of

- 13 IMT recipient stool samples were all colonised with CPE. The samples were collected before IMT and at 14 days post IMT.
- Six donor stool samples
- Six unmatched CPE colonised stool samples (grouped with the pre-IMT cohort)

Faeces were delivered from Israel to London, the transfer took less than 24 hours and samples were kept in dry ice. All samples were kept at -80 °C until sample preparation for further analysis was performed.

6.4.1.3 Polish data set

These were samples taken from primarily Haematology patients from Warsaw, Poland. The sample set consisted of.

- Thirteen patients with stool samples pre and post IMT at several time points (7,14 or 28 days), eight of these patients also had EDTA blood sent.
- Thirteen donor serum samples

These samples were Faeces and plasma in EDTA tubes were delivered from Poland to London, the transfer took less than 24 hours and samples were kept in dry ice. All samples were kept at -80 °C until sample preparation for further analysis was performed.

6.4.2 Metabolomic studies

Short-chain fatty acids

6.4.2.1 Sample preparation for Metabolomic studies.

Faecal samples were prepared by mixing 250-300 mg of homogenised faecal samples with high performance liquid chromatography (HPLC) - grade water at a ratio of 1 mg faeces to 2 µL in 2 mL safe lock tubes. Once in the tube, samples were vortexed for 10 min and centrifuged at 20,000 x g for 10 min at 4°C. The supernatant (600 µL) was mixed with 60 µL of NMR buffer in a new 1.5 mL micro centrifuge tube. The samples were vortexed and spun for 10 s before an aliquot of 570 µL was transferred into 5-mm-diameter NMR tubes.

6.4.2.2 ^{1}H-NMR spectroscopy

Solution ^{1}H-NMR spectra of all samples were acquired using a Bruker IVDr 600 MHz spectrometer (Bruker 19 BioSpin) operating at 14.1 T and equipped with a 5 mm BBI probe with 2 H decoupling probe including a z-axis gradient coil, an automatic tuning-matching (ATM), high level shimming and an automatic refrigerated sample handling robot (Sample-Jet). Temperature was regulated to 310 ± 0.1 K. The following standard one-dimensional (1D) pulse sequence was performed: RD– gz1– 90°– t190°– tm– gz2– 90°– ACQ54. The relaxation delay (RD) was set at 4 s, 90° represents the applied 90° radio frequency pulse,

interpulse delay (t1) was set to an interval of 4 µs, mixing time (tm) was 10ms, magnetic field gradients (gz1 and gz2) were applied for 1 ms and the acquisition period (AQA) was 2.7s. Water suppression was achieved through irradiation of the water signal during RD and tm. A spectral width of 12,000 Hz was used for all the samples. Prior to Fourier transformation, the free induction decays (FIDs) were multiplied by an exponential function corresponding to a line broadening of 0.3 Hz. For each patient sample, two NMR experiments were acquired in automation: a general profile 1 H-NMR water pre-saturation experiment using a one-dimensional pulse sequence (where the mixing time of the 1D-NOESY experiment is used to introduce a second pre-saturation time), and a spin echo ediprotons from using the Carr–Purcell Meiboom–Gill (CPMG) pulse sequence (which filters out signals from fast T2 relaxing protons from molecules with slow rotational correlation times such as proteins and other macromolecules). The CPMG pulse sequence had the form RD– 90°– (t– 180°– t) n– ACQ. The acquisition parameters were set using the same settings as the standard 1D pulse sequence, with the spin-echo delay (t) set at 0.3 ms and 128 loops (n) performed. Continuous wave irradiation was applied at the water resonance frequency during the relaxation delay (RD). Relaxation time was set to 4 s. Spectral width was always set onto the water resonance and optimised for every dataset in order to optimise water signal depletion. 1 H-NMR spectra were automatically corrected for phase and baseline distortions and referenced to the TSP singlet at δ 0.0 using TopSpin 3.1 software. Spectra were then digitised into 20 K data points at a resolution of 0.0005 ppm using an in-house MATLAB (MathWorks, version R2019b) script. Spectral regions corresponding to the internal standard (−0.5 to 0.5 ppm) and water (4.6–5.0 ppm) peaks were removed. In addition, the region containing urea (5.4–6.3 ppm) was removed from the plasma spectra due to its tendency to cross-saturate with the suppressed water resonance. All ^{1}H-NMR plasma spectra were of high quality and resolution, and were quality controlled using the nPYc toolbox. SMolESY and SMolESY-select were employed to aid metabolite assignment and quantification respectively, via MATLAB programming suite (MathWorks, version R2019b). For absolute quantification reference, the ERETIC57 signal was employed. For statistical

analyses, features were mean-centred and univariance scaled. Prominent glycerol peaks were visualised and manually inspected to correct for this.

6.4.2.3 Statistical analysis

In this study, in-house developed linear mixed-effects models were used to examine the relationship between selected metabolites extracted from faecal water NMR data and the following clinical variables of interest. The metabolites selected were short-chain fatty acids previously reported in the literature as having a role in the prevention of MDRO colonisation (261). The main clinical variable was development of post-IMT "culture positive" infection, i.e., infection with the causative pathogens identified from the clinical samples. The following linear mixed-effects models were used to allow for adjustment for possible confounders in the data.

1) Comparison of culture positive infection pre-IMT versus those who did not develop culture positive infection in post-IMT samples.
   Covariant selected: Sex, age, invasive infection pre-IMT, underlying condition, bowel cleansing and FMT dose.
2) Comparison of culture positive infection post-IMT versus those who did not develop culture positive infection in pre-IMT samples.
   Covariant selected: Sex, age, invasive infection pre-IMT, underlying condition, bowel cleansing and FMT dose.
3) Comparison of patients having a reduction in invasive infection with those who do not have this reduction in pre vs post-IMT samples.
   Covariant selected: Sex, age, underlying condition, timepoint, mode of IMT.

The models were fitted using the lme4 R package and adjusted for multi-omics dataset specifics. The models incorporated random effects to account for the choice donor, participant measurements (for longitudinal data), differences in recruitment site location. The NMR data was modelled as an interaction with the clinical variables of interest, while considering age, sex, country of origin, mode of IMT (i.e., enema, naso-gastric or colonoscopy route), whether they had bowel cleansing before IMT, dose of IMT and

underlying condition as covariates. Continuous covariates (age, sex, country of origin, and underlying condition etc) were scaled to mitigate extreme value effects and reduce result uncertainty. Nominal p-values were adjusted using the Benjamini-Hochberg method with a target FDR of 0.10.

6.4.3 Methods for Immunological analysis of serum

6.4.3.1 Sample collection.

A total of twenty-one serum samples were collected from Polish cohort and consisted of eight patients pre and one or more time points post IMT.

Table 22: Timepoints of serum taken from eight patients pre and post IMT from Polish cohort.

Patient	Time points pre and post IMT			
		Days post IMT		
PL4	Pre	7	14	30
PL5	Pre	7	14	30
PL8	Pre			30
PL14	Pre	7	14	
PL19	Pre	7		
PL20	Pre	7		
PL23	Pre	7		
PL24	Pre	7		

6.4.3.2 Measurement of serum FABP-2

The level of FABP-2 was measured in serum samples from the patients enrolled in the study, using a Human FABP2/I-FABP Quantikine Enzyme-Linked immunosorbent Assay (ELISA, R&D, USA) Kit, which is essentially a sandwich ELISA. All reagents were brought to RT as per manufacturer's instructions. The wash buffer, substrate solution and Human FABP-2 standard were constituted as per protocol. Firstly, 50 µL of Assay diluent RD1-63 were added to each well. Secondly, another 50 µL of standard, control or serum sample were added to each well. Sera were prepared using a 5-fold dilution and were added to the plate in duplicates, i.e., each sample was aliquot to two consecutive wells. The plate was covered with the adhesive strip provided and incubates at RT on a horizontal orbital microplate shaker set at 500 ± 50 rpm. After two hours of incubation, each well was washed with 400 µL of wash buffer for three times. After the last wash, wash buffer was completely aspirated, and the plate was inverted and blotted against clean paper towel so that any remaining buffer could be removed. At the following step, 200 µL of Human FABP-2 Conjugate was transferred to each well. The plate was then covered with a new adhesive strip and incubated at RT on the shaker. After two hours, another sequence of aspiration and wash was performed. A 200 µL of the Substrate solution was aliquoted in each well, while the plate was left for incubation for 30 minutes at RT on the benchtop, carefully protected from light. Afterwards, 50 µL of the Stop Solution was also added to each well and a change of colour from blue to yellow was noted in the wells. Finally, the 96 well plate was read at 570 nm on a Thermoscientific Multiskan machine.

6.4.3.3 Measurement of serum cytokines

The serum levels of IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8 (CXCL8), IL-10, IL-12p70, IL-13, and TNF-α were measured using a sandwich immunoassay (V-plex Proinflammatory Panel 1 (MSD). All reagents were left at RT. Calibrator dilutions, controls, detection antibody solution, wash buffer and read buffer T were constituted as per protocol. The MSD plate was washed 3 times with 150 µL of wash buffer to provide greater uniformity of the results of the assay. Fifty µL of samples, calibrators and controls were added in each well. Filtered FW samples

were loaded on the MSD after a 2-fold dilution with Diluent 2. Serial 4-fold dilutions of the standards were run to generate a 7-standard concentration set, and the diluent alone was used as a blank. The plate was then sealed with an adhesive plate seal and incubated at RT on a shaker for 2 hours. Afterwards, the plate was washed again for three times with 150 µL of wash buffer. A volume of 25 µL of detection antibody solution was transferred to each well. The plate was sealed with an adhesive seal and incubates at RO with shaking for 2 hours. Another sequence of three washes with 150 µL of wash buffer was done with the MSD plate. As final step, 150 µL of 2 X read buffer T was added to each well. The plate was then analysed on an MSD reader within 30 minutes. The standard curves for each cytokine were generated using the premixed lyophilized standards provided in the kits. Values of cytokines which were at or lower than the lower limit of detection (LLOD) were reported as LLOD for these analytes. Median LLOD were derived from the assay protocol.

6.4.3.4 Statistical analysis

The distribution of variables was explored using the Shapiro-Wilk test. Continuous variables were reported as medians and IQR, while categorical variables were expressed as relative frequencies and percentages. Univariate analysis was carried out using Mann-Whitney for continuous, and chi-square test for categorical variables respectively. Kruskal-Wallis or ANOVA with post-hoc corrections was used for comparison between multiple groups. Spearman correlation and logistic regression carried out to explore the relationship between variables. All tests were two-sided and a P value 0.05 was considered significant. Statistical analysis was performed using GraphPad Prism version 9.3.

6.5 Results

6.5.1 Metabolomic studies

Acetate, Butyrate, Formate, Isovalerate, Propionate, Succinate and Valerate were the short-chain fatty acids chosen to apply statistical modelling to.

6.5.1.1 Comparison of selected faecal metabolites in post-IMT faecal samples between patients who developed culture positive infection versus those who did not.

Post IMT samples from patients who did and did not develop culture positive infection were compared. Higher stool valerate showed a strong negative correlation with culture positive infection pre-IMT (P=0.0094).

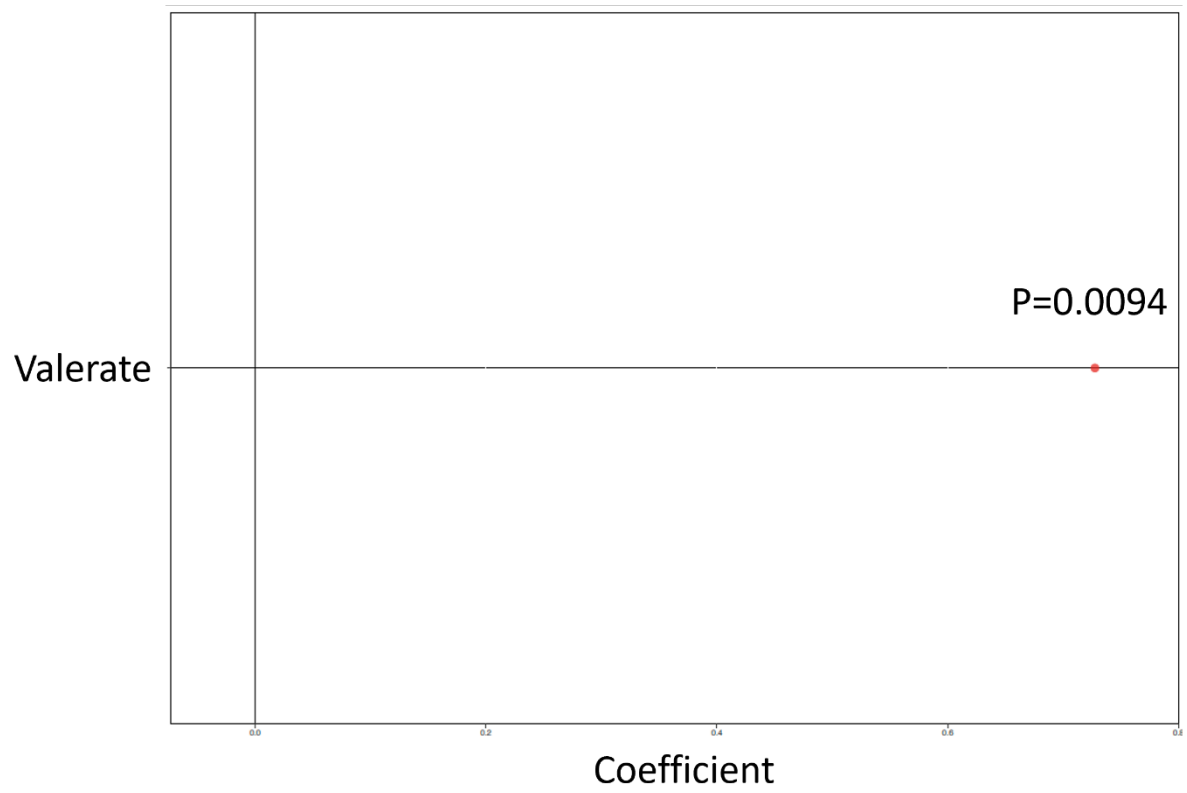

Figure 30: Linear mixed effect model for post-IMT samples comparing patients who did and did not develop culture positive infection post-IMT.
P values in the graph are adjusted with Benjamini-Hochberg with a target FDR of 0.10

6.5.1.2 Comparison of selected faecal metabolites in pre-IMT faecal samples between patients who developed culture positive infection versus those who did not.

Pre-IMT samples from patients who did and did not develop culture positive infection were compared. Valerate (P=0.0003), Propionate (P=0.095) and Isovalerate (P=0.095) all had a positive correlation with those who did not develop culture positive infection. Butyrate (Butyrate 1: P=0.062, Butyrate 2 P=0.059) and Formate both had a negative association with the same population.

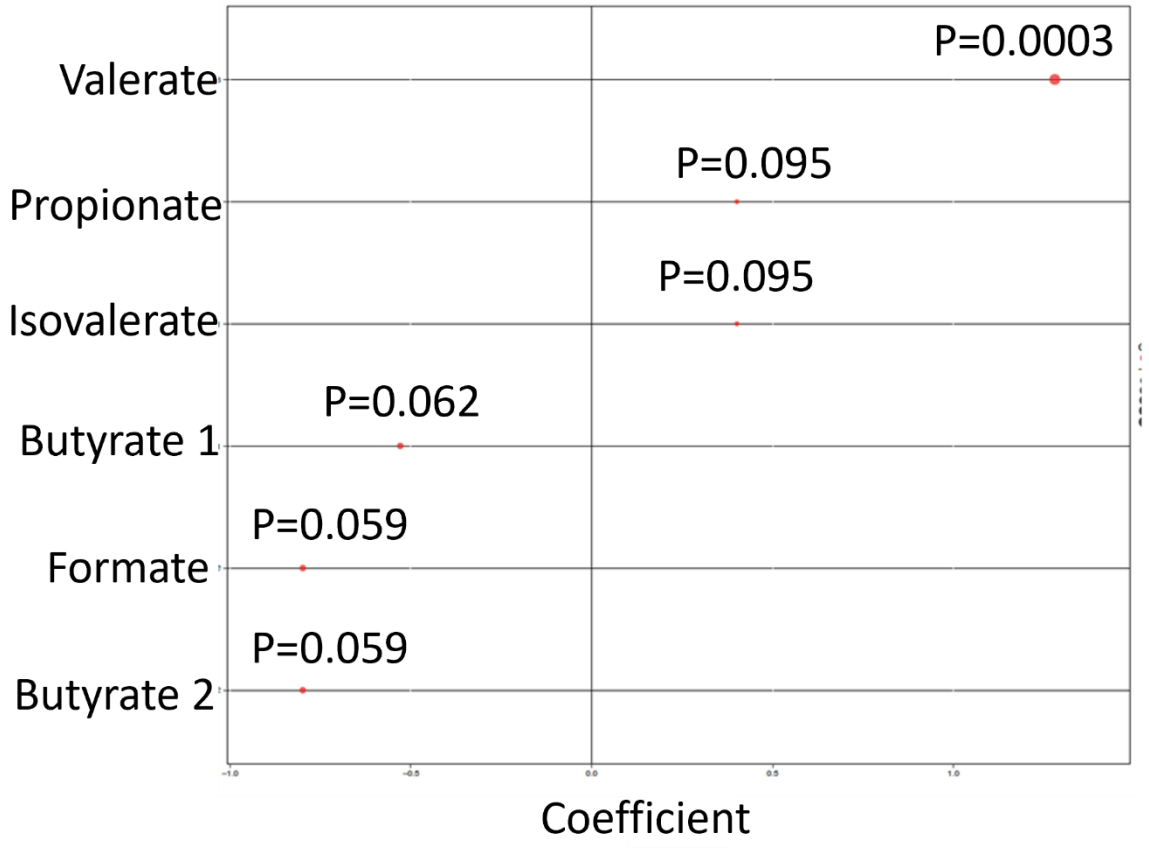

Figure 31: Linear mixed effect model for stools pre- IMT samples comparing patients who did and did not develop culture positive infection post-IMT.

P values in the graph are adjusted with Benjamini-Hochberg with a target FDR of 0.10

6.5.1.3 Reduction in invasive infection

When looking at patients where there had been a change in infection (i.e., comparing those who had an increase or decrease in infections post-IMT), higher stool valerate (P=0.05), propionate (P=0.066) and isovalerate (P=0.066) levels were correlated with reduced invasive infection between pre vs post-IMT, whilst higher stool butyrate (P=0.02) and formate (P=0.002) correlated with increased infection.

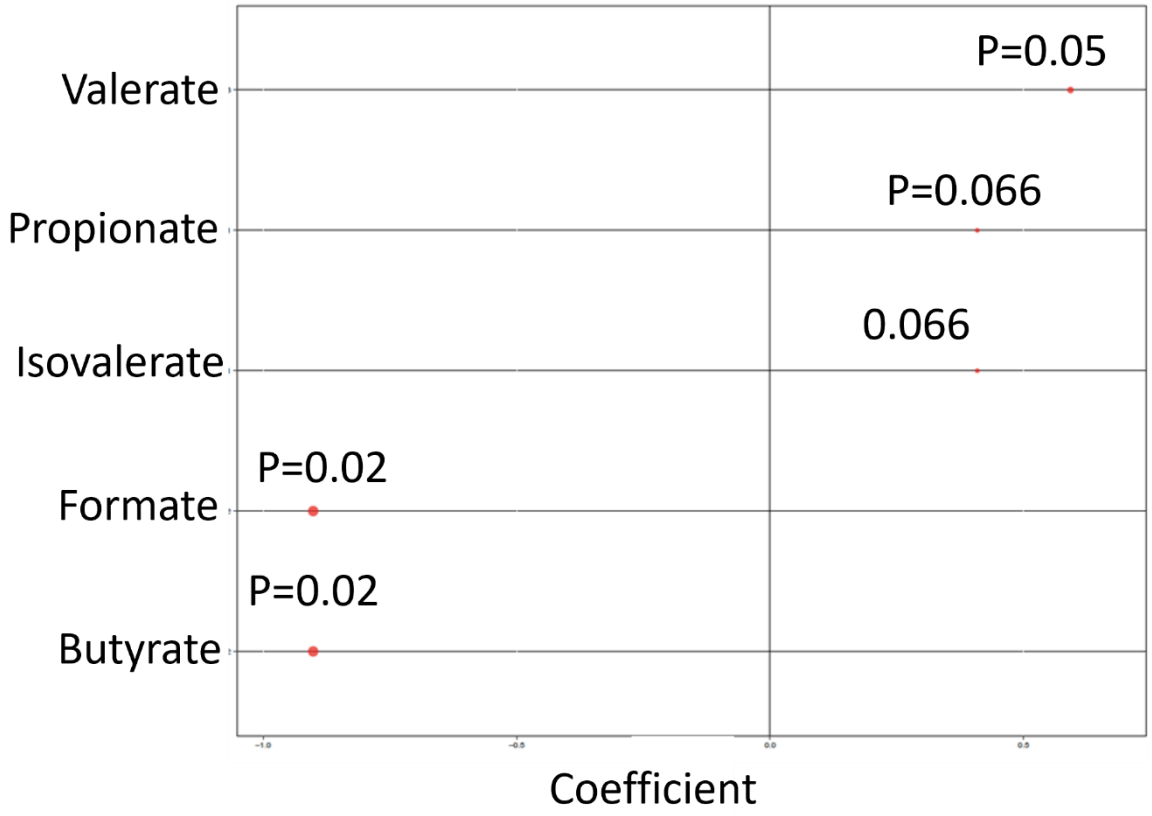

Figure 32: Linear mixed effect model looking at change in number of infections pre and post IMT.

P values in the graph are adjusted with Benjamini-Hochberg with a target FDR of 0.10

6.5.2 Immunological assay results

6.5.2.1 FABP-2 levels pre and post IMT

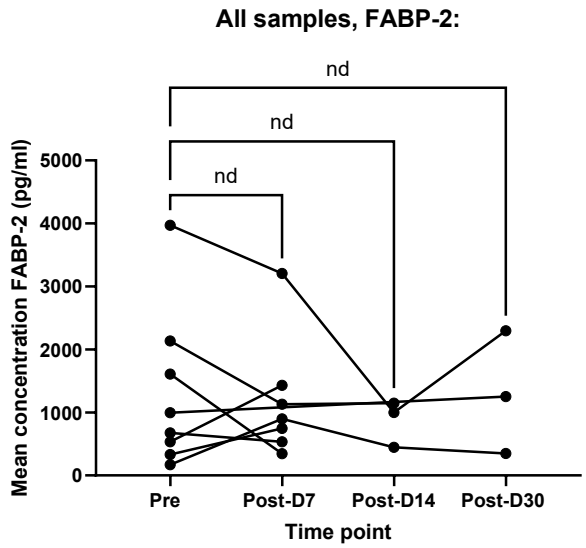

Figure 33: Fatty acid binding protein 2 measurement in serum samples in eight patients pre and post Intestinal Microbiota Transplantation

Mean serum concentration for FABP-2 was 1242.57 (170.29-2133.49) pg/ml before IMT and 1089 (345.89 -2296.06) pg/ml post IMT. Overall, there was no difference in terms of levels of serum FABP-2 across study groups.

6.5.2.2 Serum cytokine levels pre and post IMT

Overall, there was a significant decrease in the levels of TNF-α (P=0.0057, Figure 34) and Il-8 (P=0.0057, Figure 35) post IMT. There was no significant change in IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70 or IL-13.

Serum Immunological Markers		
Cytokine	**Mann-Whitney**	
IL-1beta	p=0.69	
IL-8	p=0.0057	↓
TNF-alpha	p=0.0057	↓
IL-10	p=0.1801	
IL-6	p=0.3781	

IFN-gamma	p=0.4042	
IL-2	p=0.9596	

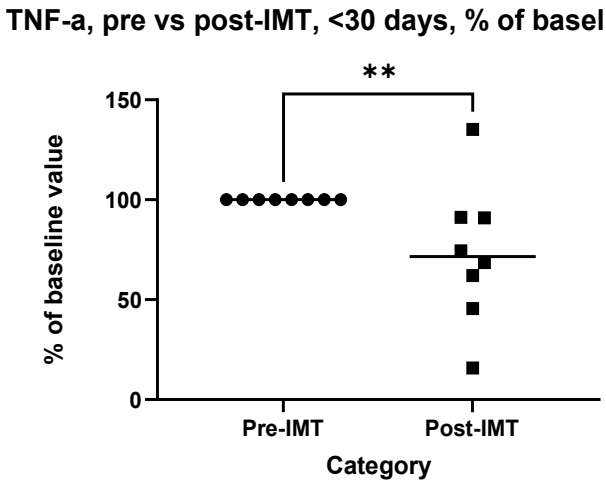

Figure 34: Change in levels of serum TNF-a levels measured pre and post IMT expressed as % change from baseline sample.

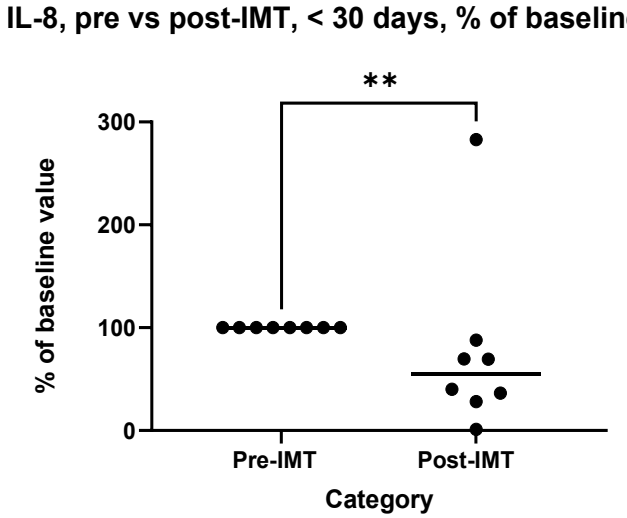

Figure 35: Changes in serum IL-8 levels pre and post IMT expressed as % change from baseline sample.

Table 23: Concentrations of serum cytokines pre- and post-IMT.

This table shows median values and related IQR of levels of serum cytokines in the study groups. Abbreviations: IQR: interquartile range, IFN-γ: interferon gamma, IL-1β: interleukin-1beta, IL-2: interleukin-2, IL-4: interleukin-4, IL-6: interleukin-6, IL-8: interleukin-8, IL-10: interleukin-10, IL-12p70: interleukin-12p70, IL13: interleukin-13, TNF-α: tumour necrosis factor alpha.

	PRE IMT MEDIAN (IQR)	POST IMT MEDIAN (IQR)
IFN-gamma pg/ml	13.25 (12.97-49.82)	11.11 (7.01-33.91)
IL-1 beta pg/ml	0.13 (0.06-1.25)	0.29 (0.11-0.71)
IL-10 pg/ml	1.09 (0.76-2.23)	1.11 (0.59-1.59)
IL-12p70 pg/ml	0.01 (0-0.64)	0.00 (0-0.35)
IL-13 pg/ml	0.00 (0-2.1)	0.00 (0-0.52)
IL-2 pg/ml	0.12 (0.12-0.29)	0.22 (0.07 -0.75)
IL-4 pg/ml	0.00 (0-0)	0.00 (0-0)
IL-6 pg/ml	4.03 (2.31-16.73)	4.75 (1.47 -11.01)
IL-8 pg/ml	91.94 (51.77-739.5)	48.92 (19.95 – 132.95)
TNF-alpha pg/ml	1.21 (1.11-5.61)	0.85 (0.71 -1.75)

6.6 Discussion

6.6.1 Metabolomic studies

In this study, ^{1}H-NMR spectroscopy was performed to evaluate the dynamic changes in SCFA expression pre- and post-IMT. SCFA are a source of fuel for colonocytes and is fundamental in maintaining epithelial hypoxia(262). Previous mouse studies have shown maintenance of an acidic environment plus with production of high concentrations of SCFA by the caecal and colonic microbiota is critical in preventing the expansion and promoting clearance of the antibiotic-resistant isolates of *K. pneumoniae*, *E. coli*, and *P. mirabilis*(263).

In pre-IMT samples, valerate was demonstrated to be a positive indicator that a patient would not develop a culture positive infection. Post IMT samples which had higher levels of valerate, propionate and isovalerate were seen in patients who did not develop a culture positive infection, and in the those who had a reduction in the frequency of culture positive infections. This may suggest that even in MDRO colonised patient, valerate may have a protective effect. Previous studies have demonstrated that valerate significantly inhibited

the growth of *C. difficile in vitro* (with minimal effect on other commensal gut bacteria) and was depleted in stool from patients with rCDI but was restored after successful IMT(75). Interestingly in other mouse studies, valerate was noted to decrease 66-fold after antibiotic treatment, suggesting a decrease in carbohydrate fermentation by members of the gut microbiota(159). One proposed mechanism of activity by valerate could be the anti-inflammatory effect on the host immune phenotype by inhibiting histones deacetylases (HDAC) (264). Exogenous valerate has been demonstrated in rodent models to reduce colitis via HDAC inhibition(265). In a recent study, mice treated with a metabolite mix which contained valerate and propionate had an average of 87% less *E. coli* CFU per gram of faeces compared to PBS-treated mice(266). Similarly in mouse models, *Bacteroides* production of propionate mediates colonization resistance to *S. typhii*(267).

Interestingly, butyrate and formate had a negative correlation with prevention of culture positive infection. Although butyrate has been cited as having a positive effect on invasive infection(264,268), Recent mice experiments have revealed that this may be dependent on the conditions (i.e., sugar concentrations) that the *Bacteroides* that produces the butyrate is exposed to (268). Interestingly formate which also fell post IMT is recognised as an energy source for bacteria, and associated with gut inflammation so a decrease in formate levels supports the decrease in invasive infection(269,270).

6.6.2 Makers of gut barrier function

In this study, FABP-2 levels did not differ among groups, suggesting the presence of an intact intestinal epithelium in the study groups. IL-8 and TNF- α were significantly reduced post-IMT. IL-8 is a proinflammatory cytokine, reduction of which indicates reduced disruption to the gut barrier, and its secretion is induced by TNF- α(271). Of note, TNF-α has been shown to disturb tight junctions and increase gut permeability, which may increase gut translocation of MDROs (272).

6.6.3 Limitations

Although faecal sampling to infer microbial SCFA production is a relatively easy and non-invasive technique in humans they may not be a reliable reflection of colonic fermentation in vivo. >95% of SCFAs are absorbed within the colon but are not quantifiable in the peripheral serum as SCFAs absorbed in the colon are transported to the liver via the portal vein. Ingestible intestinal sensors can sample from multiple regions of the intestinal tract, which may be a more representative but more costly and invasive measure(273). Serum samples were only taken pre- and post-IMT from selected patients in the Polish cohort which reduced the sample size.

6.6.4 Future Directions

To look at the expression of bile acid expression UPLC-MS bile acid profiling could also reveal changes relevant to MDRO colonisation and infection undetected by NMR based global profiling. There are many far reaching cytokines and small molecules that could are recognised to be related to gut barrier function that could be investigated with functional studies or immunohistochemistry experiments, including measuring faecal cytokines and other markers of gut barrier function such as anti-F-actin IgA antibodies and zonulin(274,275).

Chapter 7: Conclusion

7.1 Summary and key findings

- Patients who have underlying health conditions are more likely to develop invasive disease from multidrug-resistant organisms colonising the intestine with a worse outcome profile.
- Intestinal Microbiota Transplantation for patient who are colonised MDROs requires careful consideration in terms of administration and donor stool preparation.
- Patients who undergo Intestinal Microbiota Transplantation are less likely to have invasive infection from their colonising MDRO post-IMT and in certain cohort have improved survival outcomes.
- Intestinal Microbiota Transplantation increases intestinal microbiota alpha diversity and reduces the burden of enterococci but not Gram-negative pathobiont.
- *Blautia producta* and *Coprococcus catus* are commensal bacteria that are seen to increase post IMT.
- Valerate, a short-chain fatty acid, is higher in patients who do not develop invasive infection.
- IMT is seen to reduce serum levels of TNF-α and IL-8, proinflammatory cytokines associated with gut barrier permeability.

7.2 Clinical implications of this project and future directions.

This project was a novel bench to bedside look at the impact of restoration of the intestinal microbiota and the effect on MDRO colonised patients. From a clinical epidemiological perspective point of view, it confirmed that focus of AMR and rectal colonisation should be the prevention of invasive infection in at risk groups, for whom there is the most impact. The results of the study also developed a framework for future IMT procedures for MDRO colonised patients, which had not to date been published in the literature.

The clinical outcomes indicated that IMT could be utilised as a modality for reducing invasive infection, not only could this improve morbidity and mortality in these groups, but

the reduction in inpatient stay and reduction in use of antibiotics means that there is an economic benefit to the use of IMT.

Administration of IMT via NG, capsules or colonoscopically, are not created from fixed quantities and are time consuming in terms of preparation and not without risk of transmission of pathogens(181). Positive clinical outcomes were correlated with an increase in alpha diversity of the intestinal microbiota and reduction in cytokines associated with gut inflammation. On a more granular level, commensal bacteria, and selected metabolites such as valerate were seen to increase preventing development of invasive disease without necessarily "decolonising" the intestine from the MDRO. This opens the door to further work looking at whether a refined consortium of metabolites or commensal bacteria have the same impact. Finally, the initial group of 20 patients although showed promising results, opens itself up to a randomised control trial, delineating haematology and renal transplant patients and assessing clinical and economic outcomes on a wider scale. From the results in Chapter 4 and 5, a multicentre phase IIa trial, the Microbiota Transplant Prior to Allogeneic Stem Cell Transplantation (MAST) study, which has been funded by the Medical Research Council's Developmental Pathway Funding Scheme is about to commence (ISRCTN13241761 https://doi.org/10.1186/ISRCTN13241761). This study will focus in on haematology patients and samples will be collected for further large scale microbiome analysis.

Plans are also underway for a similar study looking at recurrent UTIs based on the results found in this study, collecting urine, stool and blood from patients to see if we can replicate the findings in this study in recurrent UTIs and further explore the urobiome and intestinal microbiome. The data set of pre-and post-IMT in MDRO colonised patients is one of the largest of its kind so from a mechanistic perspective, the metagenomic and metabolomic analysis is planned to be exploited further with more complex modelling, to stratify if there are any other signals which can help correlate the relationship between the clinical outcomes and the microbiome dynamics. Examples of other mechanistic studies could be the trajectory of bile acids pre- and post-IM, as well as a larger range of SCFA. Faecal cytokines and other serological markers of gut inflammation could also be further explored

in future studies, as gut barrier function, appears in part to play a role in preventing pathobionts colonising the intestinal microbiome from crossing the gut barrier and resulting in invasive disease.

Abstracts and Publications Arising from this Thesis.

Journal articles

Yip, A.Y.G., King, O.G., Omelchenko, O. et al. Antibiotics promote intestinal growth of carbapenem-resistant Enterobacteriaceae by enriching nutrients and depleting microbial metabolites. Nat Commun 14, 5094 (2023). https://doi.org/10.1038/s41467-023-40872-z

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Ghani R, Mullish BH, Roberts L, Davies FJ, Marchesi JR et al., 2022, The potential utility of fecal (or intestinal) microbiota transplantation in controlling infectious diseases, Gut Microbes, Vol: 14, Pages: 1-22, ISSN: 1949-0976

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Lythgoe MP, Ghani R, Mullish BH, Marchesi JR, Krell Jet al., 2021, The Potential of Faecal Microbiota Transplantation in Oncology, Trends in Microbiology, ISSN: 0966-842X

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Mullish BH, Ghani R, McDonald J, Marchesi JR et al., 2019, Faecal microbiota transplant for eradication of multidrug-resistant Enterobacteriaceae: a lesson in applying best practice? Re: 'A five-day course of oral antibiotics followed by faecal transplantation to eradicate carriage of multidrug-resistant Enterobacteriaceae: A Randomized Clinical Trial', Clinical Microbiology and Infection, Vol: 25, Pages: 912-913, ISSN: 1198-743X

Conference Papers

Ghani R, Blanco JM, Forlano R, Triantafyllou E, Bilinski J, Geva-Zatorsky N, Bar-Yoseph H, Thursz MR, Davies F, Mullish BH, Marchesi JR et al., 2022, Relative change of Enterococcus faecium, selected commensal bacteria and cytokines are seen in patients colonized with multidrug-resistant organisms who undergo Intestinal Microbiota Transplantation, Publisher: W B SAUNDERS CO-ELSEVIER INC, Pages: S218-S219, ISSN: 0016-5085

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Society-forSurgery-of-the-Alimentary-Tract Annual Meeting at Digestive Disease Week (DDW), Publisher: W B SAUNDERS CO-ELSEVIER INC, Pages: S168-S169, ISSN: 0016-5085

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Appendices

Appendix 1: Permissions Table

Page No.	Type of work:	Name of work	Source of work	Copyright holder and contact	permission requested on	I have permission. yes /no	Permission note
53	figure	Figure. 1. A schematic model for omics approaches to study the gut microbiome	Whon, T.W., Shin, NR., Kim, J.Y. et al. Omics in gut microbiome analysis. J Microbiol. 59, 292–297 (2021). https://doi.org/10.1007/s12275-021-1004-0	© 2023 Springer Nature Switzerland AG. Part of Springer Nature. journalpermissions@springernature.com	14.05.2023	yes	License Number 5547620614761
35	Table	Table 1. Classification of b-lactamases	Wilson H, Török ME. Extended-spectrum β-lactamase-producing and carbapenemase-producing Enterobacteriaceae. Microbial Genomics. 2018;4(7): e000197–e000197.	© 2023 Copyright Clearance Center, Inc. support@copyright.com	18.10.2023	yes	Order number 1408044-1.

Appendix 2: IMT stool donor selection for rCDI

Donors:

. Family, friends, or close contacts

-Absolute contraindications:

- ≤ 18 years or ≥ 50 years.
- BMI ≥ 30 kg/m2.
- Receipt of antimicrobials within the past three months.
- Known prior exposure to HIV and/ or viral hepatitis and known previous or latent tuberculosis.
- Risk factors for blood-borne viruses - including high risk sexual behaviours, use of illicit drugs, any tattoo/ body piercing/ needlestick injury/ blood transfusion/ acupuncture, all within the previous six months.
- Receipt of a live attenuated virus within the past six months.
- Underlying gastrointestinal conditions/ symptoms (e.g., history of IBD, IBS, chronic diarrhoea, chronic constipation, coeliac disease, bowel resection or bariatric surgery) - also including acute diarrhoea/ gastrointestinal symptoms within the past two weeks.
- Family history of any significant gastrointestinal conditions (e.g., family history of IBD, or colorectal cancer).
- History of atopy (e.g., asthma, eosinophilic disorders).
- Any systemic autoimmune conditions.
- Any metabolic conditions, including diabetes and obesity.
- Any neurological or psychiatric conditions or known risk of prion disease.

- History of chronic pain syndromes, including chronic fatigue syndrome and fibromyalgia.
- History of any malignancy.
- Taking particular regular medications, or such medications within the past three months, i.e., antimicrobials, proton pump inhibitors, immunosuppression, chemotherapy
- History of receiving growth hormone, insulin from cows, or clotting factor concentrates.
- History of receiving an experimental medicine or vaccine within the past six months.
- History of travel to tropical countries within the past six months.

Relative contraindications:

- Donors will be asked for allergies, including food allergies. Donors with food allergies will be asked to strictly avoid any trigger foods for five days prior to donation of faecal material.
- Potential donors with food allergies will still be consented to undergo blood/ stool assays to fully assess their eligibility as donors and will be allowed to join the donor register if there are no further exclusion criteria. However, at the time of transplantation, their faecal material from donors with no relative contraindications will be used in preference to those who have food allergies.

Appendix 3: Preparation and administration of IMT

Preparation of donor faecal material

Faecal material will be in the first instance passed into a clean closed plastic container. Only samples between 3 and 5 on the Bristol stool chart scale will be accepted as suitable potential transplant material. From here, faecal material will be immediately transported on ice into the laboratory.

- Staff involved in preparing the transplant will use universal precautions as appropriate throughout, e.g., a fluid-resistant gown, gloves, and a mask with goggles or eye shield.
- Material will first be weighed.
- 50g aliquots of faeces will be mixed with 250ml of sterile, non-bacteriostatic phosphate-buffered saline (PBS)/ normal saline. Material will then be homogenised and strained.
- The strained material will be centrifuged at 6000xg for 20 minutes in a rotor and washed in PBS/ normal saline.
- The resulting concentrated faecal bacteria suspension will be amended with sterile pharmacological grade glycerol to a final concentration of 10% and will then be stored frozen at -80°C until used.
- The material will be prepared with only a code number on and there will be no information (e.g., name) from which the donor may be identifiable.

Preparation of the IMT

- The frozen material that is chosen for the IMT will be selected by a clinician preparing the IMT and involved with the clinical team caring for the patient.
- The frozen material for the IMT will be thawed on the day of the procedure over 2 – 4 hours in an ice bath, starting from approximately 2 – 4 hours prior to the expected time of administration of the IMT.
- The preparation will be put into enteral syringes and transported to the ward for administration to the patient.

Administration of IMT

- Relevant antibiotics will be stopped at least 24 hours prior to IMT.
- The day before the transplant, patients will have prescribed bowel lavage with four litres of macrogol electrolyte solution (Klean-Prep), either orally or via nasogastric (NG) tube.

- If the patient does not have one already, they will have a NG tube inserted on the day before the transplant (or other enteric tube as appropriate, e.g., NJ tube). Placement will be checked by conventional means, in keeping with Trust protocol. Patients with functional PEG/PEG-J tubes may receive IMT via these.

- Patients will be fasted for six hours prior to the administration of the IMT (medications will be allowed with sips of water).

- Patients will be given 30mg lansoprazole fast-tabs po/ng daily (or equivalent PPI) from two days prior to the IMT, with the final dose on the morning of the treatment. They will also be given 10mg metoclopromide approximately two hours prior to the procedure.

- The IMT will be infused via enteric syringes attached to the NG tube (or other route of administration as appropriate) using universal precautions. The rate will be slowed, and anti-emetics will be administered if patients describe nausea. Patients will be allowed sips of fluid during the procedure for comfort.

- The tube will be flushed with at least 50ml of water after infusing the IMT. Recipients will stay nil by mouth for twenty minutes, and the NG tube (or other enteric tube) will then be removed if appropriate (assuming no other indication for NG tube, such as feeding). If there are no concerns from the clinician administering IMT, recipients will be allowed water only for forty minutes more and will be allowed to eat and drink as normal.