Utility of Screening-Bead Assay in Testing for Formation of Donor-Specific Anti-HLA Antibodies and Antibody-Mediated Rejection in Renal Allografts Post-Transplant.

by

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Abstract

Background: Present-day immunosuppressive therapy in renal allograft recipients targets cell-mediated immunity and has significantly improved outcomes over the years. However, allograft failure rates still approach 50% towards 10 years post-transplantation. Antibody-mediated rejection (ABMR) is being increasingly recognized as a major cause of poor long-term graft survival. Though it can aid in risk stratification and optimizing immune-suppression, post-transplant monitoring of anti-HLA donor specific-antibodies (DSAs) is controversial as it is costly and there is little data that early detection impacts outcomes. Moreover, the best way to test for DSAs is not well-established. Some centres use an initial screening-bead assay, claiming it is cost-effective. Others forego the screening-bead assay, as they believe it is not sensitive enough to warrant its use. This study aims to establish the screening bead assay's utility by measuring its sensitivity, specificity, and negative and positive predictive values and likelihood ratios.

Methods: The screening-bead assay's clinical utility was retrospectively determined by comparing it to single-antigen bead assays as a surrogate for detecting anti-HLA-antibodies and renal biopsies as a surrogate for detecting antibody-mediated rejection. Further, screening-bead assays were correlated to changes in serum creatinine and eGFR over time. These data had been collected from renal allograft recipients at the University of Alberta Hospital between 2013-2017.

Results: Overall, positive results had less clinical utility than did negative results. Sensitivity for anti-HLA antibodies was 90.6%, 95% CI [87.5%, 93.8%], negative predictive value was 87.1% when re-calculated for literature-reported prevalence of anti-HLA antibodies and 94.2% to 96.3% when re-calculated for literature-reported prevalence of *de novo* donor-specific anti-HLA antibodies. Negative likelihood ratio for anti-HLA antibodies was 0.345. Sensitivity for antibody-mediated rejection was 91.3%, 95% CI [79.8%, 100%], negative predictive value was 89.1% to

92.8% when re-calculated for literature-reported prevalence of antibody-mediated rejection and negative likelihood ratio was 0.70. When recalculated for *de novo* donor-specific anti-HLA antibodies that were identified by screens and single-antigen bead assays, these values improved to 94.7%, 95% CI [84.7%, 100%], 97.4% to 98.3% and 0.15 respectively. Further, positive screens correlated with a more aggressive decline in renal function, though degree of positivity of the screen was not prognostic.

Conclusions: The screening-bead assay's negative predictive value and sensitivity were high enough to warrant its use as a screening measure for formation of *de novo* anti-HLA donor-specific antibodies in previously unsensitized renal transplant recipients and those with low clinical suspicion of antibody-mediated rejection. Its use is less compulsory in those who are already sensitized and those who have high pre-clinical suspicion of antibody-mediated rejection.

Preface

The research project, of which this thesis is a part, was approved by the University of Alberta's Research Ethics Board (Pro00083783).

Dedicated to my family.

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Chapter 1: Introduction

1.1 Chronic Kidney Disease and Renal Replacement Modalities

Chronic kidney disease, defined as abnormalities in kidney structure or function for >3months [1], is associated with significant morbidity and mortality [2, 3]. It is widespread, occurring in 10-15% of the global population, with end-stage renal disease representing 0.1% [4]. Canadian prevalence of chronic kidney disease, as estimated from primary care data, is between 68.4 to 86.2 cases per 1,000 people [5]. In the US, 690,000 patients have end-stage renal disease, with incidence and prevalence being projected to rise [6].

Though dialysis, which includes hemodialysis and peritoneal dialysis, has drastically improved the clinical course for patients with end-stage renal disease, it limits their quality of life [7], does not replicate normal physiologic function of native kidneys [8] and has significant economic impact. Patients must adhere to strict dietary restrictions on potassium, phosphate, sodium and fluid intake. Those requiring in-centre intermittent hemodialysis must budget significant time towards it, including travel to and from their facility; are spatially confined with little privacy; and lose control over normal physiologic functions, such as thermal regulation and hemodynamics, leaving many with fatigue, nausea and muscular cramps during and after treatments. Moreover, as dialysis cannot remove uremic toxins with 100% efficiency, patients are predisposed to progressive cardiovascular disease, neuropathy, bone disease and amyloidosis, limiting their life expectancy. Dialysis is further limited by expenses incurred by facility space, equipment, water and its purification, and personnel, including physicians, nurses and technicians. One study showed that costs range between Canadian $40,000 to $65,000 depending on dialysis modality [9].

Kidney transplantation, despite risks of surgery, immune-suppression and new-onset diabetes after transplantation, is the optimal renal replacement modality due to its improvement of quality of life [10], morbidity, mortality, and long-term cost-effectiveness.

In their systematic review of 110 studies, Gill, J. *et al* found that mortality was improved amongst renal transplant recipients, with a significant decrease in cardiovascular events also being observed [11]. Port, F. K. *et al* showed that though relative risk of death within 2 weeks post-transplant was 2.8 times that of chronic dialysis patients, by 18 months it had decreased to 0.32, 95% CI (0.30, 0.35). Further, long-term mortality rate was 48% to 82% lower amongst transplant recipients [12].

Canadian data show that transplant recipients cost between Canadian $70,000 to $80,000 1 year post-transplant and Canadian $20,000 to $22,000 in the second year post-transplant [13] (not adjusted for inflation). American data are similar, with Medicare analyses showing that transplant recipients 1-year post-transplant cost approximately US $63,000 in in-patient management and US $20,000 in out-patient management; combined costs were approximately US $24,000 per year thereafter [14] (not adjusted for inflation). Further, cost-savings persist regardless of donor-risk (deceased donors, ABO-incompatibility and HLA-mismatch) [15] and time from transplant [16].

Though present-day immunosuppression has significantly improved outcomes over the years, 10-year graft survival rates still range between 60-80% for living donors and 45-75% for deceased donors [17]. Antibody-mediated rejection (ABMR) is being increasingly recognized as a major cause of poor long-term graft survival. Given transplantation's impact, studying it is crucial to improving long-term graft survival.

1.2 Epidemiology

Several studies have examined the incidence and prevalence of ABMR. Matas, A. J. *et al* showed evidence of antibody-mediated injury in the majority of kidney allografts with late-onset dysfunction. Their cross-sectional study analyzed 173 kidney transplant recipients with late-onset graft dysfunction (mean time post-transplant 7.3+/-6 years), showing that 57% of patients had circulating donor-specific antibodies (DSAs), C4d positivity on biopsy, or both [18]. However, while these findings may indicate antibody-mediated injury, they do not correlate late graft dysfunction with ABMR. In another study, Taube, D. *et al* retrospectively studied 469 ABO-compatible, cross-match negative kidney transplant recipients between 2005-2010. They had been induced with alemtuzumab and maintained with tacrolimus monotherapy. Of the 100 patients who experienced graft rejection, 48 had acute ABMR while the remainder had acute cellular rejection [19]. One limitation of this study is it only identified patients with acute ABMR, leaving those with chronic ABMR unaccounted for. In another example, Lefaucheur, C. *et al* identified 278 patients with ABMR requiring treatment, out of a total of 1,978 patients transplanted between 2008-2014 [20]. Halloran, P. F. *et al* prospectively followed 315 kidney allograft recipients who underwent clinically-indicated kidney biopsy for a median follow-up period of 31.4 months. 60 of these allografts failed, of which 56 were included in the study. 36/56 of these allografts

demonstrated rejection, of which all demonstrated some form of ABMR: 28 had ABMR, 5 had probable ABMR and 3 had mixed, cellular and antibody-mediated rejection. Other causes of graft failure included recurrent glomerulonephritis, polyomavirus nephropathy and acute kidney injury from other events [21]. Though methodical consistency between studies is hampered by ABMR's evolving definition, small population size and lack of standardization of induction and treatment protocols, it remains a significant barrier to long-term graft survival.

1.3 Antibody Formation and Antigenic Targets

Antibody formation requires exposure of the innate immune system to antigen, classically human-leukocyte antigen (HLA), which can occur before, during and after transplantation. Pre-transplant, exposure events include pregnancy, blood transfusions, and prior transplants. Molecular mimicry from exogenous pathogens and commensal organisms has also been implicated. Peri-transplant, organ ischemia alters expression of donor vascular endothelial proteins, triggering release of damage-associated molecular patterns. Reperfusion exposes the recipient's innate immune system to these signals, activating pro-inflammatory intracellular signaling cascades, release of cytokines and recruitment of alloreactive T and B Cells [22]. Post-transplant, non-compliance with immune-suppression is the most significant risk factor [21].

Once exposed, antibody formation requires that professional antigen-presenting cells present the antigen to CD4+ T-helper cells. This process requires three key signals: first, the T-Cell receptor must engage both the antigen and HLA molecule on which it is presented; second, there must be a co-stimulatory signal; third, cytokines subsequently direct T-Cell differentiation in into several effector types, including subtypes that are capable of stimulating B-Cell function. Current immune-suppression therapies prevent formation of antibodies through interfering with this pathway [23].

As the vascular endothelium forms the interface between donor and recipient, various antigenic targets have been identified on it. These include components of the glomerular, tubular and vascular basement membranes, such as perlecan, agrin and collagen, and proteins on vascular endothelial cells, such as angiotensin type 1 receptor and MHC Class I-related chain antigens (MICA) [24]. However, human-leukocyte antigen (HLA) remains the most important antigenic target.

1.3.1 Human-Leukocyte Antigen (HLA)

HLA is highly immunogenic because of heavy and constitutive surface expression on most cells, genetic polymorphism and ubiquitous sensitizing events such as pregnancy, blood transfusions and prior transplants. Further, it is unique in that it also undergoes direct allorecognition, a process whereby T-Cell receptors directly recognize it from recipient and donor professional antigen-presenting cells, as opposed to indirect allorecognition, which refers to antigen-presentation on MHC molecules after intracellular processing, and is used for all other peptides.

It is integral to activating the adaptive immune system. Two classes exist: I and II. HLA Class I is expressed by all nucleated cells. It is responsible for presenting intracellular antigens, typically self- and viral antigens, to T-cell receptors of CD8+ T-cells. HLA Class I is comprised of □ and □2 microglobulin chains, with □1 and □2 forming the peptide binding groove. The peptide binding groove is comprised of two □-helices that are conjoined at either end, limiting the size of peptide that can be presented. The floor of the peptide binding groove is made of a □-pleated sheet. Below the peptide binding groove is another extracellular domain comprised of □3 and □2 microglobulin, a transmembrane domain and a cytoplasmic tail. HLA Class I is loaded with antigen during its processing in the endoplasmic reticulum, from where it is shuttled to and expressed on the cell membrane. Engaged CD8+ T-cells have already undergone negative and positive thymic selection to regulate their cytotoxicity. When activated, they clear infected, dysfunctional and senescent somatic cells [25].

Contrastingly, HLA Class II is expressed constitutively on professional antigen-presenting cells, which include macrophages, dendritic cells and B-cells and intermittently on atypical antigen-presenting cells, which include myeloid, innate lymphoid, epithelial, endothelial and stromal cells [26]. It presents extracellular proteins to T-cell receptors of CD4+ T-cells. HLA Class II is a heterodimer of □ and □ chains. Unlike HLA Class I, its peptide binding groove is comprised of □1 and □1, and its □-helices are not conjoined, allowing larger peptides to be presented. Beneath this peptide binding groove are an extracellular domain, transmembrane domain and two cytoplasmic tails, each comprised of the □ and □ chains. Antigen-loading is also distinct from HLA Class I: vesicles containing unloaded HLA Class II bud from the endoplasmic reticulum, and eventually fuse with endosomes containing exogenous peptide and lysosomal enzymes. Once fused, HLA Class II is loaded with antigen and expressed on the cell surface,

where it stimulates CD4+ T-cells, which are crucial to propagating the adaptive immune response [25].

HLA is highly immunogenic because of its genetic polymorphism. In fact, advances in gene typing technologies continue to increase the number of identified alleles exponentially – to the point that more alleles were identified in the first three months of 2019 than have been over the last century [27]. To date, over 20,000 alleles have been identified [27]. Genetic polymorphism is thought to be driven by point mutation, recombination and gene conversion [28]. HLA Class I and II are primarily encoded by the 'Major Histocompatibility Complex' gene cluster on chromosome 6. Several distinct genes exist for Class 1, with HLA-A, B and C being capable of presenting antigen. Of note, □2 microglobulin is encoded separately on chromosome 15. Unlike Class I, Class II's a and b chains are encoded on chromosome 6, with HLA-DP, Q and R having antigen-presenting capability. Genes are inherited as haplotypes from parents, and are co-dominantly expressed [25].

Up to 30% of renal transplant recipients have been shown to have anti-HLA antibodies post-transplant [29], with antibodies against HLA Class II portending a worse prognosis [30].

1.3.2 Anti-HLA Donor-Specific Antibodies (Anti-HLA DSAs)

'Anti-HLA DSAs' refer to recipient anti-HLA antibodies with a specificity for donor HLA antigen. Anti-HLA DSAs first gained notoriety in kidney transplantation in the 1960s, when various groups described hyperacute rejection in ABO-compatible allografts. Milgrom, F. *et al* described leukocytic infiltration of glomerular and peritubular capillaries with thrombosis on biopsies taken within one hour of transplantation, with severe cortical necrosis, in their case series on 7 ABO-matched renal transplant recipients. Patients with prior renal transplants were particularly predisposed to this form of hyperacute rejection [31]. Other groups later correlated lymphocytotoxic antibodies to HLA-mismatch [32], showing that positive cross-matches were a contraindication to transplantation [33]. Taube *et al* showed that while patient survival was unaffected, acute ABMR from anti-HLA DSAs limited allograft survival when compared to patients with no evidence of rejection (70.2% compared to 97.0% at 54 months of follow-up, $p<0.0001$), with no significant difference being found when compared to cell-mediated rejection (84.6% at 54 months of follow-up, $p = 0.07$) [19]. Further, anti-HLA DSAs forming *de novo* post-transplant also worsen prognosis. Between 10-15% of renal transplant recipients have been shown

to develop *de novo* anti-HLA DSAs [29, 34, 35], with median graft survival 10 years post-transplant being found to be 56% in those with them, compared to 96% in those without them ($p < 0.0001$) [35]. In fact, *de novo* anti-HLA DSAs portend a poorer prognosis than pre-existing ones: Halloran, P. F. *et al* showed that graft survival 8 years after rejection was 34% and 63% respectively ($p<0.001$) [36].

Anti-HLA DSAs are known to bind to particular regions on HLA, which have been termed 'epitopes'. Each epitope has multiple regions that can bind anti-HLA DSAs, which are defined by their amino acid sequence in the folded protein, and are termed 'eplets' [37]. Notably, the number of eplet mismatches between donor and recipient have been correlated with formation of *de novo* anti-HLA DSAs, with optimal thresholds for maximum number of mismatches also having been suggested [38].

1.3.3 Preventing Anti-HLA-DSAs

Preventing formation of anti-HLA-DSAs is the best method of curtailing ABMR. It is done by assessing for sensitizing events in the patient's medical history and three laboratory methods: HLA-typing, screening for pre-existing anti-HLA antibodies and cross-matching.

HLA-typing has evolved significantly since its introduction in 1964. Originally, target lymphocytes would be incubated with antisera of known specificity, then with complement and dye, with a positive reaction being indicated by uptake of dye [39]. Now, DNA-typing prevails. Two categories exist: low-resolution and high resolution typing. Low-resolution typing can be done through sequence-specific oligonucleotides (SSOs) or sequence-specific primers (SSPs). The former relies on amplifying the gene of interest, after which the amplified product is identified by hybridization to fluorescently-labeled probes of known HLA-type. The latter amplifies the gene of interest using SSPs, then uses gel electrophoresis to compare the size of the amplified product against controls of known HLA-type. Since low-resolution typing is limited by our arsenal of probes and primers, it cannot identify the genetic sequence. Unlike low-resolution typing, high-resolution typing determines the nucleotide sequence of the HLA-gene.

Solid-phase assays, where microspheres are loaded with HLA, are used pre- and post-transplant to screen for anti-HLA-antibodies. These will be described in further detail under "1.6.1 Serologic Evidence: the Luminex Assay". Pre-transplant, this assay is used to identify specificities

of pre-existing anti-HLA-antibodies and approximate the percentage of compatible donors in the population.

Cross-matching was first canonized in 1969, when Patel, R. and Terasaki, P. I. showed that 24/30 renal transplant recipients with a positive cross-match suffered hyperacute rejection, with 1 additional patient rejecting at 4 months [33]. Since then, cross-matching has evolved from using the complement-dependent cytoxicity assay to using flow cytometry. Two methods exist: virtual and flow-based cross-matching. Virtual cross-matching requires identifying pre-existing anti-HLA-antibodies in the recipient using the solid-phase assay, and cross-referencing specificities with donor HLA-type. Flow-based cross-matching requires combining donor lymphocytes with recipient serum, then adding fluorescently labeled anti-human-immunoglobulin to identify bound anti-HLA-DSAs.

Yet, despite rigorous preventative methods, anti-HLA DSAs still form from ubiquitous sensitizing events such as blood transfusions, pregnancies and prior transplants, non-adherence to immune-suppression and titration of immune-suppression for various clinical indications.

1.4 Mechanisms of Antibody-Mediated Rejection

ABMR occurs primarily through two mechanisms: complement-dependent and complement-independent [40]. Understanding its pathophysiology has refined our diagnostic approach and identified several potential therapeutic targets.

1.4.1 Complement-Dependent Antibody-Mediated Rejection

Complement-activating antibodies have been shown to significantly limit renal allograft survival. In their prospective analysis of 1,016 renal transplant recipients, enrolled between 2005-2011, Loupy, A. *et al* showed that 5-year graft survival rates for those with complement-binding antibodies was 54%, as compared to 93% and 94% for patients with non-complement-binding DSAs and no DSAs respectively ($P<0.001$ for both comparisons) [41].

Antibodies' ability to activate complement is determined by several factors. IgG, particularly isotype 3, has been shown to be a potent activator of complement [42]. Others have also shown that complement activation occurs through hexamerization of IgG, which is dependent on antibody titre and independent of isotype [43]. Bound antibody activates complement through the classical pathway, culminating in the formation of cytotoxic membrane-attack complexes. By-

products of the complement activation cascade are also pro-inflammatory. Others, namely C1q, C3d and C4d, have been explored in non-invasive diagnostic methods [44], with C4d staining forming one of the hallmarks of ABMR's histological diagnosis [45].

Complement-dependent ABMR has also been linked with activation of natural killer cells, which then release cytotoxic granules and recruit other inflammatory cells [40].

1.4.2 Complement-Independent Antibody-Mediated Rejection

ABMR can also occur independently of complement activation, primarily through recruitment of monocytes. Its clinical significance is derived from biopsies consistent with ABMR but negative for C4d staining and the failure of complement inhibitor therapies in some patients with ABMR. Little else is known about it, as it has been mostly studied in animal models [40].

1.5 Clinical Presentation

Definitions of the clinical presentation of ABMR have changed with advancing knowledge. The optimal approach integrates ABMR's pathophysiology, which is characterized histologically.

Histologically, the Banff 2017 Classification has defined ABMR according to 'active' and 'chronic' presentations. Active ABMR refers to ongoing antibody-mediated inflammation without findings of chronic vascular injury. It is characterized by evidence of acute tissue injury, such as microvascular inflammation, and evidence of interaction of antibodies with the vascular endothelium, including staining for C4d [45]. Active ABMR can present at varying time points. Hyperacute rejection presents within minutes of graft anastomosis. It is caused by high titres of pre-existing antibodies against major antigens such as ABO and HLA. Due to advances in screening, it is very rare today. Delayed hyperacute rejection, also known as accelerated acute rejection, occurs within several days of transplantation. It is caused by activation of memory B-cells that formed from prior sensitizing events. Other forms of active rejection can occur at any point during the course of transplant. They are broadly categorized into early active rejection, which is ascribed to increasing levels of pre-existing DSA, and late active rejection, which is ascribed to formation of *de novo* DSAs [46].

Chronic ABMR refers to ongoing antibody-mediated inflammation with findings of chronic vascular injury, which include transplant glomerulopathy, peritubular capillary basement membrane multilayering and new-onset arterial intimal fibrosis [45].

Other descriptors of ABMR include 'subclinical ABMR', which refers to histologic evidence of antibody-mediated inflammation with normal graft function. Both active and subclinical ABMR are risk factors for developing chronic ABMR [46].

Clinically, ABMR presents with deteriorating graft function and sub-nephrotic range proteinuria. However, since these findings are highly non-specific, other diagnostic methods must be utilized. These include solid-phase assays and biopsy. Lefaucheur *et al*, in their development of a prognostic tool for ABMR, described high-risk clinical features in 278 patients with anti-HLA-DSA-mediated ABMR requiring treatment with plasma exchange, intravenous immunoglobulin and rituximab. The mean eGFR was 34.9+/-18.4 ml/min per 1.73m^2 with mean proteinuria being 0.86+/-1.23 g/g. 132/278 patients had multiple anti-HLA-DSAs, with the mean being 2.1+/-1.6. 176/278 had *de novo* anti-HLA-DSAs; 76/278 had antibody against Class 1, 127/278 against Class II and 75/278 against both. Mean fluorescence intensity for anti-HLA DSAs was 5222.5+/-317.9. Independent predictors for allograft loss at time of biopsy included eGFR (HR 0.97, 95% CI 0.95-0.98, P<0.001), *de novo* anti-HLA DSA (HR 2.45, 95% CI 1.34-4.47; P=0.004) and biopsy findings of chronic allograft glomerulopathy (HR 2.25, 95% CI 1.29-3.92, P=0.004) and interstitial fibrosis with tubular atrophy (HR 2.93, 95% CI 1.62-5.29; P<0.001) [20].

1.6 Diagnosis

ABMR is classified according to the Banff Classification, which was first conceived in 1991 and published in 1993. Rejection of kidney allografts was originally categorized chronologically, according to hyperacute, acute and chronic rejection, as opposed to mechanistically [47]. ABMR was recognized as a distinct entity in 1997 and was described as either hyperacute or delayed acute [48]. Subsequent iterations of the Banff Classification have refined ABMR's diagnosis as new insights have developed.

The latest Banff Classification was published in 2017, and recognizes two categories of ABMR: active and chronic. Now, its diagnosis consists of three components: histologic evidence, C4d staining and serologic evidence [45].

Histologic evidence is used to differentiate between active and chronic ABMR. Active ABMR is characterized by either of: microvascular inflammation (defined by glomerulitis and/or peritubular capillaritis), intimal or transmural arteritis, acute thrombotic microangiopathy or acute tubular injury. Chronic ABMR, on the other hand, is characterized by either of: transplant

glomerulopathy, peritubular capillary basement membrane multilayering or new onset arterial intimal fibrosis [45].

C4d staining, representing by-products from activation of the classical pathway of complement, forms the second component of diagnosis. However, as antibody-mediated injury to kidney allografts has been shown to be independent of complement in some cases, its inclusion is no longer compulsory. It can be replaced by evidence of microvascular inflammation, if other histologic features are present [45]. Of note, 'C4d staining without evidence of ABMR' forms a third diagnostic category, in addition to active and chronic ABMR, in the 2017 Banff Classification [45]. Though its clinical significance is unknown, emerging evidence is linking it with increased risk of ABMR [49].

Serologic evidence of anti-HLA DSAs is the final component. Its inclusion is also non-compulsory, as not all ABMR is HLA-related. It can be replaced by C4d staining, as long as two distinct histologic lesions are identified. Regardless, testing is strongly advised, as HLA is the dominant antigen against which antibodies form [45].

1.6.1 Serologic Evidence: The Solid Phase Assay

The solid phase assay has become integral to diagnosing anti-HLA antibodies. It consists of microscopic beads loaded with various HLA-antigens. Two types exist: screening and single-antigen bead assays. Screening-bead assays are loaded with various HLA antigens from one class. Single-antigen bead assays are loaded with a specific HLA allele, and are codified fluorescently. When these beads are incubated with recipient serum, anti-HLA antibodies will bind to their corresponding antigen. Anti-human immunoglobulin is then added, followed by a streptavidin-phycoerythrin complex. The bead-antibody complexes are analyzed by the Luminex assay, a specialized flow cytometer. Excitation wavelengths trigger emission of light from the streptavidin-phycoerythrin complex, identifying bound anti-HLA antibodies. The single-antigen beads are triggered by a different excitation wavelength, allowing their identification. The resulting 'mean fluorescence intensity' (MFI) is quantified by the flow cytometer, and interpreted against a negative control [50].

Despite its utility in detecting anti-HLA antibodies, the solid phase assay has several limitations. Foremost, MFI is a poor estimate of anti-HLA antibody titre, as it is influenced by several factors. It must be standardized against negative controls, which can have high variance

due to laboratory technique and binding of non-specific serum proteins. Antigen-density between beads is also variable, making comparison between them inaccurate. Moreover, solid phase assays are marred by non-specific binding, which results from shared epitopes between antigens, conformational changes in antigen when conjugated to the bead and interference of binding of detection reagents when high-titre anti-HLA antibodies activate other serum components [51]. Lastly, antibody potency is determined by several factors, include antibody avidity, affinity, quantity and isotype [42, 43], neither of which is effectively measured by the bead assay.

1.6.1.1 Utility in Monitoring Anti-HLA DSAs

The clinical utility of the solid-phase assay has been examined in several different contexts. Pre-transplant data is robust. Even low-titre anti-HLA DSAs, detectable by solid-phase assay but not flow cytometry, have been shown to nearly double the risk of antibody-mediated rejection [52].

Post-transplant, *de novo* anti-HLA-DSAs have been shown to worsen graft survival [35, 36], with several groups correlating a reduction in MFI after treatment for ABMR with improved graft survival [20, 53, 54]. Yet, using the solid phase assay to monitor anti-HLA DSAs post-transplant is limited by its cost and little data establishing early detection's impact on outcomes [55]. Consensus guidelines recommend using the solid phase assay for screening transplant recipients post-transplant depending on risk level, though recommendations are not unanimously supported. Moreover, no recommendation is made on whether the screening-bead assay should be used as a precursor to the single-antigen bead assay [56]. Clinically, some centres use an initial screening-bead assay, claiming it is cost-effective. Others forego the screening-bead assay, as they believe it is not sensitive enough to warrant its use. Thus, the screening-bead assay's utility in screening patients for formation of *de novo* anti-HLA-DSAs post-transplant remains to be elucidated.

1.6.2 Emerging Diagnostic Methods

Several other diagnostic methods are under development, each targeting various steps from formation of antibodies to their activity. Molecular evidence of antibody-mediated injury has been the most widely-accepted of these methods, and is being gradually integrated into the Banff Classification since its introduction in 2013. Various groups are characterizing gene expression

profiles of antibody-mediated injury, such as endothelial cell damage and NK cell activation, in an attempt to improve the Banff Classification's sensitivity and specificity [45].

Complement-dependent assays, designed to detect by-products of the classical pathway of activation, are a modern adaptation of the solid phase assay. Unlike current solid-phase assays, which detect anti-HLA antibodies using anti-human immunoglobulin, complement-dependent assays use immunoglobulin against complement. C1q, C3d and C4d have been investigated as targets. However, implementation of such assays is hindered by their cost, labour and complement activation being partly dependent on antibody-titre, which can be affected by operator technique. Moreover, complement-independent antibody-mediated inflammation is also deleterious [44]. Other trials, investigating the clinical utility of detecting immunoglobulin subtypes (NCT04026087) and donor-specific B-cells (NCT02133248), are underway.

1.7 Treatment

Treatment is initiated when ABMR has been confirmed by decreased renal function, serologic evidence of anti-HLA DSAs and biopsy. However, there is little evidence to guide preventative treatment based off detection of anti-HLA DSAs. Though *de novo* DSAs have been shown to worsen prognosis [35, 36], small-scale studies in pediatric and adult populations have not shown improvement of allograft survival with pre-emptive treatment [57, 58]. Similarly, subclinical ABMR has also been shown to affect graft outcome [59], but studies regarding its treatment have produced conflicting results [60].

Further, development of standardized treatment protocols is hindered by small numbers of patients and the lengthy follow-up period required. Currently, the KDIGO Clinical Practice Guidelines for the Care of Transplant Recipients recommends any combination of plasma exchange, intravenous immunoglobulin, rituximab and/or lymphocyte-depleting antibodies, with or without steroids [61]. Targets for clinical trials include the complement cascade, B-cells, plasma cells and inflammatory cytokines [40].

1.8 Study Objectives

I aim to establish whether the screening bead assay is an effective tool in post-transplant screening of renal allograft recipients for formation of *de novo* DSAs, and if it should be used before single-antigen bead assays in establishing serologic evidence of DSAs for the diagnosis of

ABMR. I will determine its specificity, sensitivity, positive and negative predictive values and positive and negative likelihood ratios, as compared to single-antigen bead assays and biopsy results. Screens will be further correlated to changes in serum creatinine, eGFR and graft failure rates over time.

1.9 Hypotheses

I hypothesize that the screening-bead assay will have enough clinical utility to warrant its use before the single-antigen bead assay when diagnosing ABMR. Further, it will also likely be useful in screening renal transplant recipients for post-transplant formation of DSAs.

Chapter 2: Methods

1.1 Study Approval

Approval for the project was obtained from the Health Research Ethics Board, and was deemed to comply with requirements of the Health Information Act.

1.2 Protocols

1.2.1 Donor Genotyping

Donor HLA genotyping was performed by reverse sequence specific oligonucleotide (rSSO) typing, per product insert from One Lambda A Thermo Fisher Scientific Brand®, California, USA.

1.2.2 Screening-Bead Assay

Between 2013 to 2017, the University of Alberta's Histocompatibility lab switched between two screening-bead assays: Immucor LIFECODES LifeScreen®, Georgia, USA and One Lambda A Thermo Fisher Scientific Brand®, California, USA. The assays differ in their conjugation of HLA antigen to the microscopic beads: the former requires lysis of donor lymphocytes, thereby releasing HLA antigen, which is bound to the beads using capture antibodies [62]. Beads in the latter are pre-bound to HLA antigen [63].

Luminex 200 was used to measure the mean fluorescence intensity (MFI). Controls included unstained samples, to establish the MFI of non-specific, background staining. Results were interpreted using SystemLink's HistoTrac Software®, Virginia, USA.

Screen results were determined as borderline, positive or strongly positive, based on comparison of the MFI curves of test beads and negative controls.

1.2.3 Single-Antigen Bead Assay

Single-antigen bead assay was performed per the product insert for Luminex® single-antigen beads (One Lambda A Thermo Fisher Scientific Brand®, California, USA) and analyzed by flow cytometry. Controls included unstained samples, to establish the MFI of non-specific, background staining. Results were interpreted using SystemLink's HistoTrac Software®, Virginia, USA.

High-risk antigens were defined as those with an MFI of over 3,000, moderate-risk antigens with an MFI between 500 and 3,000 and low-risk with an MFI below 450. Important interpretive considerations for MFI thresholds, performed by trained personnel, included accounting for lot-to-lot variability of beads, various HLA loci, conformational changes in HLA when conjugated to the bead and patient sensitization history.

Donor-specificity was determined by matching specificity of moderate- and high-risk anti-HLA antibodies, as determined from recipient serum, to donor genotype (1.2.1 Donor Genotyping).

1.2.4 Biopsy and Histology

Biopsy and histology were performed per local laboratory guidelines, as established by the University of Alberta's renal pathologists. They were interpreted according to the Banff Criteria at time of biopsy.

1.3 Data Collection

Patient data from the University of Alberta's clinics and HLA laboratory were collected in OTTR® CompleteOrgan software and imported into a free, secure, streamlined, university-supported database known as 'Research Electronic Data Capture' (REDCap). Data was formatted to anonymously display patients' demographics of dates and results of HLA-typing, graft function, rejection, longevity and histology. Patients with multiple anti-HLA DSAs were displayed as separate entries. Once inputted into REDCap, data was extracted into Microsoft Excel, from where it was analyzed.

1.4 Using the Single-Antigen (Ag) Bead Assay as a Gold Standard to Detect Anti-HLA Abs

1.4.1 Inclusion and Exclusion Criteria

Results were included if the screen was followed-up by a corresponding single-Ag bead assay within 15 days (arbitrarily chosen to reduce inclusion of *de novo* DSAs, as median onset to their formation is 3.8 to 68 months [51]).

1.4.2 Formulae

*See Appendix for formulae.

The total number of positive and negative screens was determined by counting test results.

True positive screens were defined as those with a corresponding positive single-antigen bead assay. True negative screens were defined as those with a corresponding negative single-antigen bead assay.

False positive screens were defined as positive screens that had a corresponding negative single-antigen bead assay. They were calculated by subtracting the number of true positive screens from the total number of positive screens. Analogously, false negative screens were defined as negative screens that had a corresponding positive single-antigen bead assay. They were calculated by subtracting the number of true negative screens from the total number of negative screens.

1.5 Using Biopsy as a Gold Standard to Detect ABMR

Biopsy samples were regraded according to the Banff 2017 Classification, which was the latest iteration at the time of the study. Single-antigen bead assays were used to demonstrate serologic evidence of ABMR. Molecular testing was not included as a criterion for classification, as it is not widely clinically implemented.

Of note, a positive screen was defined by its doublet (i.e. positive screen = [LSMIX 1 or 2 positive] or [LC-LMX 1 or 2 positive]), as each doublet had one corresponding biopsy (in '1.4 Using the Single-Antigen Bead Assay as a Gold Standard', positive screens were defined individually (i.e. positive screen = [LSMIX1 positive], [LSMIX 2 positive], [LC-LMX1 positive] or [LC-LMX2 positive]).

1.5.1 Inclusion and Exclusion Criteria

Biopsies were included if they were done within 1 month of drawing the blood sample used for the screening-bead assay (as blood samples are frozen, screening-bead assays can be done several months after the initial draw). 1 month was arbitrarily chosen to reduce inclusion of *de novo* DSAs (median onset to formation has been shown to be 3.8 to 68 months [51]), while maximizing number of biopsies included. Moreover, at this point, antibody profiles and biopsy

changes can be assumed to be stable. If a biopsy was inadequate or marginal, it was included only if it gave adequate information to diagnose ABMR. Implantation biopsies were excluded.

1.5.2 Formulae

*See Appendix for formulae.

The total number of positive and negative screens was determined by counting test results.

True positive screens were defined as those with a corresponding positive biopsy. True negative screens were defined as those with a corresponding negative biopsy.

False positive screens were defined as those that had a corresponding negative biopsy. They were calculated by subtracting the number of true positive screens from the total number of positive screens. Analogously, false negative screens were defined as those that had a corresponding positive biopsy. They were calculated by subtracting the number of true negative screens from the total number of negative screens.

1.6 Determining Change in Creatinine and eGFR for Screening-Bead Assays

*See Appendix for formulae.

Changes in serum creatinine and estimated glomerular filtration rate (eGFR, as calculated by CKD-EPI), over time, were correlated to positive and negative screens. Again, a positive screen was defined by its doublet (i.e. positive screen = [LSMIX 1 or 2 positive] or [LC-LMX 1 or 2 positive]), since each doublet had one corresponding blood test. The overall strength of the doublet was defined by the strongest result.

Changes in creatinine over time were calculated by subtracting the creatinine at biopsy (within 2 weeks of biopsy) from the most recently recorded serum creatinine (up to 2018), then dividing the difference by the elapsed time.

Similarly, changes in eGFR over time were calculated by subtracting the eGFR at biopsy (within 2 weeks of biopsy) from the most recently recorded eGFR (up to 2018), then dividing the difference by the elapsed time.

1.6.1 Inclusion and Exclusion Criteria

Results were included if the screen was followed-up by measurement of serum creatinine and eGFR within 1 month (arbitrarily chosen to reduce the effect of de novo DSAs, as median

onset to their formation is 3.8 to 68 months [1]). Results were excluded if testing was done near transplantation, without stabilization of post-transplant creatinine.

1.7 Statistics

Exact Clopper Pearson Method was used to determine the 95% confidence intervals (95% CI) for the derived sensitivities, specificities and prevalence.

Receiver Operatory Characteristic curves were formed for the screening-bead assay and its corresponding single-Ag bead assays and biopsies. Sp and Sn were derived for each of the screen's 4 thresholds: "Borderline", "Weak Positive", "Positive" and "Strong Positive". MFI was not used, as it is a poor estimate of antibody titre (Chapter 1: Introduction, 1.6.1). Further, separate curves for LS-MIX and LC-LMX were also made, as the latter tended to underperform due to differing MFI thresholds.

Linear regression was applied to analyzing changes in creatinine and eGFR over time for different screening groups.

Chapter 3: Results.

3.1 Preface

The screening bead assay's utility in monitoring recipients for ABMR post-transplant was determine through:

1) Comparing it to the single-antigen bead assay as a gold standard to detect anti-HLA antibodies.
2) Comparing it to biopsy as a gold standard to detect ABMR.
3) Examining changes in serum creatinine and estimated glomerular filtration rate (eGFR) over time.

3.2 Using the Single-Antigen (Ag) Bead Assay as a Gold Standard to Detect Anti-HLA Abs

Results of screens and corresponding single-Ag bead assays, ordered by transplant nephrologists at University of Alberta to investigate ABMR as a cause of graft dysfunction, done between 2013 to 2017, were pulled from the HLA laboratory's data repository and formatted in Microsoft Excel. 1,655 samples, including screening-bead assays and single-antigen bead assays, from 338 patients were pulled. After inclusion criteria were applied, 688 samples were included.

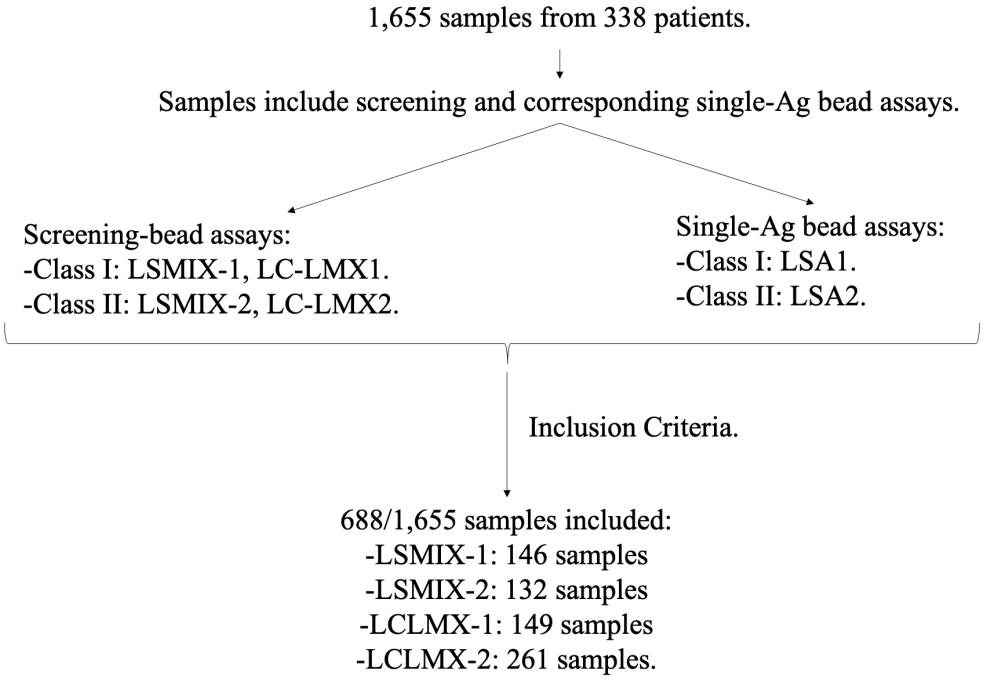


Figure 1: Schematic of sample selection of screening- and single-Ag bead assays.

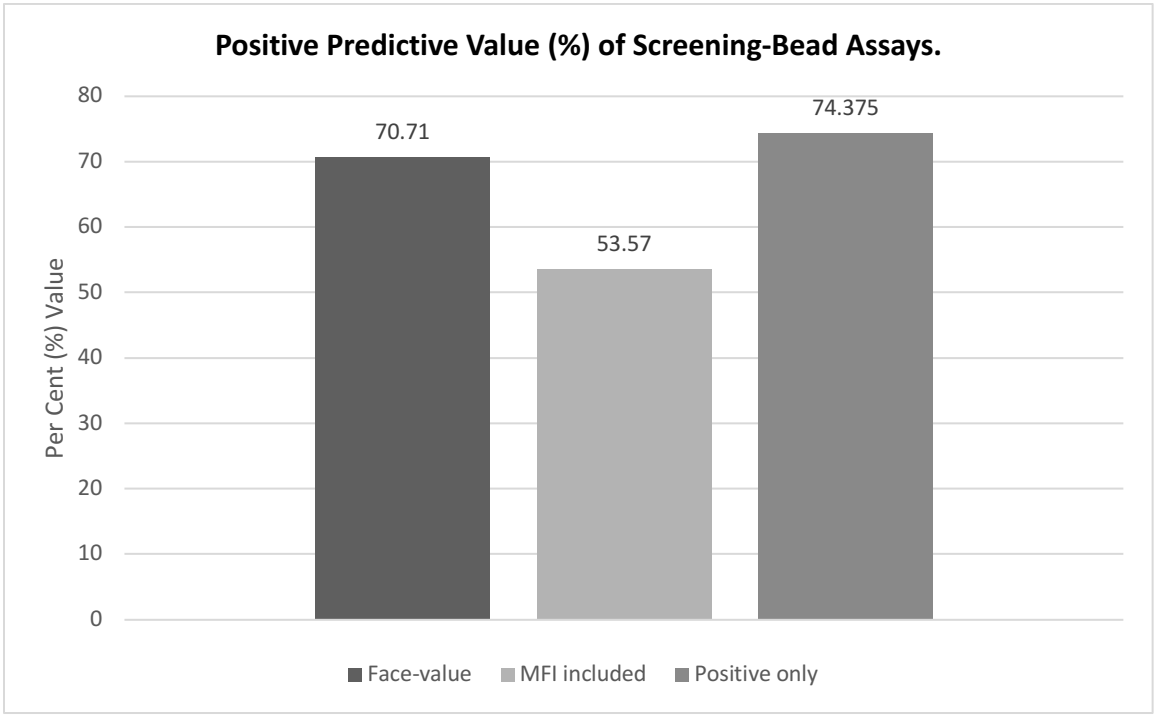
3.2.1 Test Characteristics

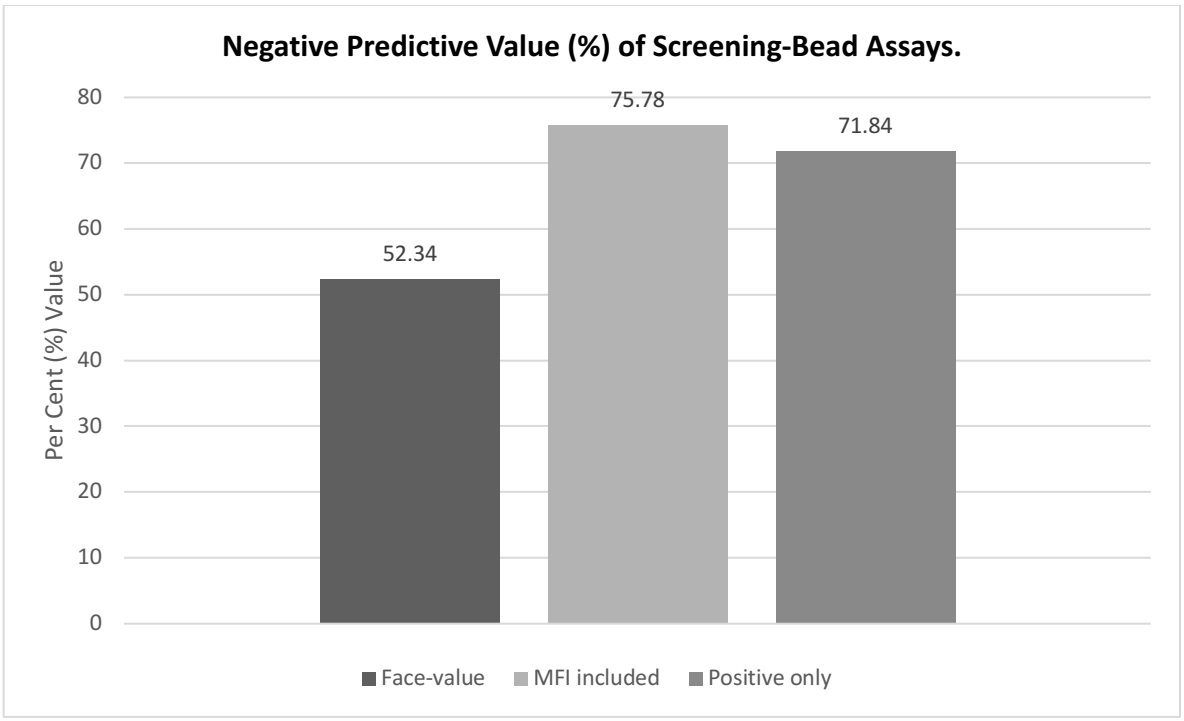
Specificity (Sp), sensitivity (Sn), positive and negative predictive values (PPV and NPV), positive and negative likelihood ratios (+/-LR) and pre- and post-test probabilities (formulae in Chapter 2: Methods, Appendix) were derived from three data sets. Positive screens were defined as those that were "borderline" or above.

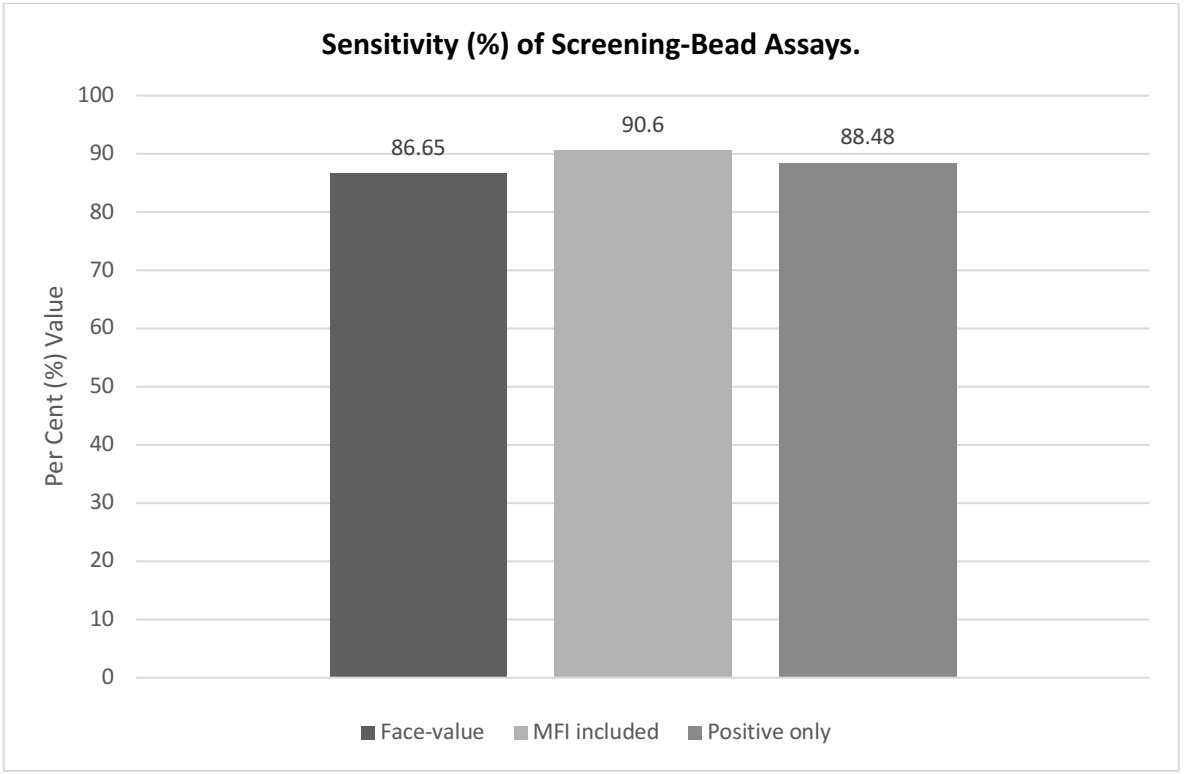
i) "Face-value": single-antigen beads were defined as positive if they fluoresced above background, regardless of the degree of reactivity (as indicated by mean fluorescence intensity (MFI)).

ii) "MFI included": if the MFI of single-Ag bead assay was <1,000, it was counted as a negative result. This group accounts for the effect of human interpretation of test results, specifically, its effect on the negative predictive value. It also accounts for DSAs, which can be present in higher quantities (though this does neglect low-titre, high-affinity and cross-reactive antibodies).

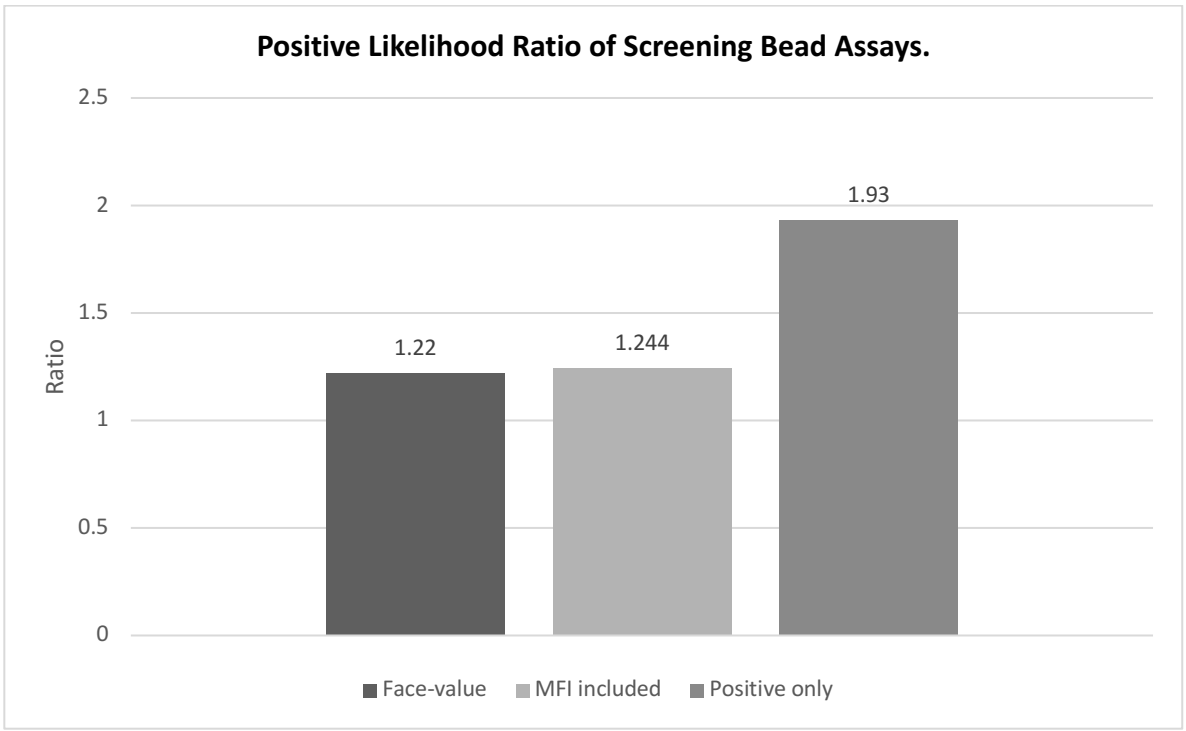
iii) "Positive only": this group looked at the metrics of only those screens that were "positive" or "strongly positive", and compared them to single-Ag bead assays with an MFI of >1,000, to establish the value of positive results and human interpretation and DSAs, with the aforementioned caveats.

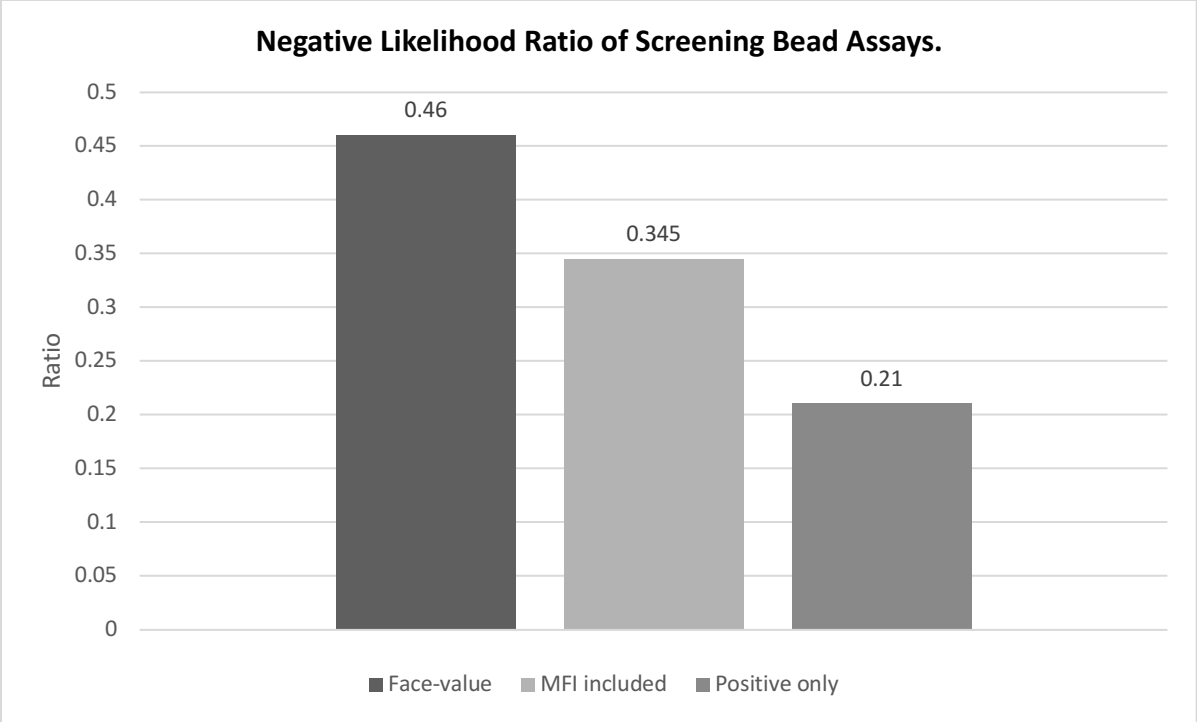



Negative Predictive Value (%) of Screening-Bead Assays.
Per Cent (%) Value
80
70
60
50
40
30
20
10
0
52.34
75.78
71.84
Face-value
MFI included
Positive only


Sensitivity (%) of Screening-Bead Assays.
Per Cent (%) Value
100
90
80
70
60
50
40
30
20
10
0
86.65
90.6
88.48
Face-value
MFI included
Positive only


Specificity (%) of Screening-Bead Assays.
Per Cent (%) Value
60
50
40
30
20
10
0
29
27.17
54.19
Face-value
MFI included
Positive only


Positive Likelihood Ratio of Screening Bead Assays.
Ratio
2.5
2
1.5
1
0.5
0
1.22
1.244
1.93
Face-value
MFI included
Positive only



Figures 2-7: PPV, NPV, Sp, Sn and +/-LR of LS-MIX and LC-LMX combined, as determined by comparing to single-Ag beads at i) Face Value, with ii) MFI included and of iii) Positive only results. PPV, NPV, Sp, Sn, pre- and post-test probabilities and +/-LR of LS-MIX and LC-LMX individually are in Appendix: Figure 1.

From 'ii) MFI included', sensitivity and specificity of LS-MIX and LC-LMX combined were 90.6%, 95% CI [87.5%, 93.8%] and 27.2%, 95% CI [22.6%, 31.8%] respectively.

Applying +/-LR from ii) 'MFI included' to an externally-validated prevalence of anti-HLA-Abs shows that for any given transplant recipient, who has a 30% chance of having anti-HLA-Abs [29], a negative screen reduces their likelihood of having anti-HLA-Abs to 12.9%, while a positive screen raises their likelihood marginally to 34.8%. In an alternate scenario, where there is a pre-test probability of 100%, a negative screen would reduce the likelihood of anti-HLA-Abs to 35.2%.

3.2.2 Correlating Screens to DSA, as detected by Single-Ag Bead Assays

Screens were also correlated to DSA. However, due to limitations of the data set, correlations were examined regardless of antibody class (i.e. if DSA was listed, and one of the screens for either Class I or II was positive, it was assumed that the screen doublet identified the

DSA). The major caveat is that the screening bead assay's ability to pinpoint DSA is inflated. Of the 152 samples that had DSA, 88.2% (134) of doublets were 'Borderline' or greater. To circumvent the problem, a correlation was made to MFI of the screen, as DSA often present in higher titres: 61.2% (93) of doublets were 'Positive' or greater. However, this data set neglects those patients with low-quantity DSA that can have clinical significance (though unknown), due to cross-reactivity and differing levels of immunogenicity.

3.2.3 PPV and NPV as Functions of Prevalence

One of the drawbacks of our dataset is that only patients with adverse changes in renal function were tested. As graft rejection and transplant glomerulopathy are important causes for adverse changes in renal function of transplant recipients, our population has an uncharacteristically high prevalence of anti-HLA Abs. Consequently, our results overestimate the screening-bead assay's PPV – and more importantly for screening tests – underestimate its NPV.

To determine how the NPV and PPV vary with prevalence, a 2x2 epidemiological table was constructed using the data for LS-MIX and LC-LMX combined, from ii) "MFI included". This data set was chosen as it best represented clinical interpretation of single-Ag bead assays:

	Disease + (Single-Ag Bead +)	Disease - (Single-Ag Bead -)
Test + (Screen +)	300	260
Test - (Screen -)	31	97

Table 1: 2x2 epidemiological table of ii) MFI included.

Using this table, the prevalence of anti-HLA Abs in our population is 48.1%, 95% CI [0.444, 0.518]. Prevalence of anti-HLA Abs in patients post-transplant has been reported to be up to 30% [29], with prevalence of *de novo* anti-HLA DSAs being between 10-15% [29, 34, 35].

Thus, PPV and NPV were recalculated as functions of prevalence (Appendix, Table 1).

Prevalence	PPV	NPV
0.05	0.06147259	0.98218161
0.1	0.12147815	0.96311369
0.15	0.1800686	0.94266001

0.2	0.23729339	0.92066382
0.25	0.29319974	0.89694379
0.3	0.34783268	0.87128901
0.35	0.40123523	0.84345269
0.4	0.45344849	0.81314423
0.45	0.50451177	0.78001899
0.48110465	0.53571429	0.7578125
0.5	0.55446262	0.74366517
0.75	0.78873723	0.4916243
0.99	0.9919487	0.02847021

Table 2: PPV and NPV as functions of varying prevalence. Blue font indicates the PPV and NPV of our study population; red font indicates the PPV and NPV for literature-reported prevalence of anti-HLA Abs and *de novo* anti-HLA DSAs.

The results are also graphed below:

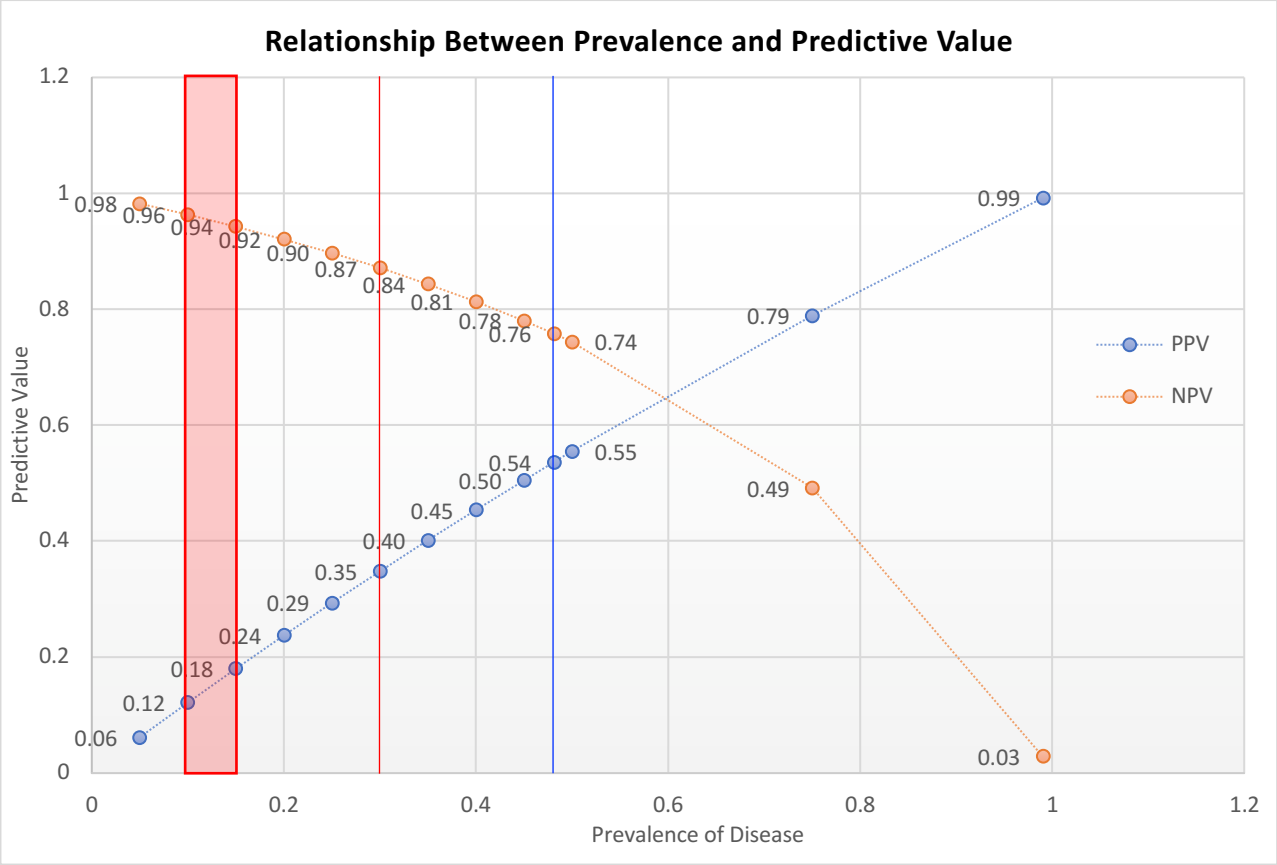


Figure 8: Graph of PPV and NPV as functions of varying prevalence, using single-Ag bead assay as a gold standard for detecting anti-HLA Abs. Blue line represents the prevalence of anti-HLA-Abs in our study population, at 48.1%; red line represents literature-reported prevalence of anti-HLA Abs, at 30% and red box represents literature-reported range of prevalence of *de novo* anti-HLA-Abs.

Thus, the screening-bead assay's PPV and NPV for anti-HLA Abs post-transplant are 34.8% and 87.1% respectively, while its PPV and NPV for anti-HLA DSAs are between 12.1%-18.0% and 94.2% to 96.3% respectively.

3.2.4 Receiver Operating Characteristic (ROC) Curves

An ROC curve was constructed to further characterize the screening-bead assay's clinical utility.

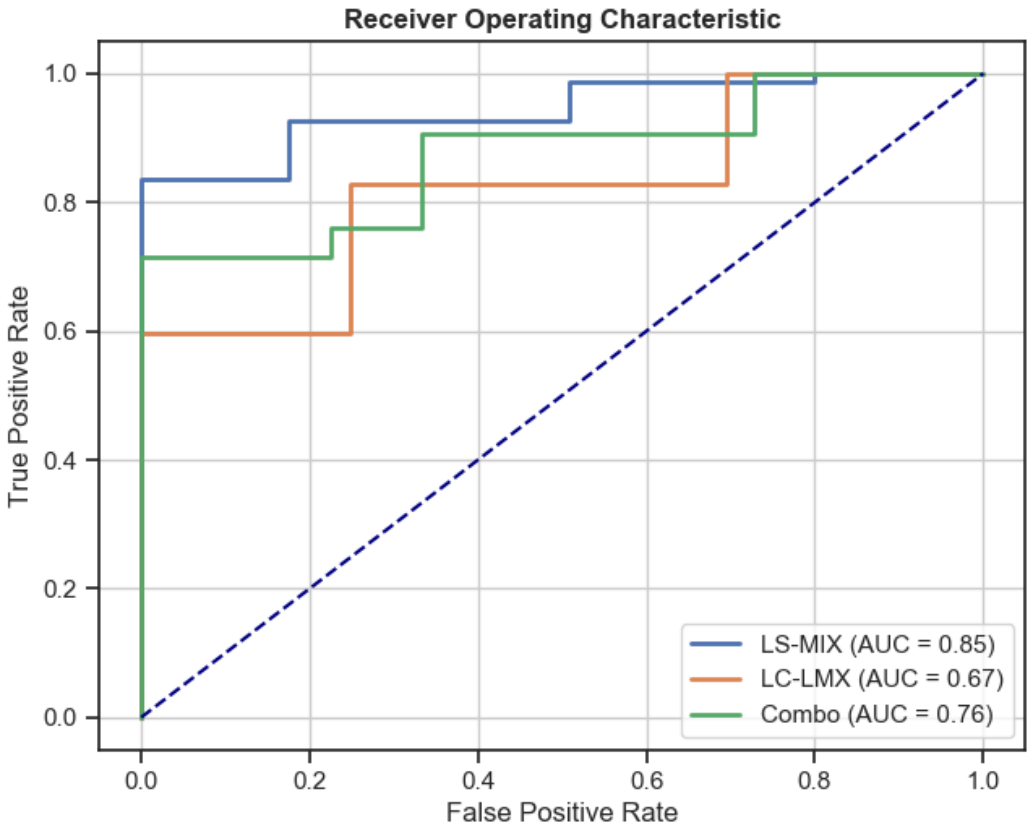


Figure 9 ROC curve of LS-MIX, LC-LMX and LS-MIX and LC-LMX combined, using single-Ag bead assays as a gold standard for detection of anti-HLA Abs.

The ROC curve showed fair performance of the screening-bead assay when using the single-Ag bead assay as a gold standard. Differing MFI thresholds for positivity explain the difference between LS-MIX and LX-LMX, with LS-MIX having good performance, and LC-LMX having marginal performance.

3.3 Using Biopsy as a Gold Standard to Detect ABMR

Biopsies, ordered by transplant nephrologists at University of Alberta to investigate cause of graft dysfunction, done between 2013 to 2017, were pulled from the HLA laboratory's data repository and formatted in Microsoft Excel. A total of 849 biopsies, from 338 patients, were pulled. 311 of these biopsies, from 183 patients, were done between 2013 to 2017. After inclusion and exclusion criteria were applied, 95 biopsies, from 87 patients, were included.

3.3.1 Clinical Characteristics of Biopsies

Biopsies were done soon after blood draws for corresponding screening-bead assays, with median and average elapsed time being 1 and 4.83 days respectively, with interquartile range of 0 to 6.25 days. Screening-bead assays were typically done after biopsies, with median and average elapsed time being 6.00 and 8.09 days respectively, with interquartile range of 1 to 8.75 days.

Median and average times between biopsy and transplant were 5.28 years and 9.86 years respectively, with interquartile range of 2.0 to 11.6 years. Subgroups analyses demonstrated median and average times between biopsy and transplant for recipients with negative screens to be 5.89 and 5.72 years respectively with interquartile range of 2.97 to 7.2 years; 10.4, 18.0 and 2.03 to 13.4 years for recipients with positive screens and 7.41, 10.2 and 2.9 to 17.8 years for recipients with anti-HLA DSAs.

Average number of glomeruli obtained was 19.4, 95% CI [16.6, 22.1], with average number of sclerotic glomeruli being 3.3, 95% CI [2.1, 4.5]. 24% (23) demonstrated ABMR, 34% (32) demonstrated T-Cell mediated rejection, 23% (22) had recurrent glomerulonephritis, 5.3% (5) had acute tubular necrosis, 21% (20) had calcineurin-inhibitor toxicity and 1% (1) had thrombotic microangiopathy. Average 2017 Banff Classification scores are tabulated below (Table 3).

Banff Criterion	Average Score [95% CI]
Glomerulitis (*g*)	0.40 [0.30, 0.50]
Peritubular capillaritis (*ptc*)	0.50 [0.40, 0.60]
Intimal arteritis (*v*)	0 [0,0]
C4d (*C4d*)	0.25 [0.16, 0.34]
Glomerular basement membrane double contour (*cg*)	0.20 [0.12, 0.28]

Vascular fibrous intimal thickening (*cv*)	1.00 [0.82, 1.18]
Interstitial inflammation (*i*)	0.20 [0.12, 0.28]
Tubulitis (*t*)	0.20 [0.12, 0.28]
Interstitial fibrosis (*ci*)	1.20 [1.05, 1.35]
Tubular atrophy (*ct*)	1.40 [1.30, 1.50]
Mesangial matrix expansion (*mm*)	0.60 [0.44, 0.76]
Arteriolar hyalinosis (*ah*)	1.60 [1.39, 1.81]
Total inflammation (*ti*)	0.20 [0.12, 0.28].

Table 3: Average 2017 Banff Classification scores.

Changes in creatinine (Cr) and estimate glomerular filtration rate (eGFR) were calculated for 77 biopsies. 8 biopsies were excluded as they were duplicates from the same patient; 10 more were lost as their GFRs were not available in our registry. They are tabulated (Tables 4 and 5) and graphed (Figures 10-13) below.

Changes in Cr (±95% CI)						
	n	**Cr at bx**	**Cr at last f/u**	**Avg length of f/u (days)**	**Total days of f/u**	**Avg change in Cr/yr**
Overall	76 (28 DSA+)	175.72 (±20.83)	248.00 (±50.46)	958.05 (±126.73)	72812	79.30 (±64.86)
ABMR+	19 (15 DSA+)	166.16 (±13.85)	351.26 (±75.37)	897.05 (±123.36)	17044	83.28 (±23.66)
DSA+, ABMR+	15	162.73 (±13.62)	387.53 (±82.30)	1003 (±126.58)	15045	89.25 (±24.14)
DSA-, ABMR+	4	179.00 (±16.45)	215.25 (±30.23)	499.75 (±56.89)	1999	60.89 (±24.43)
Acute ABMR+	3 (3 DSA+)	196.67 (±24.78)	560.33 (±92.71)	709.00 (±109.28)	2127	137.94 (±24.39)
Chronic ABMR+	16 (12 DSA+)	160.44 (±11.71)	312.06 (±71.78)	932.31 (±127.48)	14917	73.03 (±23.60)
DSA+ cABMR+	12	154.25 (±10.24)	344.33 (±80.93)	1076.50 (±129.43)	12918	77.08 (±24.35)
DSA-, cABMR+	4	179.00 (±16.45)	215.25 (±30.23)	499.75 (±56.89)	1999	60.89 (±24.43)

ABMR-	57 (13 DSA+)	178.91 (±22.75)	213.58 (±36.66)	978.39 (±128.59)	55768	77.98 (±73.85)

Changes in eGFR (±95% CI)						
	n	**eGFR at bx**	**eGFR at last f/u**	**Avg length of f/u (days)**	**Total days of f/u**	**Avg change in GFR/yr**
Overall	76 (28 DSA+)	42.25 (±3.94)	38.93 (±5.31)	957.61 (±126.57)	72778	-7.27 (±8.83)
ABMR+	19 (15 DSA+)	42.58 (±3.54)	35.11 (±6.35)	895.26 (±122.59)	17010	-5.56 (±1.61)
DSA+, ABMR+	15	42.73 (±3.62)	34.00 (±6.87)	1000.73 (±125.71)	15011	-5.90 (±1.71)
DSA-, ABMR+	4	42.00 (±3.72)	39.25 (±4.46)	499.75 (±56.89)	1999	-4.26 (±1.32)
Acute ABMR+	3 (3 DSA+)	47.00 (±6.17)	28.67 (±8.20)	709.00 (±109.28)	2127	-10.40 (±1.06)
Chronic ABMR+	16 (12 DSA+)	41.75 (±3.12)	36.31 (±6.24)	930.19 (±126.61)	14883	-4.65 (±1.63)
DSA+ cABMR+	12	41.67 (±3.08)	35.33 (±6.89)	1073.67 (±128.43)	12884	-4.78 (±1.78)
DSA-, cABMR+	4	42.00 (±3.72)	39.25 (±4.46)	499.75 (±56.89)	1999	-4.26 (±1.32)
ABMR-	57 (13 DSA+)	42.14 (±4.10)	40.21 (±4.95)	978.39 (±128.59)	57024	-7.85 (±10.17)

Tables 4 and 5: Changes in Cr and eGFR for biopsies. Time of observation started at biopsy and was tracked through to the last made measurement, all of which were in 2018.

Linear regression was used to analyze these data (Figures 14-17, linear regression for eGFR is presented in Appendix: Figures 2-5). They were plotted as change in serum creatinine, where each point represents the difference between the serum creatinine at any given time point and the initial serum creatinine at biopsy (labeled as time 0). The first point in each plot, the y-intercept, represents the difference between the creatinine at biopsy and the second measurement of creatinine. Slope of the graphs represent the rate of change in the difference between serum creatinine and serum creatinine at screen.

Note, 10% of outliers were removed from Figures 14, 16, and 17 and Appendix: Figures 2-5, as their values were so high they were obscuring patterns. They were not removed from Figure 15, as they represented the majority of acute ABMRs. Differences that arose after outliers were removed are labeled.

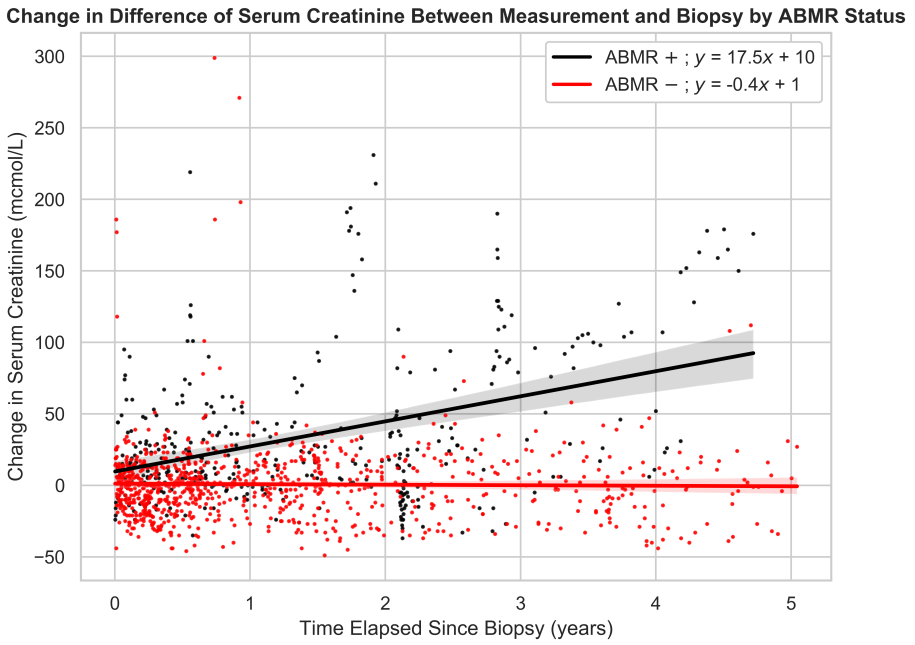


Figure 10: Y-intercept and slope were higher amongst ABMR+ patients (p = 0.046224 and 0.000000 respectively). Before outliers were removed, y-intercept was also higher amongst ABMR+ patients (p = 0.000000), though there was no statistically significant difference between slopes (p = 0.689821).

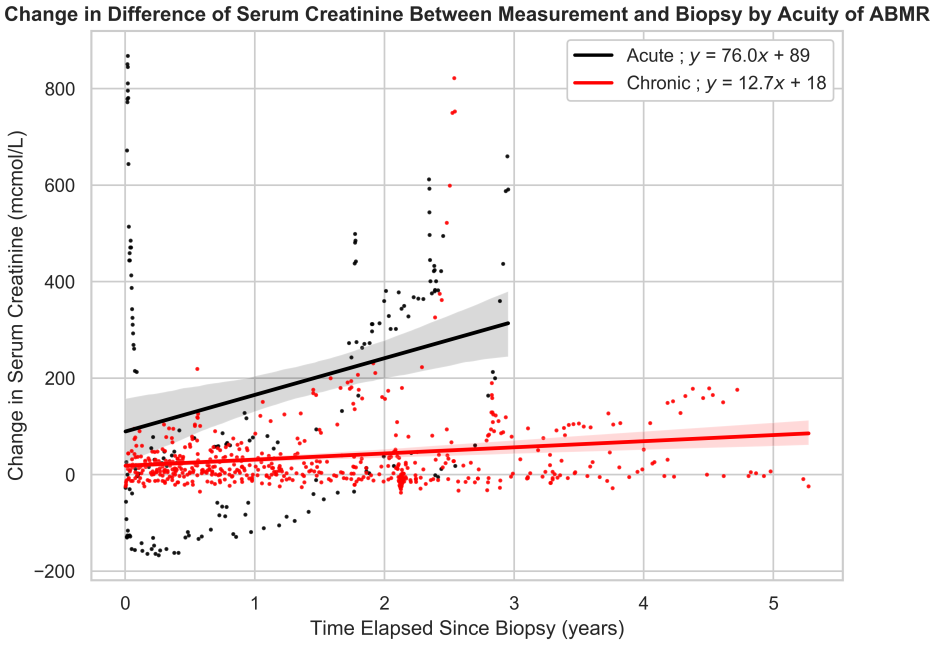


Figure 11: Y-intercept and slope were higher amongst patients with acute ABMR (p = 0.014182 and 0.001650 respectively). Outliers were not removed from this group.

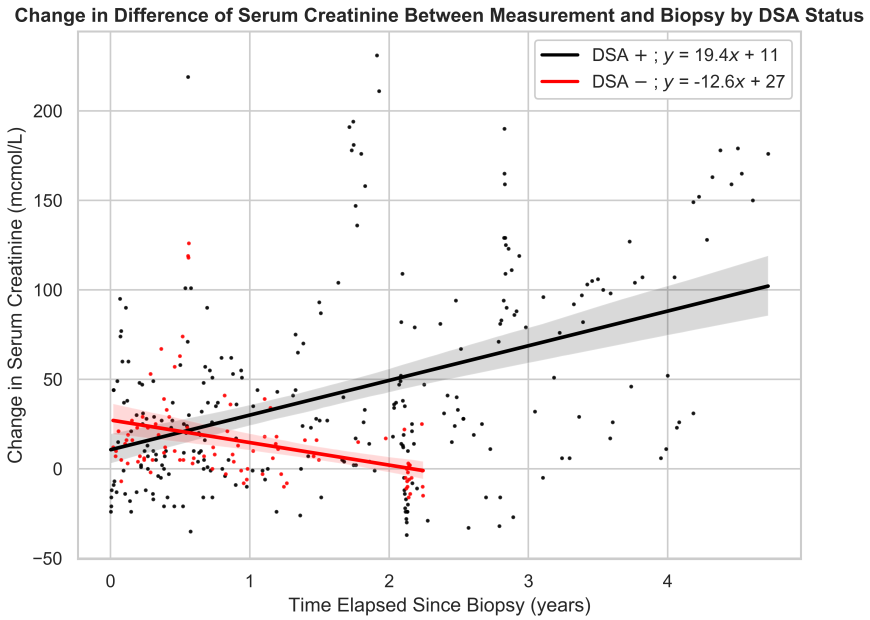


Figure 12: Y-intercept was higher amongst DSA- patients (p = 0.013586) whereas slope was higher amongst DSA+ patients (p = 0.000000). Before outliers were removed, y-intercept was also higher amongst DSA+ patients (p = 0.024363), though there was no statistically significant difference between slopes (p = 0.067416).

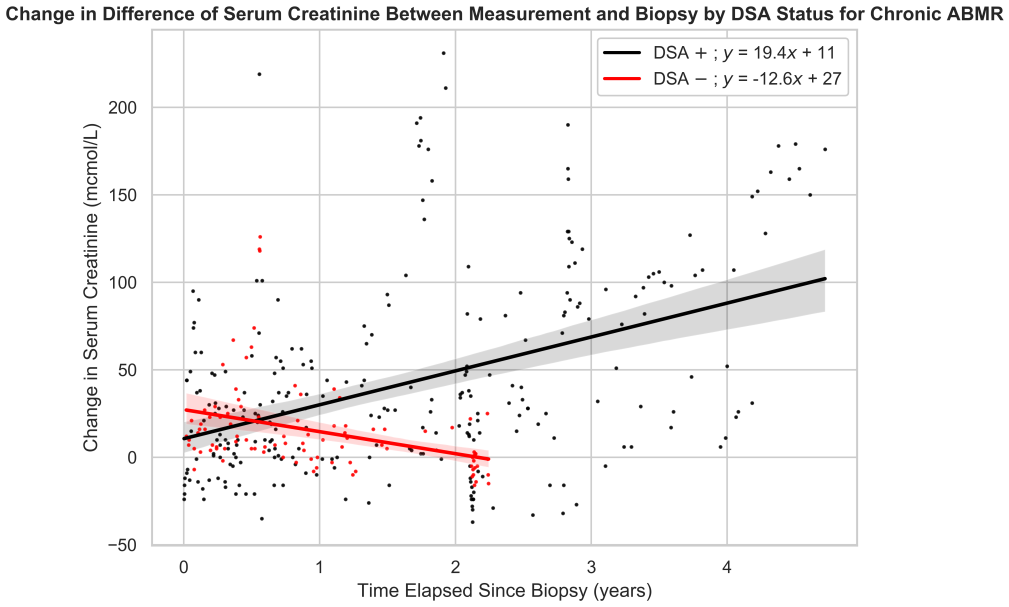


Figure 13: Y-intercept was higher amongst patients without DSAs (p = 0.013586). However, slope was higher amongst DSA + patients (p = 0.000000). Before outliers were removed, y-intercept

was not higher amongst DSA+ patients (p = 0.267006), though the statistically significant difference between slopes persisted (p = 0.002579).

3.3.2 Test Characteristics

Sp, Sn, PPV and NPV (formulae in "Chapter 2: Methods, Appendix) were derived from two data sets.

The first data set examined concordance between screen doublets and biopsies. A screen doublet refers to a corresponding pair of Class I and II screening-bead assays, as every biopsy had both these screens done. A true positive screen was defined as a positive doublet (borderline screens were counted as positive) and positive biopsy, regardless of DSA status. True negative screens were defined as a negative doublet and biopsy.

	Disease + (Biopsy +)	Disease - (Biopsy -)
Test + (Screen +)	21	63
Test - (Screen -)	2	9

Table 5: 2x2 table when true positive screen was defined as a positive doublet and biopsy, regardless of DSA status, and true negative screen was defined as a negative doublet and biopsy.

84/95 doublets were positive; 25.0% (21/84) correlated with the biopsy, of which 14.3% (3/21) of the rejections were acute and 85.7% (18/21) were chronic. 11/95 screens were negative, of which 22.2% (2/11) had rejection, 50% (1/2) of which were acute, and 50% (1/2) chronic. Sensitivity and specificity of LS-MIX and LC-LMX combined were 91.3%, 95% CI [79.8% to 100%] and 12.5%, 95% CI [4.9% to 20.1%] respectively. PPV and NPV were 25% and 82.0% respectively.

Applying +/-LR to an externally-validated prevalence of anti-HLA-Abs shows that for any given transplant recipient, who has a 10-15% chance (we will use 15% for calculations) of having ABMR [19-21], a negative screen reduces their likelihood to 11.0%, while a positive screen raises it marginally to 15.5%. In an alternate scenario, where there is a pre-test probability of 100%, a negative screen would reduce the likelihood of ABMR to 70.2%.

The second data set examined concordance between DSAs, as identified by screens, and biopsies. A true positive screen was defined by a positive doublet, presence of DSA and positive

biopsy. True negative screens were defined by a negative doublet, absence of DSA and negative biopsy.

	Disease + (Biopsy +)	Disease - (Biopsy -)
Test + (Screen, DSA +)	18	15
Test – (Screen, DSA -)	1	8

Table 6: 2x2 table when true positive screen was defined as a positive doublet, DSA and biopsy, and true negative screen was defined as a negative doublet with no DSAs and a negative biopsy.

84/95 doublets were positive; 39.3% (33/84) had DSA, of which 54.5% (18/33) correlated with the biopsy. 88.9% (16/18) of the rejections were chronic and 11.1% (2/18) were acute. 9/95 screens were negative, of which 11.1% (1/9) had chronic rejection on biopsy. Sensitivity and specificity of LS-MIX and LC-LMX combined were 94.7%, 95% CI [84.7% to 100%] and 34.8% [15.3% to 54.2%] respectively. PPV and NPV were 54.5% and 88.9% respectively.

Applying +/-LR to an externally-validated prevalence of anti-HLA-Abs shows that for any given transplant recipient, who has a 10-15% chance (we will use 15% for calculations) of having ABMR [19-21], a negative screen with single-Ag bead assay showing no DSAs reduces their likelihood to 2.58%, while a positive result raises it marginally to 20.4%. In an alternate scenario, where there is a pre-test probability of 100%, a negative result would reduce the likelihood of ABMR to 15.1%.

3.3.3 PPV and NPV as Functions of Prevalence

As in '3.2.1 PPV and NPV as Functions of Prevalence', one of the drawbacks of our dataset is that only patients with adverse changes in renal function were tested. As graft rejection and transplant glomerulopathy are important causes for adverse changes in renal function of transplant recipients, our population has an uncharacteristically high prevalence of ABMR. Consequently, our results overestimate the screening-bead assay's PPV – and more importantly for screening tests – underestimate its NPV.

Using Tables 5 and 6, the prevalence of ABMR is 24.2% and 22.1% respectively, which are uncharacteristically high. In fact, prevalence of ABMR in renal transplant recipients has been reported to be 10-15% [20, 21]. Thus, PPV and NPV are recalculated as functions of prevalence,

for when true positive was defined by a positive doublet and biopsy (Table 7; Appendix, Table 2 and Figure 8) and for when true positive was defined by a positive doublet, presence of DSA and positive biopsy (Table 8; Appendix, Table 3; and Figure 9).

	Predictive Value	
Prevalence	**PPV**	**NPV**
0.05	0.05206074	0.96467991
0.1	0.1038961	0.92825112
0.15	0.15550756	0.89066059
0.2	0.20689655	0.85185185
0.24210526	0.25	0.81818182
0.25	0.25806452	0.81176471
0.3	0.30901288	0.77033493
0.35	0.35974304	0.72749392
0.4	0.41025641	0.68316832
0.45	0.46055437	0.6372796
0.5	0.5106383	0.58974359
0.75	0.75789474	0.32394366
0.99	0.99041267	0.01431238

Table 7: PPV and NPV as functions of varying prevalence, using biopsy as a gold standard for diagnosing ABMR and defining true positives by a positive doublet and biopsy. Blue font indicates the PPV and NPV of our study population; red font indicates the PPV and NPV for literature-reported prevalence of ABMR in renal transplant recipients.

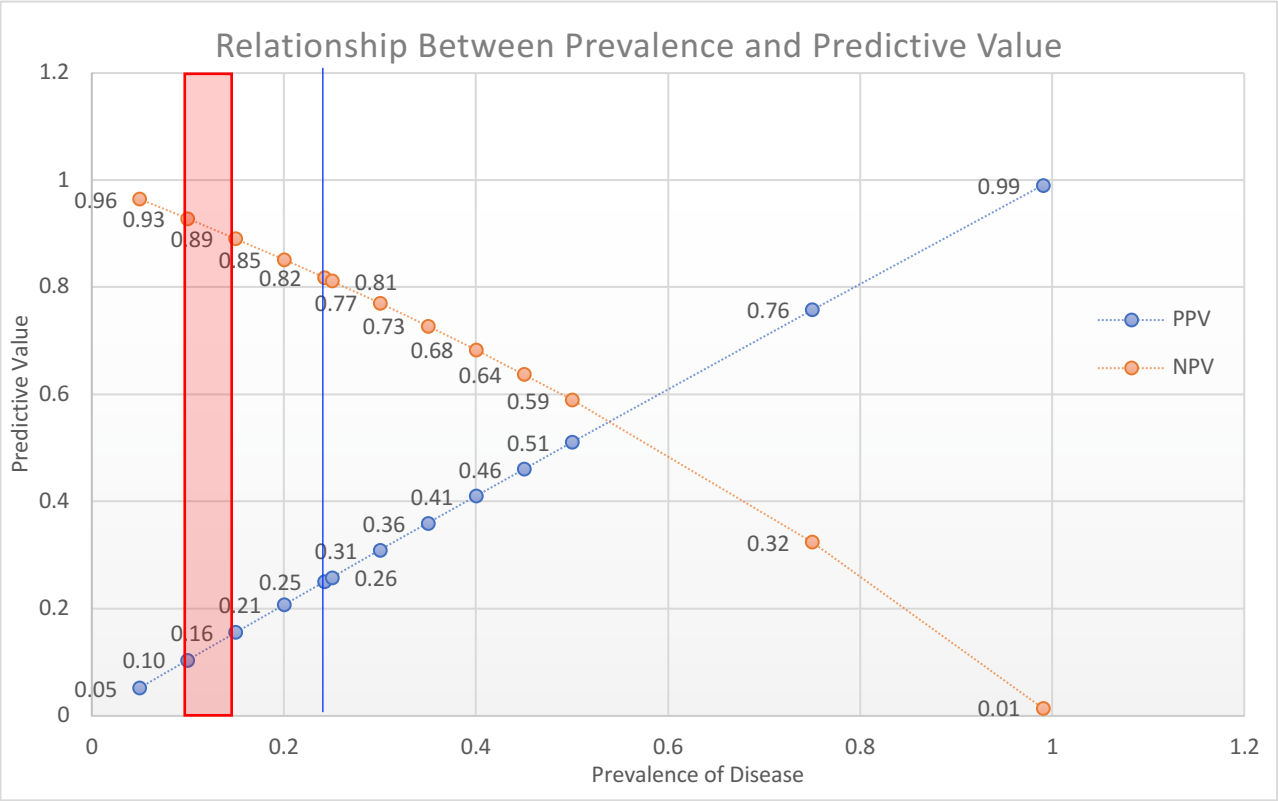


Figure 14: Graph of PPV and NPV as functions of varying prevalence, using biopsy as a gold standard for diagnosing ABMR and defining true positives by a positive doublet and biopsy. Blue line represents the prevalence of ABMR in our study population, at 24.2% and red box represents literature-reported range of prevalence of ABMR.

Prevalence	PPV	NPV
0.05	0.07102419	0.99209894
0.1	0.13897281	0.98346513
0.15	0.2040414	0.97399171
0.2	0.26640927	0.96354992
0.45238095	0.54545455	0.88888889
0.25	0.32624113	0.9519833
0.3	0.3836886	0.93909974
0.35	0.43889141	0.92466074
0.4	0.49197861	0.90836653
0.45	0.54306952	0.88983502

0.5	0.59227468	0.86857143
0.75	0.81335953	0.68778281
0.99	0.99309442	0.06257719

Table 8: PPV and NPV as functions of varying prevalence, using biopsy as a gold standard for diagnosing ABMR and defining true positives by a positive doublet, presence of DSA and positive biopsy. Blue font indicates the PPV and NPV of our study population; red font indicates the PPV and NPV for literature-reported prevalence of ABMR in renal transplant recipients.

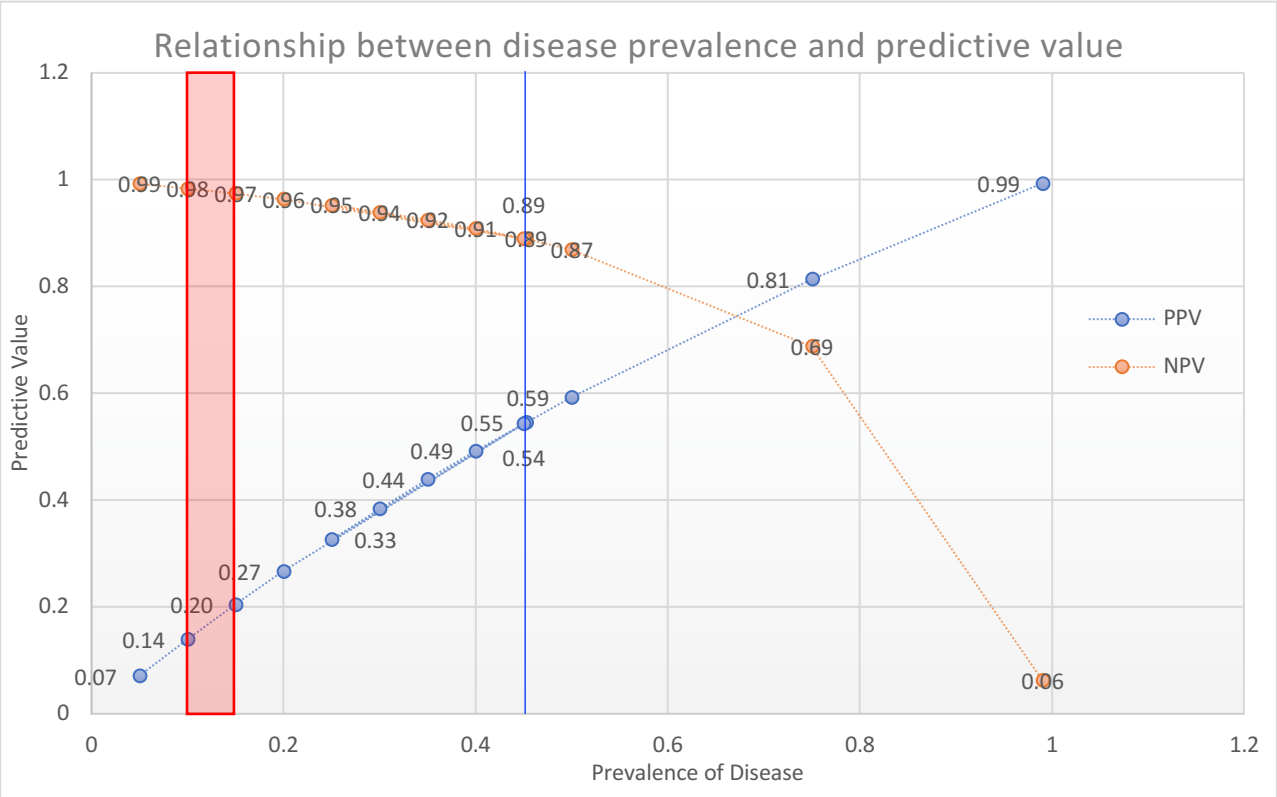


Figure 15: Graph of PPV and NPV as functions of varying prevalence, using biopsy as a gold standard for diagnosing ABMR and defining true positives by a positive doublet, presence of DSAs and positive biopsy. Blue line represents the prevalence of ABMR in our study population, at 45.2% and red box represents literature-reported range of prevalence of ABMR.

Thus, the screening-bead assay's PPV and NPV for ABMR are between 10.4% to 15.6% and 89.1% to 92.8% respectively. The PPV and NPV of DSA's, as identified by the screening-bead assay, for ABMR, were 13.9% to 20.4% and 97.4 to 98.3% respectively.

3.3.4 ROC Curves

Two ROC curves were constructed to further characterize the screening-bead assay's clinical utility (Appendix, Figures 6a and 6b). The first defines true positive as a positive doublet and biopsy; the second as a positive doublet, presence of DSA and biopsy. Unfortunately, a shortcoming of these curves is the small sample size. For example, in Appendix: Figure 6a, when going from "Weak Positive" to "Positive", "Weak Positive" results counted as negative. As it turns out, every "Weak Positive" in the data set was a false positive (i.e. only the right column on the 2x2 table was affected by this change). When counted as a negative, it increased the number of true negatives only. However, we know that a "Weak Positive" screen does not actually have that poor of a PPV – likely, with more "Weak Positive" samples, more true positives would have converted to false negatives.

3.4 Examining changes in serum Cr and eGFR over time

Screening-bead assays, ordered by transplant nephrologists at University of Alberta to investigate ABMR as a cause of graft dysfunction, done between 2013 to 2017, were pulled from the HLA laboratory's data repository and formatted in Microsoft Excel. 1,655 samples, including screening-bead assays and single-antigen bead assays, from 338 patients were pulled. After inclusion and exclusion criteria were applied, 279 doublets were included.

Changes in Cr (±95% CI)						
	n	**Cr at test**	**Cr at last f/u**	**Avg f/u (days)**	**Total days of f/u**	**Avg change in Cr/yr**
Overall	278	144.86 (±15.63)	211.68 (±45.29)	1005.93 (±115.56)	280582	57.32 (±90.44)
Anti-HLA Ab +	250	143.92 (±15.72)	214.01 (±46.49)	1036.71 (±116.86)	260047	61.17 (±95.28)
Class I Anti-HLA	27	137.11 (±10.67)	172.04 (±23.12)	1285.19 (±124.71)	34700	11.19 (±6.20)
Class II alone Anti-HLA	76	144.14 (±14.80)	215.43 (±45.99)	849.79 (±89.53)	64584	58.34 (±49.44)
Class II with I Anti-HLA	147	144.58 (±17.04)	220.62 (±49.98)	1094.75 (±121.76)	161683	69.88 (±119.49)
DSA+	78	158.31 (±19.84)	265.04 (±60.17)	1088.58 (±113.23)	84909	119.24 (±162.47)
Doublet Borderline	75	154.28 (±16.38)	202.51 (±38.09)	743.69 (±90.73)	56116	25.10 (±20.28)
Doublet Weak Positive	26	144.81 (±10.63)	205.19 (±38.10)	1484.69 (±67.53)	38602	17.06 (±11.21)
Doublet Positive	141	136.74 (±16.02)	212.23 (±50.03)	1091.56 (±118.04)	154680	85.10 (±125.17)
Doublet Strong Positive	17	180.18 (±23.91)	383.82 (±103.00)	1166.24 (±123.16)	19826	425.36 (±354.58)
Anti-HLA Ab -	28	153.11 (±14.91)	191.00 (±33.20)	733.39 (±82.41)	20535	23.09 (±15.19)

Changes in eGFR (±95% CI)						
	n	**eGFR at test**	**eGFR at last f/u**	**Avg f/u (days)**	**Total days of f/u**	**Avg change in eGFR**
Overall	278	52.23 (±5.23)	44.79 (±5.69)	1005.66 (±115.48)	278666	-3.99 (±3.24)
Anti-HLA Ab +	250	52.59 (±5.27)	44.80 (±5.77)	1036.40 (±116.78)	258131	-4.19 (±3.36)
Class I Anti-HLA	27	48.00 (±4.22)	43.96 (±5.08)	1283.89 (±124.38)	34665	-3.13 (±2.43)

Class II alone Anti-HLA	76	50.57 (±4.92)	43.32 (±5.55)	849.78 (±89.53)	64583	-4.60 (±3.39)
Class II with I Anti-HLA	147	54.72 (±5.61)	45.87 (±6.03)	1094.48 (±121.72)	159803	-4.03 (±3.49)
DSA+	78	50.24 (±5.48)	39.76 (±6.06)	1089.55 (±113.43)	84985	-4.48 (±1.89)
Doublet Borderline	75	48.89 (±4.46)	45.61 (±5.30)	746.86 (±91.61)	56351	-3.01 (±4.55)
Doublet Weak Positive	26	48.81 (±3.71)	44.58 (±5.75)	1484.69 (±67.53)	38602	-1.01 (±1.54)
Doublet Positive	141	55.93 (±5.82)	45.31 (±6.09)	1089.35 (±118.26)	152529	-5.20 (±2.90)
Doublet Strong Positive	17	43.53 (±4.99)	38.59 (±6.63)	1173.41 (±125.02)	19948	-5.27 (±3.10)
Anti-HLA Ab -	28	49.04 (±4.91)	44.68 (±5.07)	733.39 (±82.41)	20535	-2.30 (±1.84)

Tables 9 and 10: Changes in Cr and eGFR for all patients who had a screening-bead assay. Time of observation started at the date of Cr/eGFR measurement (within one month of the screening-bead assay) and was tracked through to the last made measurement, all of which were in 2018. Correction factor for weighted change was determined by dividing days of observation by total days of follow-up overall.

Linear regression was used to analyze these data (Figures 22-25, linear regression for eGFR is presented in Appendix: Figures 7-10). They were plotted as change in serum creatinine, where each point represents the difference between the serum creatinine at any given time point and the serum creatinine at the time of the screen (labeled as time 0) for all bloodwork done on the patient included within the group. The fist point in each plot, the y-intercept, represents the difference between the creatinine when the screen was conducted and the second measurement of creatinine. Slope of the graphs represent the rate of change in the difference between serum creatinine and serum creatinine at screen.

Further, 10% of outliers were removed, as their values were so high they were obscuring any patterns. Differences that arose after outliers were removed are labeled.

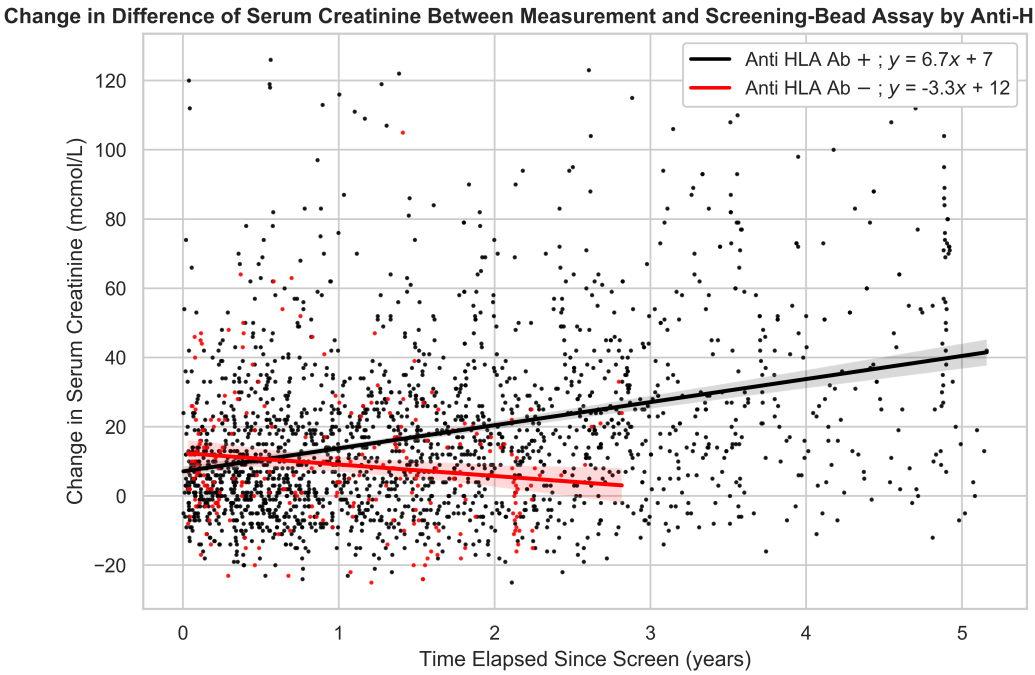


Figure 16: Y-intercept was higher amongst patients without anti-HLA-Abs (p = 0.012093). However, the slope was higher amongst anti-HLA-Ab + patients (p = 0.000000). r^2 values for anti-HLA-Ab + and – patients were 0.113 and 0.019 respectively. Before outliers were removed, y-intercept was lower amongst patients with anti-HLA-Abs (0.000008), and there was no statistically significant difference between slopes (p = 0.525618).

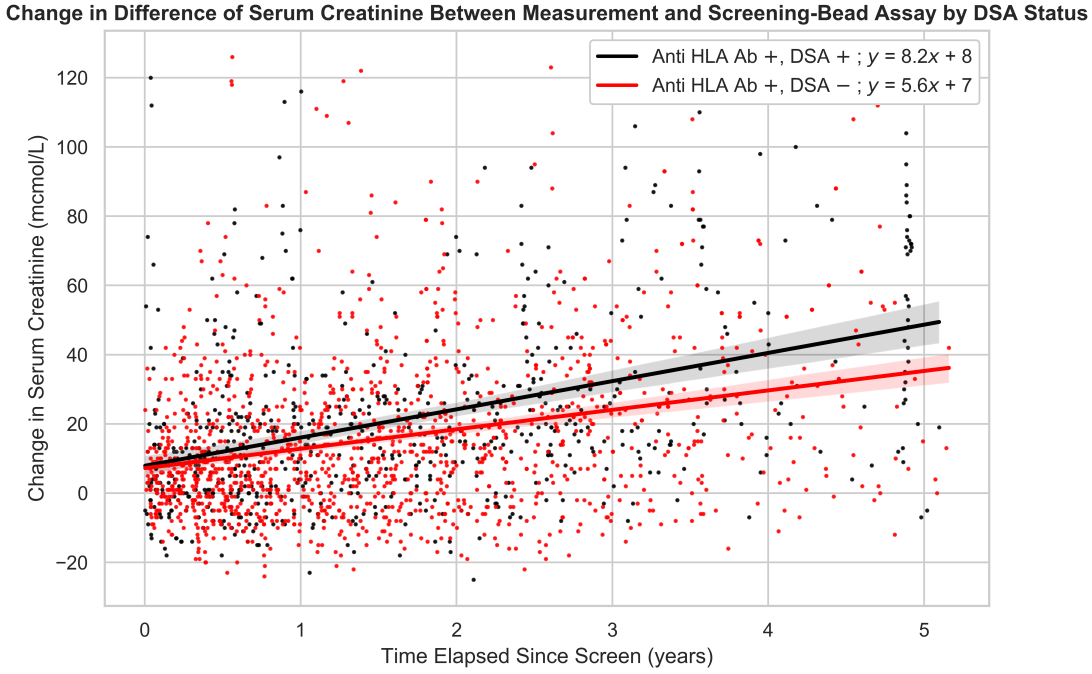


Figure 17: Y-intercepts were not different between groups (p = 0.702163). However, slope was higher amongst DSA+ patients (p = 0.003208). r^2 values for DSA+ and – patients were 0.171 and 0.081 respectively. Before outliers were removed, there was no statistically significant difference between y-intercepts and slopes (p = 0.112468 and 0.187801 respectively).

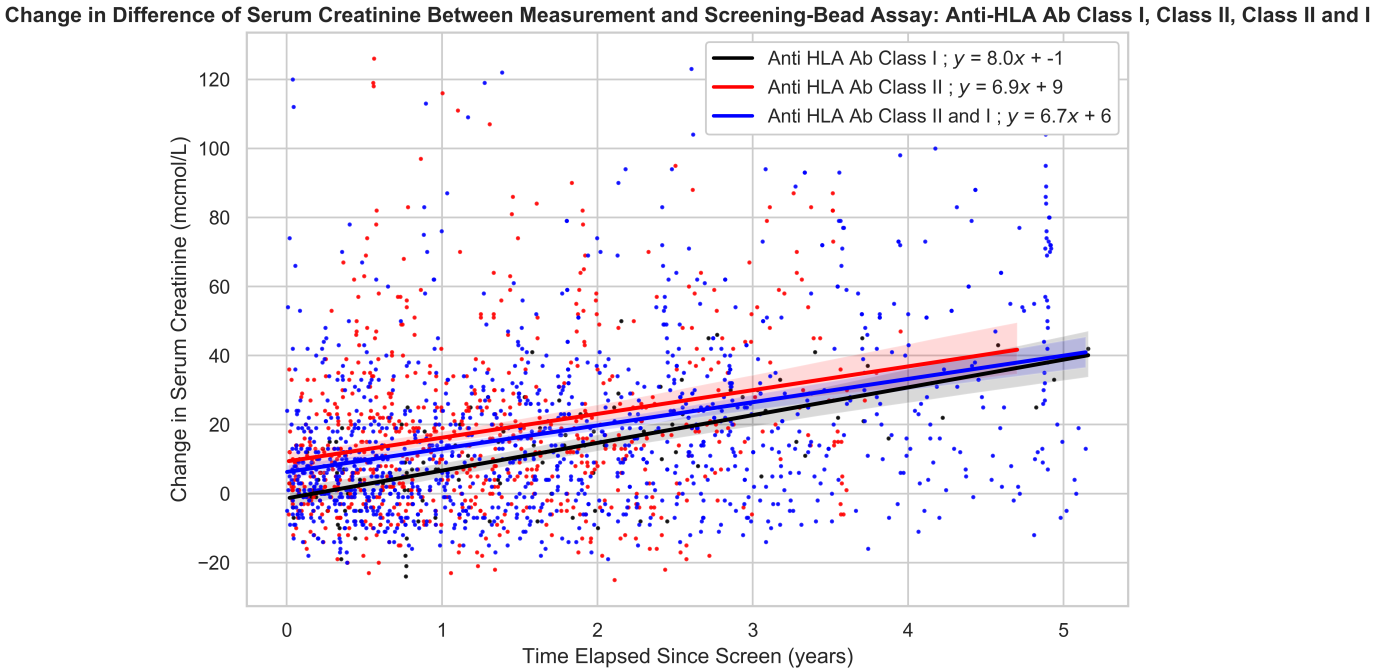


Figure 18: When comparing patients with Class II anti-HLA-Abs to those with Class I anti-HLA-Abs, y-intercept was higher in the former (p = 0.000006). However, slope was not statistically significant (p = 0.373257). When comparing patients with Class II anti-HLA-Abs to those with Class II+I anti-HLA-Abs, y-intercept and slope were similar (p = 0.098968 and 0.892164 respectively). 3-way ANOVA for slope and y-intercept showed p = 0.7961 and p = 0.0118 respectively. r^2 values for patients with Class I, II and II+I anti-HLA-Abs were 0.391, 0.070 and 0.134 respectively.

Before outliers were removed, differences for y-intercept and slope persisted, when comparing patients with Class II anti-HLA-Abs to those with Class I anti-HLA-Abs (p = 0.000000 and p = 0.196373 respectively). When comparing patients with Class II anti-HLA-Abs to those with Class II+I anti-HLA-Abs, y-intercept was higher in the former (p = 0.021630), though differences between slopes did not reach statistical significance (p = 0.050294).

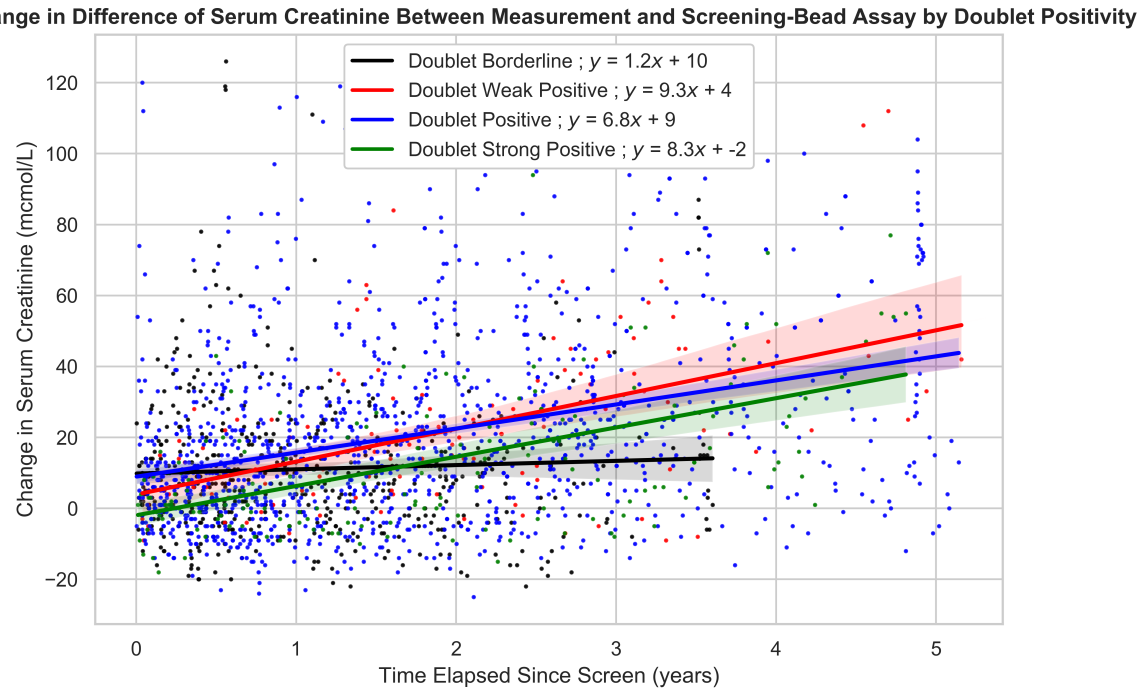


Figure 19: Each group was compared to 'Borderline' results. Y-intercept was not different when compared to 'Weak Positive' and 'Positive' results (p = 0.111760 and 630337 respectively), but was lower for 'Strong Positive' results (p = 0.000060). Slope was higher in 'Weak Positive', 'Positive' and 'Strong Positive' results (p = 0.00007, 0.00001 and 0.00003 respectively). 3-way ANOVA between 'Weak Positive', 'Positive' and 'Strong Positive' results showed p = 0.0024 for y-intercepts and no significant difference in the slopes (p = 0.2559). r^2 values for patients with 'Borderline', 'Weak Positive', 'Positive' and 'Strong Positive' screens were 0.003, 0.253, 0.116 and 0.251 respectively.

Before outliers were removed, y-intercept was higher for 'Bordelrine' results when compared to 'Weak Positive', 'Positive' and 'Strong Positive' results (p = 0.002665, 0.008240 and 0.020825 respectively). Slope was significantly higher amongst patients with 'Weak Positive' results (p = 0.003879), though it did not reach statistical significance for 'Positive' and 'Strong Positive' results (p = 0.262330 and 0.241801 respectively).

Chapter 4: Discussion.

4.1 Using the Single-Antigen (Ag) Bead Assay as a Gold Standard to Detect Anti-HLA Abs

Overall, positive screens had less clinical utility than negative ones. Specificity, at 27.2%, 95% CI [22.6%, 31.8%], and PPV, at 53.57%, were poor. PPV remained poor when recalculated for literature-reported post-transplant prevalence of anti-HLA antibodies and DSAs (34.8% and 12.1 to 18.0% respectively). Low specificity and PPV result from high false positive test rates. This is likely a consequence of setting low MFI thresholds for positivity. Other possible explanations for a high false positive rate include screening-bead assays detecting low-affinity, high-titre anti-HLA antibodies and non-specific binding of serum components, though this is less likely as washing steps in the preparation of the assay should overcome their weaker affinity for HLA.

Contrastingly, negative screens had more clinical utility, with sensitivity being 90.6%, 95% CI [87.5%, 93.8%] and NPV being 75.78%. NPV improved when recalculated for literature-reported post-transplant prevalence of anti-HLA antibodies and DSAs (87.1% and 94.3-96.3% respectively), which were lower than those in our population (48.1%, 95% CI [0.444, 0.518] in our population versus up to 30% for anti-HLA-Abs and 10-15% for anti-HLA-DSAs [29, 34, 35]). These data suggest that the screening-bead assay must be interpreted with pre-test probability in mind. For patients with a low clinical suspicion of ABMR, a negative result can be relied upon; for those with high clinical suspicion, further testing is warranted. False negative screens can result from high-affinity, low-titre anti-HLA antibodies, which can still be deleterious due to cross-reactivity and ability to activate compliment. Single-Ag beads, being studded with more antigen of one type, may better detect and retain high-affinity, low-titre antibodies from the serum.

An interesting phenomenon is the change in test characteristics of the screening-bead assay when MFI threshold of interpretation for single-Ag bead assays was altered. When weakly-fluorescent single-Ag bead results (MFI<500) were counted as negative, PPV decreased more than specificity, indicating that more true positive screens were now being labeled as false positives. Conversely, NPV increased more than sensitivity, meaning previously false negative screens were now being labeled as true negative. The former group likely indicates high-titre antibodies with low-affinity while the latter group likely indicates low-titre antibodies of medium-high affinity. These antibodies' clinical significance is unknown.

An ROC curve was constructed due to the limitations of the 2x2 epidemiological table, including evaluating the screening-bead assay as a binary test, especially since it reports results

with varying degrees of positivity based on MFI. Overall AUC was 76% (indicating fair performance), with AUC for LS-MIX and LC-LMX being 85% (good performance) and 67% (marginal performance) respectively [64]. Unfortunately however, there are several limitations to our curve. Foremost, there were only four test cut-offs – fewer inflection points can artificially lower the AUC. Second, the MFI threshold for positivity differed between LS-MIX and LC-LMX. Being lower for LC-LMX, its AUC was also lower, thereby also lowering the screening-bead assay's overall AUC.

Most of the literature for the clinical utility of the screening-bead assay is from its pre-transplant use. It has been shown to detect deleterious antibodies that were missed by the cell-dependent cytotoxicity (CDC) assay, though concomitant use with the CDC assay is still widely supported [65-67]. Literature for the clinical utility of the screening-bead assay post-transplant is extremely limited, as identified by the Sensitization in Transplant Assessment of Risk (STAR) Working Group in 2017 [68]. Since then, Crespo, M. *et al* sought to establish whether screening-bead assays should precede single-antigen bead assays in post-transplant monitoring for DSAs. They prospectively screened 118 renal transplant recipients between 2011-2015, followed by single-Ag bead assays from the same manufacturer if screens were positive. They then retrospectively analyzed frozen peri-biopsy samples with a single-Ag bead assay from a different manufacturer. They found that the latter method identified 13 additional patients with DSAs (n = 49) who had been missed by the former (n = 36), and that it fully satisfied Banff 2017 Criteria in 17% of patients who had gone on to have a biopsy after a negative screen. Overall, they report that the Sn, Sp, PPV and NPV for the latter method were 78.8%, 87.8%, 88.8% and 75.6% respectively. For the former, they were lower, at 61.5%, 93.9%, 83.6% and 84.1% respectively. However, there are some limitations to their study. As we have shown, different kits have differing test characteristics. In fact, Crespo, M. *et al* showed that of the 13 patients identified by the second single-Ag kit, the first single-Ag kit was only able to identify 6. Moreover, the retrospective component of the study was done on peri-biopsy samples, which can have more DSA present than the prospective arm – making it easier for the single-Ag bead assay to detect DSA [69]. Our study differs in that we have significantly more samples. Moreover, test characteristics were also calculated differently – they were calculated for the screen, using the single-Ag bead assay as a gold standard, rather than for the process of screening followed by single-Ag bead assays. One

relative shortcoming of our study is that we did not examine the utility of single-Ag beads in isolation.

These data show that the screening-bead assay reliably detects anti-HLA Abs, though not as well as the single-Ag bead assay. Positive screens overcall anti-HLA Abs, due to low MFI thresholds, which are necessary to ensure that negative screens are accurate. In turn, negative screens, though accurate, can miss low-affinity, low-titre anti-HLA DSAs. Though their clinical significance is unknown, they can be deleterious, due to cross-reactivity and complement activation. Regardless, as a screening measure whose value is in its ability to rule-out anti-HLA antibodies, the screening-bead assay performs relatively well.

4.2 Using Biopsy as a Gold Standard to Detect ABMR

Screening-bead assays were compared to biopsies through two data sets: the first examined concordance between screens and biopsies while the second examined concordance between DSAs, as identified by screens, and biopsies. Similar to '4.1 Using the Single-Antigen (Ag) Bead Assay as a Gold Standard to Detect Anti-HLA Abs', positive screens had less clinical utility than negative ones. When comparing screens to biopsies, specificity and PPV were 12.5%, 95% CI [4.9% to 20.1%] and 25% respectively. PPV remained poor (10.4 to 15.6%) when recalculated for literature-reported post-transplant prevalence of ABMR. Again, this could be a consequence of setting low MFI thresholds for positivity, thus overcalling positive tests. It could also result from identification of non-DSAs, which are not as harmful as DSAs. Though specificity and PPV increased when DSAs were accounted for (34.8% [15.3% to 54.2%] and 54.5% (13.6% to 20.4% when calculated for literature-reported post-transplant prevalence of ABMR) respectively), they remained poor. One important caveat to interpreting false positives from both groups is that the screen could be detecting deleterious antibodies, but renal function could be deteriorating from other causes – hence necessitating a biopsy before the pathology of ABMR can manifest.

Increased specificity and PPV when including DSA status indicate the prognostic significance of anti-HLA DSAs. They also indicate that patients are over-biopsied, highlighting the complexity behind ABMR's diagnosis. Likely, the decision to biopsy patients should be based on a composite of multiple risk factors for ABMR, such as compliance to immune-suppressive therapy, rate of change in serum creatinine and eGFR [34], presence of proteinuria, MFI of anti-HLA DSAs, degree of HLA-mismatch between donor and recipient [70] and prior sensitizing

events. In fact, Lefaucheur, C. *et al* have demonstrated the utility of an analogous model in prognostication of ABMR: they identified several variables with differing risk, including eGFR at diagnosis of ABMR, presence of interstitial fibrosis/tubular atrophy on biopsy, relative change in eGFR, *ptc* score and change in MFI of anti-HLA DSAs after treatment [20].

Negative screens, on the other hand, had more clinical utility. When comparing screening-bead assays to biopsies, sensitivity and NPV were 91.3%, 95% CI [79.8% to 100%] and 82.0% respectively. Further, NPV improved (89.1%-92.8%) when recalculated for literature-reported post-transplant prevalence of ABMR. However, there were still 2 false negative screens, which could result from low-affinity, low-titre, cross-reactive anti-HLA DSAs that are not identified by the screening-bead assay or by non-HLA DSAs. It is difficult to estimate what the true prevalence of such patients is, given the limited number of screen-negative patients that were biopsied. When accounting for DSA-negative screens, sensitivity increased further to 94.7%, 95% CI [84.7% to 100%] and NPV to 88.9% (97.4%-98.4% when recalculated for literature-reported post-transplant prevalence of ABMR). Interestingly, there was still one false negative screen, and three patients with positive screens but no DSAs had features of ABMR on their biopsies. These findings could result from non-specificity of the biopsy, or non-HLA DSAs causing ABMR.

These data show that negative screens correlate well with biopsies. However, they must be interpreted with caution in patients with risk factors for ABMR, where confirmatory testing should be pursued with a lower threshold. Positive results, on the other hand, have less correlation with biopsy. Those with DSAs warrant closer monitoring and follow-up. Overall, as a screening measure whose value is in its ability to rule-out ABMR, the screening-bead assay performs relatively well.

4.3 Examining changes in serum Cr and eGFR over time

Our results demonstrate that anti-HLA-Abs, particularly anti-HLA-DSAs, portended a poorer prognosis for renal allograft function, which is consistent with literature-reported findings [34-36]. There was inconsistency in that Class II anti-HLA-Abs did not portend a worse prognosis, though this could be because they had a higher initial change to start with.

When examining screening-bead assay results by positivity, two trends emerge. First, the rate of change in the difference between serum creatinine at any given point in time and serum creatinine at screen was lower for ‘Borderline’ results than for ‘Weak Positive’, ‘Positive’ and

'Strong Positive' results (p = 0.00007, 0.00001 and 0.00003 respectively). As discussed in '4.1 Using the Single-Antigen (Ag) Bead Assay as a Gold Standard to Detect Anti-HLA Abs' and '4.2 Using Biopsy as a Gold Standard to Detect ABMR', this is likely from setting a low MFI threshold for defining positive results. In fact, the mild slope could be explained by other causes of chronic graft dysfunction, such as transplant glomerulopathy and calcineurin-inhibitor toxicity. Second, there was no significant difference between 'Weak Positive', 'Positive' and 'Strong Positive' results (ANOVA for y-intercepts p = 0.0024; for slopes p = 0.2559), suggesting that allograft function does not correlate with MFI of the screening-bead assay. This can be from several factors. First, MFI of the screening-bead assay depends on the variety of anti-HLA-Abs present. The more variety, the higher the MFI will be. However, we know that the pathogenicity of these antibodies depend on many factors that are not represented by the screening-bead assay. These include donor-specificity, whether they formed pre- or post-transplant and their mechanism of action, which is in part dependent on their quantity. Another factor is the small number of data points in 'Weak Positive' and 'Strong Positive' groups. Significant intra-group variability can outweigh inter-group variability, nullifying ANOVA results. It is also possible that the slope of the trendline could change if we had more data points.

Overall, these data show that positive screens are related to more significant decline in graft function, though the strength of positive screens does not correlate.

4.4 Clinical Application of the Screening-Bead Assay in Post-Transplant Monitoring of Renal Transplant Recipients

Compared to the single-Ag bead assay, the screening-bead assay did not perform well enough to mandate its antecedent use in every clinical scenario. Instead, these data show that there are particular scenarios in which the screening-bead assay should precede the single-Ag bead assay.

It can be used to screen unsensitized renal transplant recipients for development of anti-HLA-Abs. Here, low prevalence of anti-HLA-Abs significantly increases the NPV. Taken with the screen's high Sn, negative results are reassuring. This can translate to screening unsensitized patients on a regular basis, for example, biannually or annually. One argument against using the screening-bead assay in this context is that, due to its poor Sp and PPV, it will unnecessarily commit many patients to further time-consuming, costly, and risky testing. However, these are

still acceptable, as missed ABMR can cause patients to suffer sequelae of chronic kidney disease, require dialysis and have reduced access to re-transplantation due to HLA-sensitization. Moreover, confirmatory testing includes single-antigen bead assays, which are minimally invasive, and renal biopsy, which despite clinical risk of pain, infection and bleeding, is mandatory in diagnosing ABMR. Positive results can still change clinical care, as they can warrant closer follow-up and further investigation, depending on renal function. If further investigation with single-Ag-bead assays shows development of new anti-HLA-DSAs, this can help us gauge compliance to and titrate immune-suppressive therapy, though confirmatory studies are required.

Unfortunately, the screen cannot be applied to recipients who have pre-existing anti-HLA-Abs, as it is unable to differentiate antibodies of differing specificity. Further, as our results did not show any correlation between the strength of screening results and change in renal function over time, screens are unlikely to indicate pre-sensitized patients who are mounting a progressive antibody response against their allograft.

Its use as a diagnostic test is also less compulsory. Such patients will present with compromised renal function, which will raise the pre-test probability of ABMR, meaning the screening-bead assay is less likely to change the course of further clinical testing. Positive results will always require a confirmatory single-antigen bead assay. Negative results will still warrant follow-up with single-antigen bead assays, as they can miss low-affinity, low-titre anti-HLA DSAs, which, though of unknown clinical significance, can potentially be deleterious due to cross-reactivity and complement activation.

4.5 Proposed Algorithm for Post-Transplant Testing

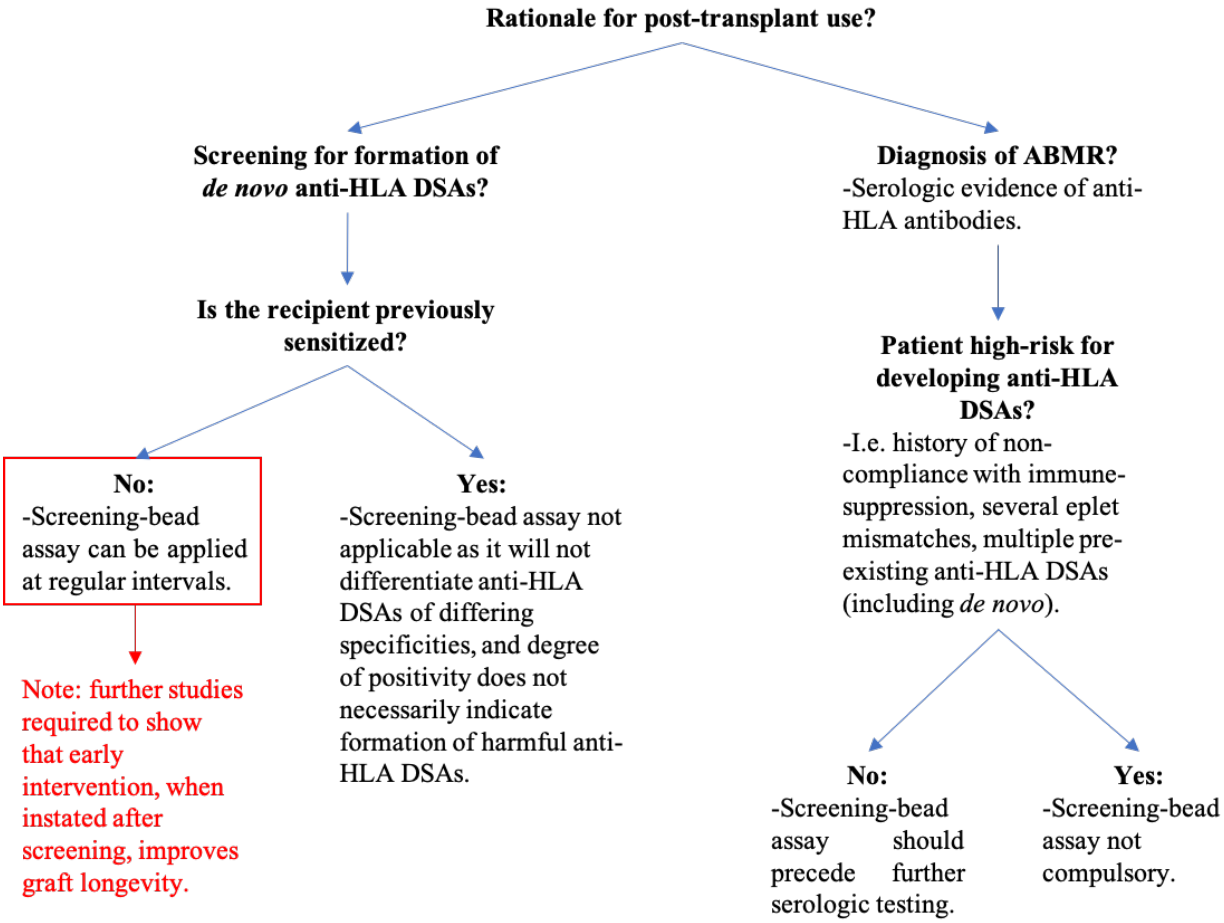


Figure 20: Proposed algorithm for post-transplant application of the screening-bead assay.

4.6 Study Limitations

Some limitations of the study include use of a patient population that had graft dysfunction, and thus a higher pre-clinical suspicion of pre-existing and *de* novo anti-HLA DSAs and ABMR. Though positive and negative predictive values were adjusted for these, specificity and positive likelihood ratios may be overstated and sensitivity and negative likelihood ratios may be understated. However, as a screening study whose clinical value lies in its ability to exclude patients with pre-existing and *de* novo anti-HLA DSAs and ABMR, negative screening results showed strong clinical utility regardless of the population's predisposition.

Other weaknesses include the use of two different assays, LC-LMX and LS-MIX, as the HLA laboratory changed its kit supplier midway in the time frame of the study. The figures in this

study represent averages from both kits, as each kit had differing inherent properties and thresholds for defining positivity.

Lastly, positive and negative predictive values were recalculated for literature-reported prevalence for anti-HLA-Abs and *de novo* anti-HLA-Abs, as it was elevated in our own study population. Unfortunately, these literature-reported values are from studies where mean time between transplant and monitoring is ~5 years. It is difficult to say how predictive and negative values would vary at differing time points, as there is a lack of data showing how percentages of renal transplant recipients with anti-HLA DSAs change with time.

4.7 Future Studies

Future studies include prospectively recalculating these parameters by applying the screening-bead assay to renal-transplant recipients, beginning immediately post-operatively, and comparing it to the single-antigen bead assay in the same population.

If still consistent with this study, we would then have to show that screening patients changes outcomes, before actually applying this to the recipient population at large. This would include a randomized-controlled trial in which patients developing *de novo* DSAs are randomized to treatment with any approved therapy for ABMR, such as PLEX, IVIG or rituximab, with a comparison against unscreened and untreated patients to establish effect.

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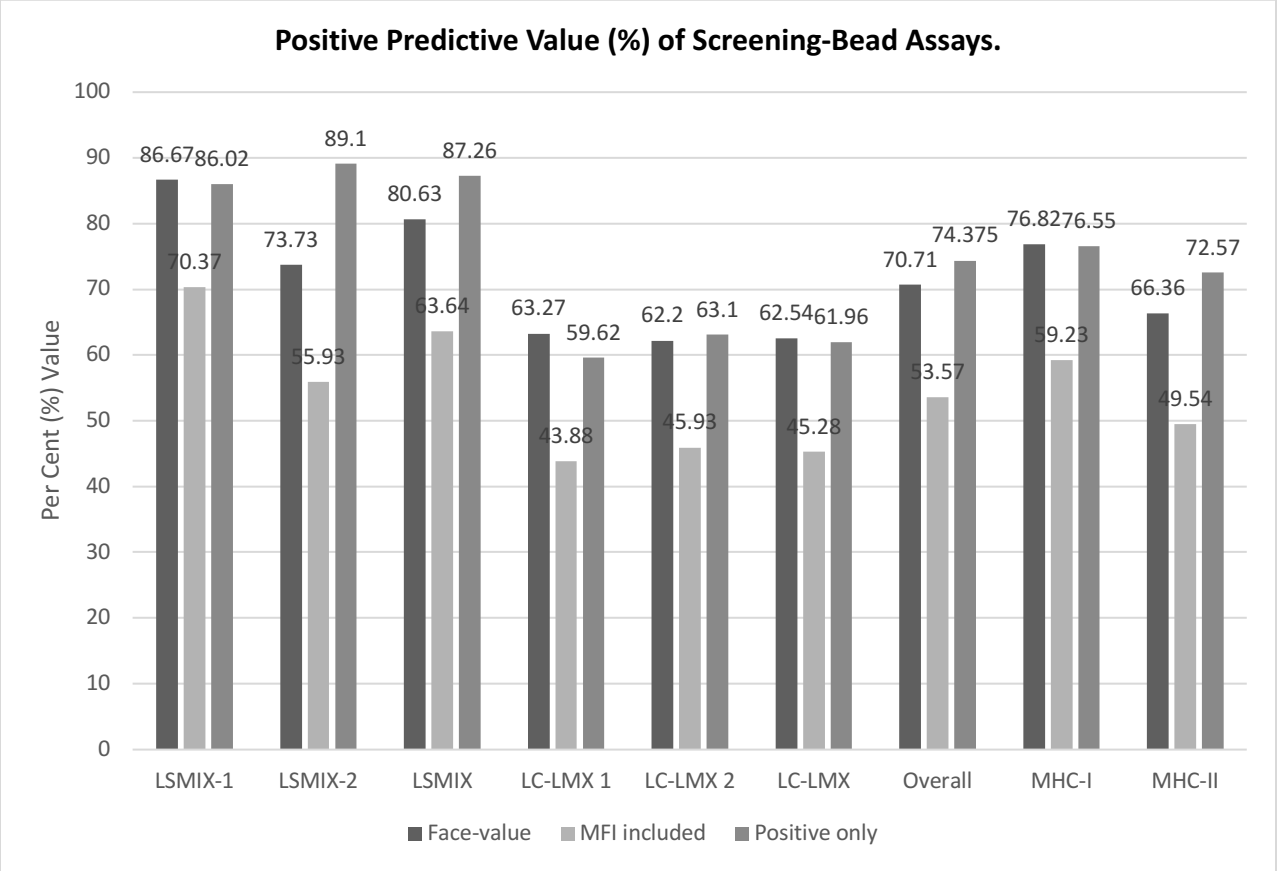
Appendix: Methods

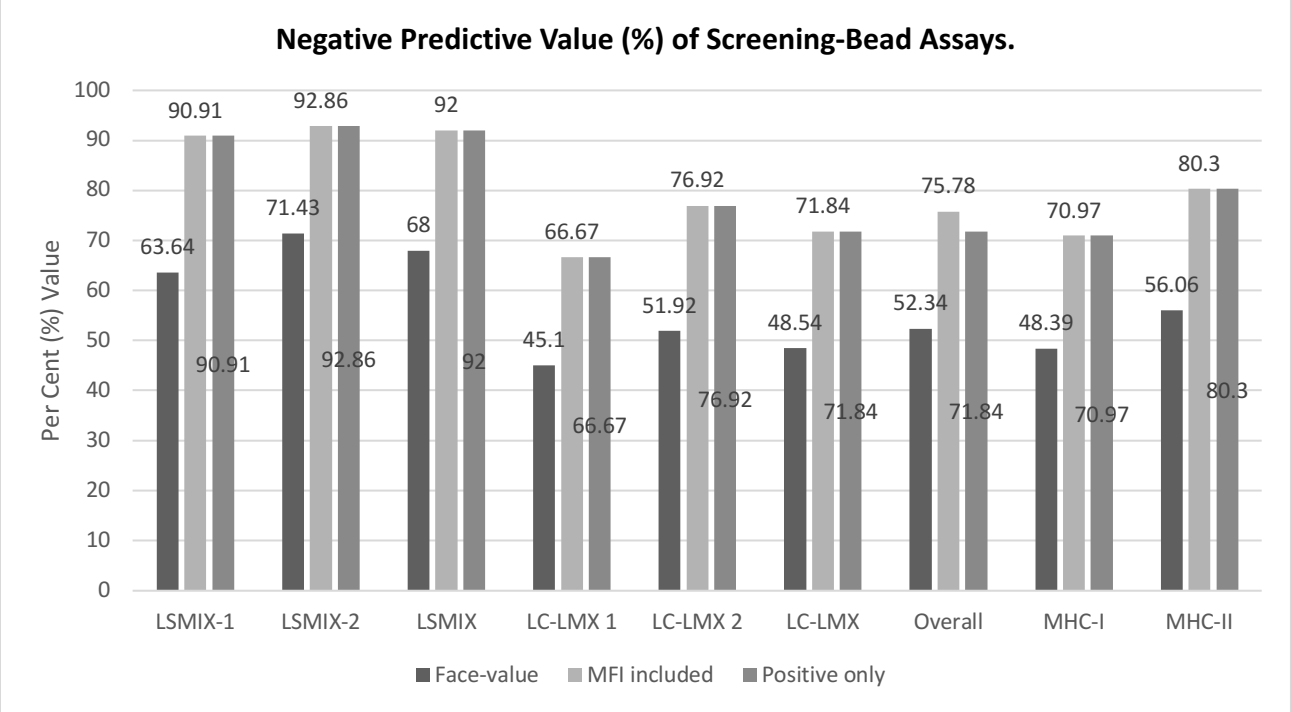
- **Positive Predictive Value** = $\frac{True\ positives}{True\ positives+False\ positives}$
- **Negative Predictive Value** = $\frac{True\ negatives}{True\ negatives+False\ negatives}$
- **Specificity** = $\frac{True\ positives}{True\ positives+False\ negatives}$
- **Sensitivity** = $\frac{True\ negatives}{True\ negatives+False\ positives}$
- **Positive likelihood ratio (+LR)** = $\frac{Sn}{1-Sp}$
- **Negative likelihood ratio (-LR)** = $\frac{1-Sn}{Sp}$
- **Pre-test probability** = $\frac{True\ positive+False\ negative}{Total}$
- **Pre-test odds** = $\frac{pre-test\ probability}{1-(pre-test\ probability)}$
- **Post-test odds** = $(pre-test\ odds) \times (\pm LR)$
- **Post-test probability** = $\frac{post-test\ probability}{1+(post-test\ probability)}$

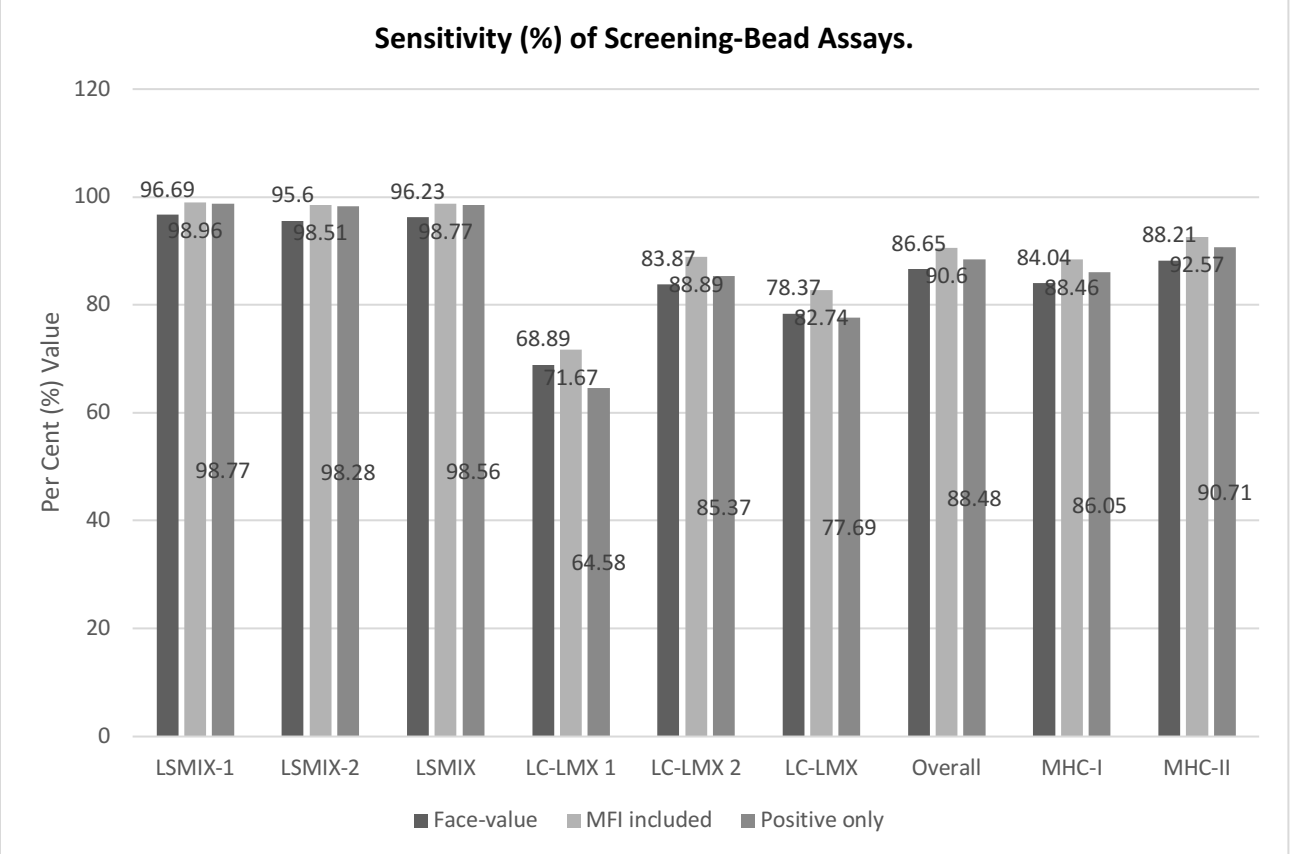
- **Change in serum creatinine over time** = $\frac{(Last\ measured\ creatinine)-(Creatinine\ at\ biopsy)}{(Elapsed\ time)}$
- **Change in eGFR over time** = $\frac{(Last\ measured\ eGFR)-(eGFR\ at\ biopsy)}{(Elapsed\ time)}$

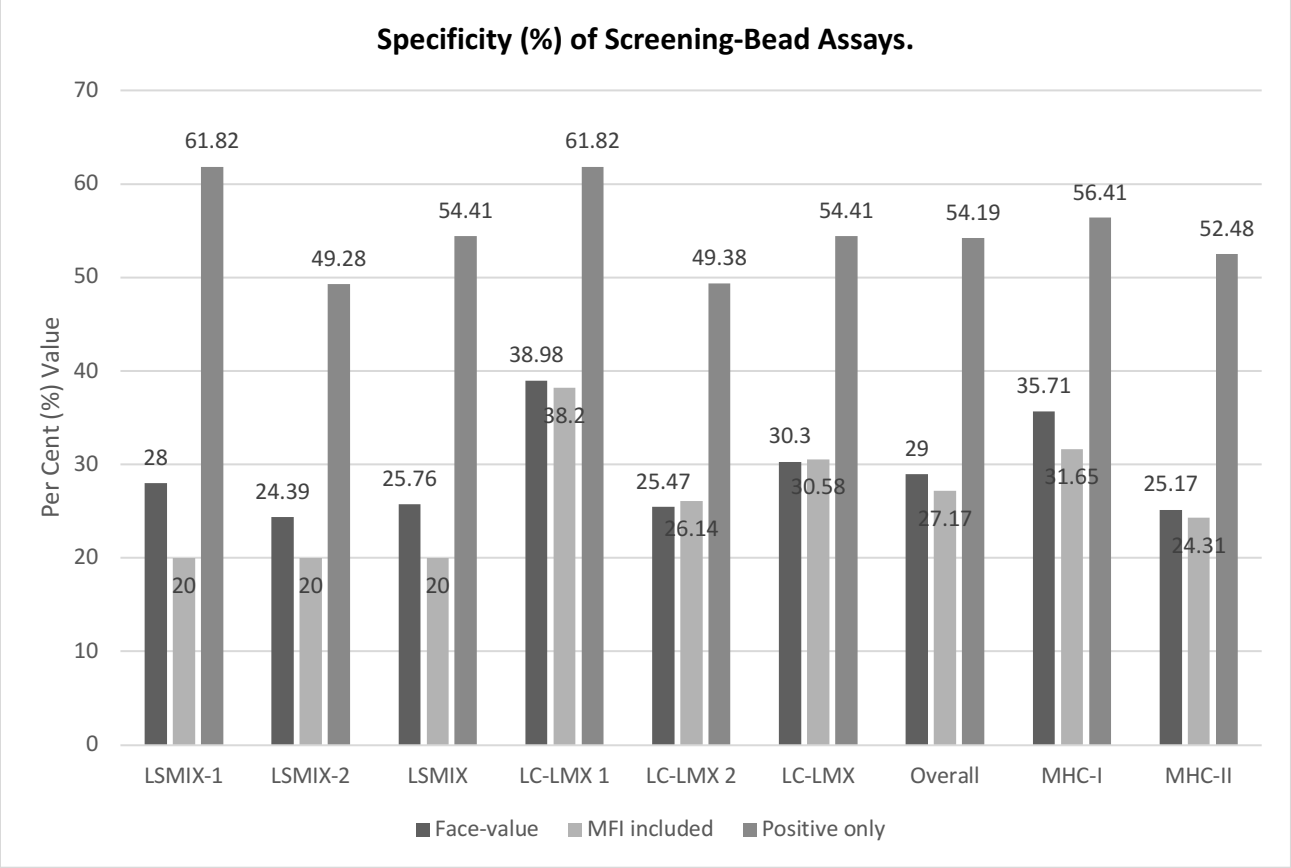
Appendix: Results

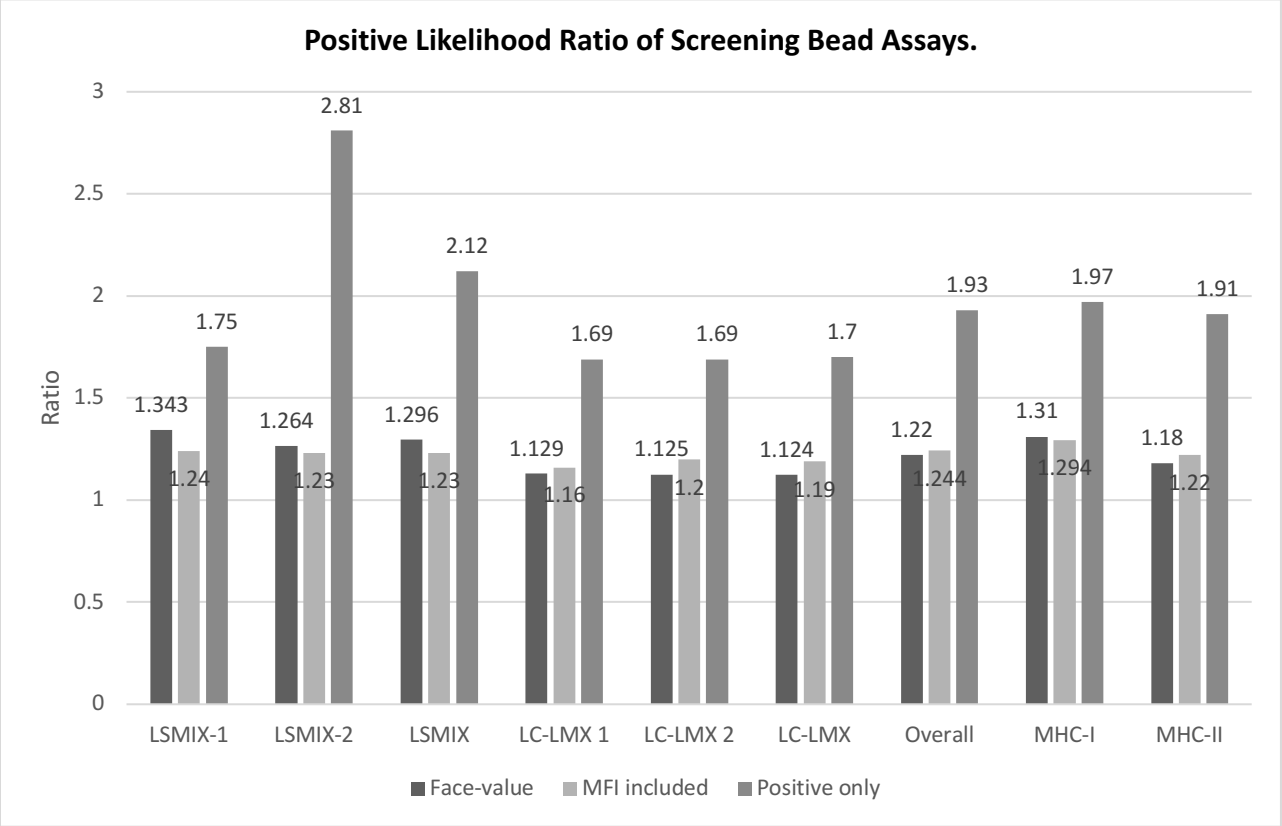
Figure 1: PPV, NPV, Sp, Sn, pre- and post-test probabilities and +/-LR of LS-MIX and LC-LMX individually.

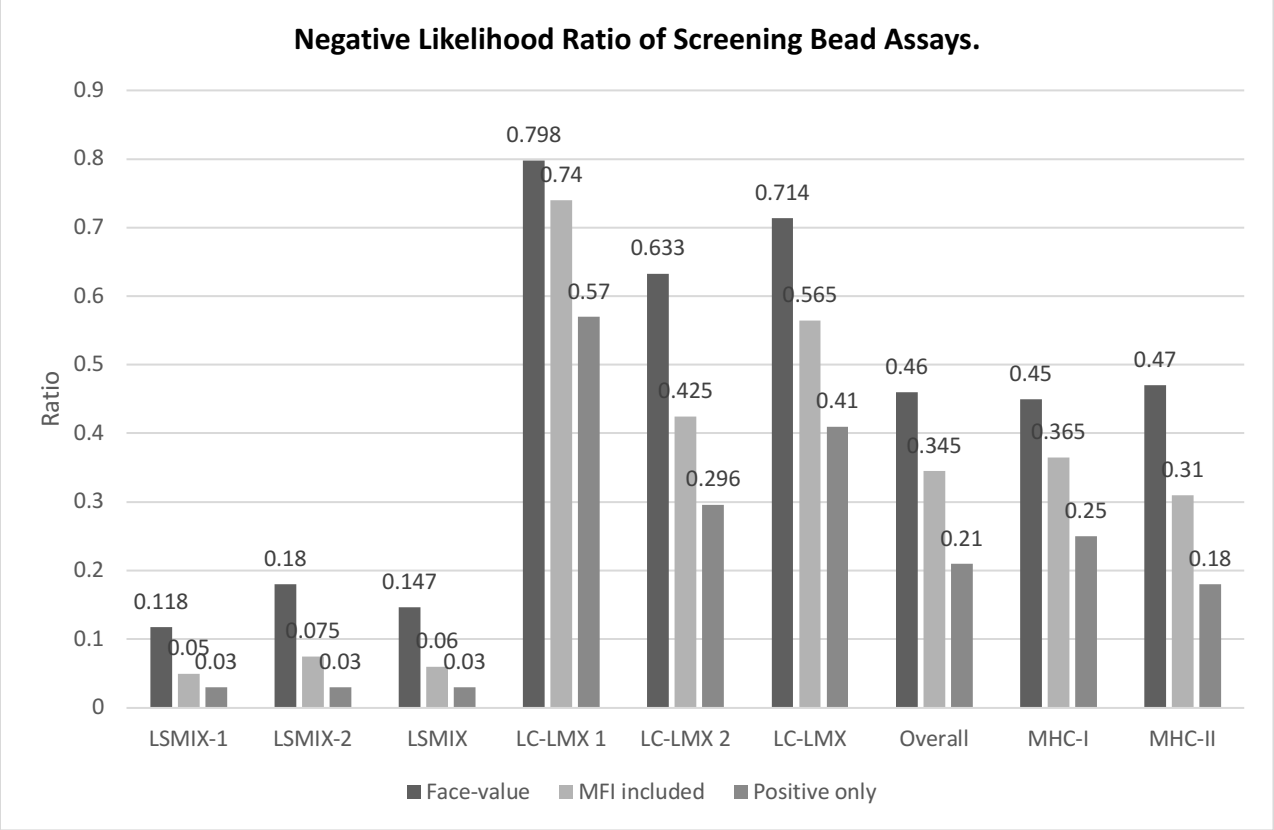


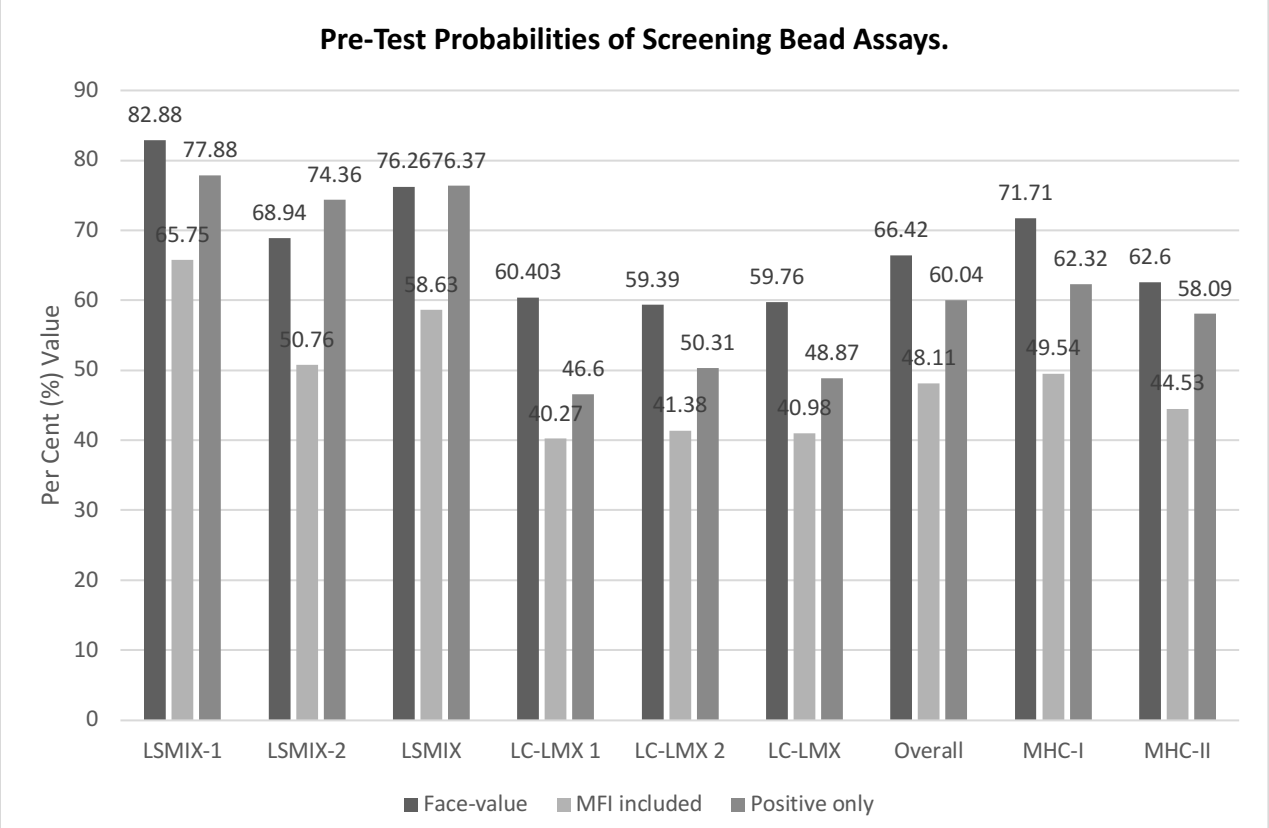

Negative Predictive Value (%) of Screening-Bead Assays.
Per Cent (%) Value
100
90
80
70
60
50
40
30
20
10
0
63.64
90.91
90.91
71.43
92.86
92.86
68
92
92
45.1
66.67
66.67
51.92
76.92
76.92
48.54
71.84
71.84
52.34
75.78
71.84
48.39
70.97
70.97
56.06
80.3
80.3
LSMIX-1
LSMIX-2
LSMIX
LC-LMX 1
LC-LMX 2
LC-LMX
Overall
MHC-I
MHC-II
Face-value
MFI included
Positive only

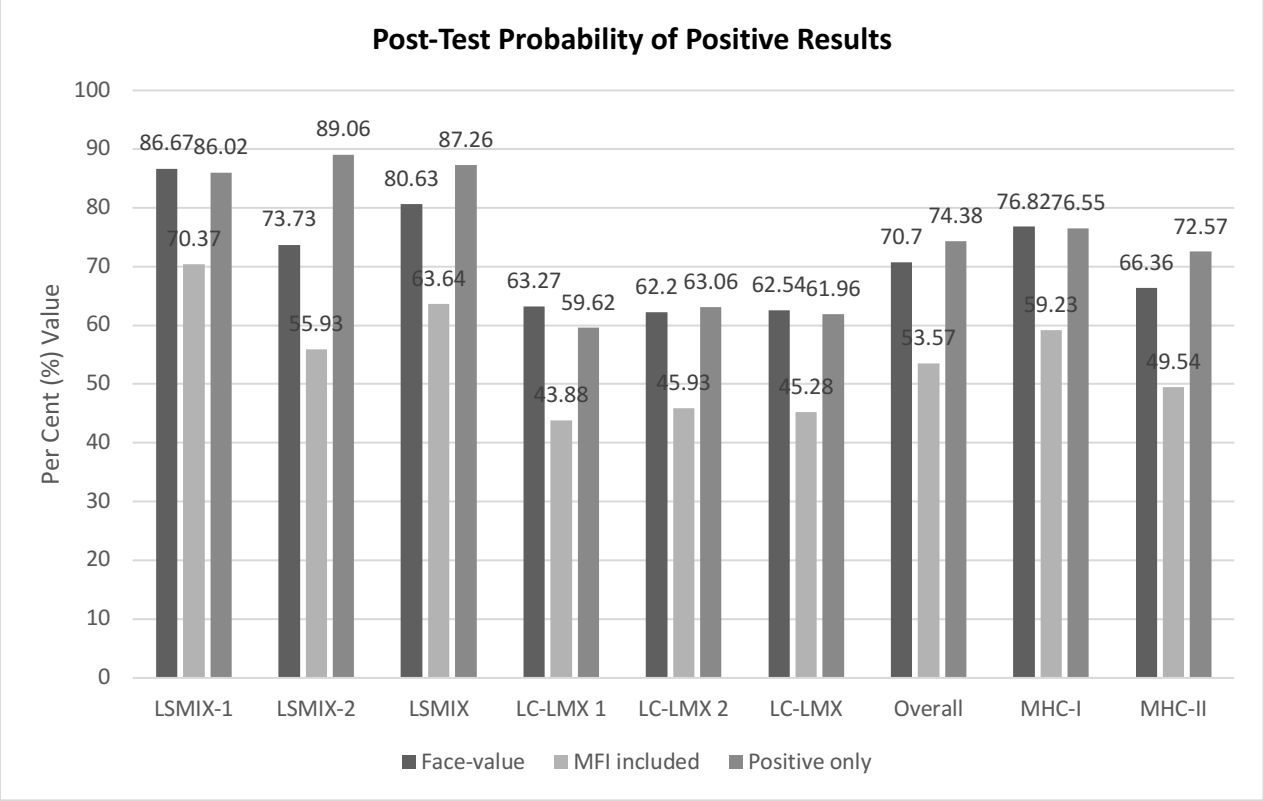

Sensitivity (%) of Screening-Bead Assays.
Per Cent (%) Value
120
100
80
60
40
20
0
96.69
98.96
98.77
95.6
98.51
98.28
96.23
98.77
98.56
68.89
71.67
64.58
83.87
88.89
85.37
78.37
82.74
77.69
86.65
90.6
88.48
84.04
88.46
86.05
88.21
92.57
90.71
LSMIX-1
LSMIX-2
LSMIX
LC-LMX 1
LC-LMX 2
LC-LMX
Overall
MHC-I
MHC-II
Face-value
MFI included
Positive only

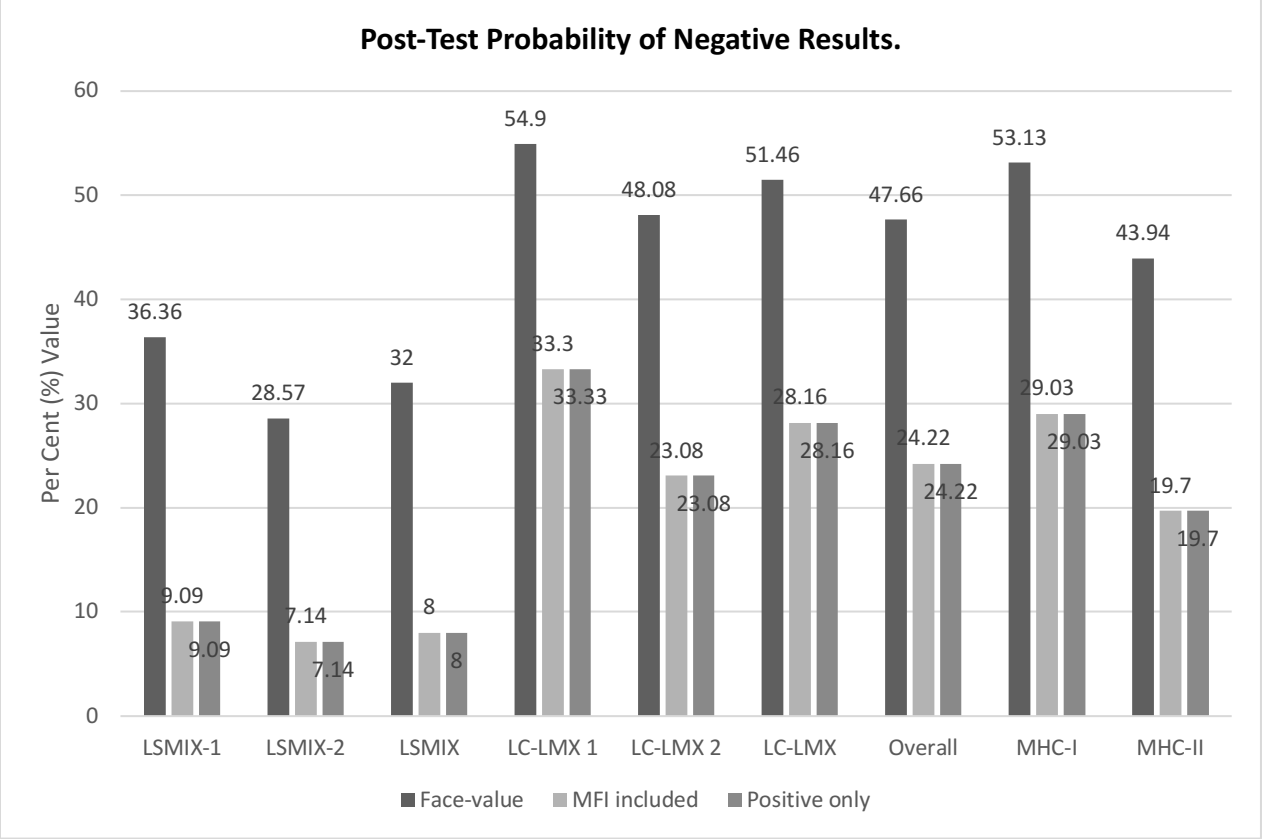

Specificity (%) of Screening-Bead Assays.
Per Cent (%) Value
70
60
50
40
30
20
10
0
61.82
61.82
54.41
49.28
54.41
49.38
54.41
54.19
56.41
52.48
38.98
38.2
35.71
31.65
30.3
30.58
29
27.17
28
20
24.39
20
25.76
20
25.47
26.14
25.17
24.31
LSMIX-1
LSMIX-2
LSMIX
LC-LMX 1
LC-LMX 2
LC-LMX
Overall
MHC-I
MHC-II
Face-value
MFI included
Positive only


Positive Likelihood Ratio of Screening Bead Assays.
Ratio
3
2.5
2
1.5
1
0.5
0
2.81
2.12
1.75
1.69
1.69
1.7
1.93
1.97
1.91
1.343
1.24
1.264
1.23
1.296
1.23
1.129
1.16
1.125
1.2
1.124
1.19
1.22
1.244
1.31
1.294
1.18
1.22
LSMIX-1
LSMIX-2
LSMIX
LC-LMX 1
LC-LMX 2
LC-LMX
Overall
MHC-I
MHC-II
Face-value
MFI included
Positive only


Negative Likelihood Ratio of Screening Bead Assays.
Ratio
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.118
0.05
0.03
0.18
0.075
0.03
0.147
0.06
0.03
0.798
0.74
0.57
0.633
0.425
0.296
0.714
0.565
0.41
0.46
0.345
0.21
0.45
0.365
0.25
0.47
0.31
0.18
LSMIX-1
LSMIX-2
LSMIX
LC-LMX 1
LC-LMX 2
LC-LMX
Overall
MHC-I
MHC-II
Face-value
MFI included
Positive only


Pre-Test Probabilities of Screening Bead Assays.
Per Cent (%) Value
90
80
70
60
50
40
30
20
10
0
82.88
77.88
65.75
68.94
74.36
50.76
76.26
76.37
58.63
60.403
46.6
40.27
59.39
50.31
41.38
59.76
48.87
40.98
66.42
60.04
48.11
71.71
62.32
49.54
62.6
58.09
44.53
LSMIX-1
LSMIX-2
LSMIX
LC-LMX 1
LC-LMX 2
LC-LMX
Overall
MHC-I
MHC-II
Face-value
MFI included
Positive only


Post-Test Probability of Positive Results
Per Cent (%) Value
100
90
80
70
60
50
40
30
20
10
0
86.67
70.37
86.02
73.73
55.93
89.06
80.63
63.64
87.26
63.27
43.88
59.62
62.2
45.93
63.06
62.54
45.28
61.96
70.7
53.57
74.38
76.82
59.23
76.55
66.36
49.54
72.57
LSMIX-1
LSMIX-2
LSMIX
LC-LMX 1
LC-LMX 2
LC-LMX
Overall
MHC-I
MHC-II
Face-value
MFI included
Positive only


Post-Test Probability of Negative Results.
Per Cent (%) Value
60
50
40
30
20
10
0
36.36
9.09
9.09
28.57
7.14
7.14
32
8
8
54.9
33.3
33.33
48.08
23.08
23.08
51.46
28.16
28.16
47.66
24.22
24.22
53.13
29.03
29.03
43.94
19.7
19.7
LSMIX-1
LSMIX-2
LSMIX
LC-LMX 1
LC-LMX 2
LC-LMX
Overall
MHC-I
MHC-II
Face-value
MFI included
Positive only

Figures 2-5: Variation in eGFR for different groups of biopsied patients.

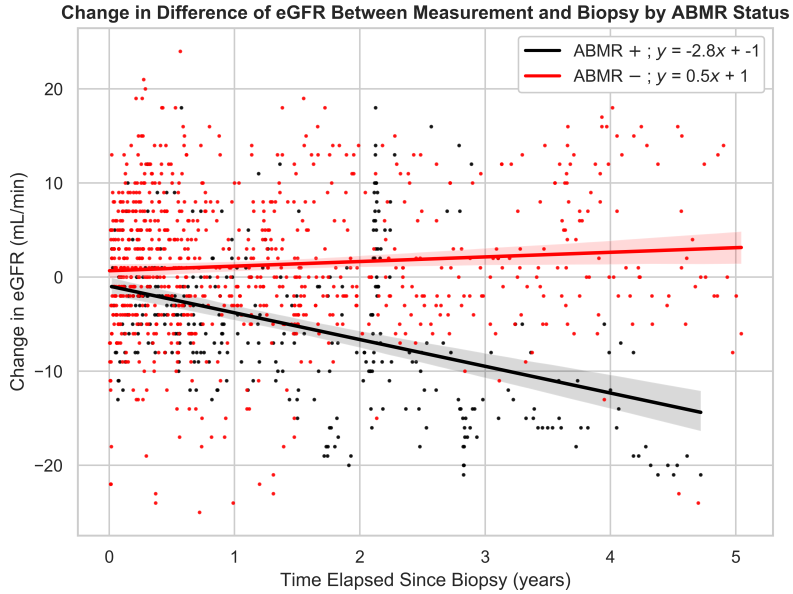


Figure 2: Y-intercept was higher amongst patients without ABMR (p = 0.024414). However, slope was higher amongst ABMR + patients (p = 0.000000). Before outliers were removed, y-intercept was higher amongst patients with ABMR (p = 0.000000), though there was no statistical difference in slopes (p = 0.689821).

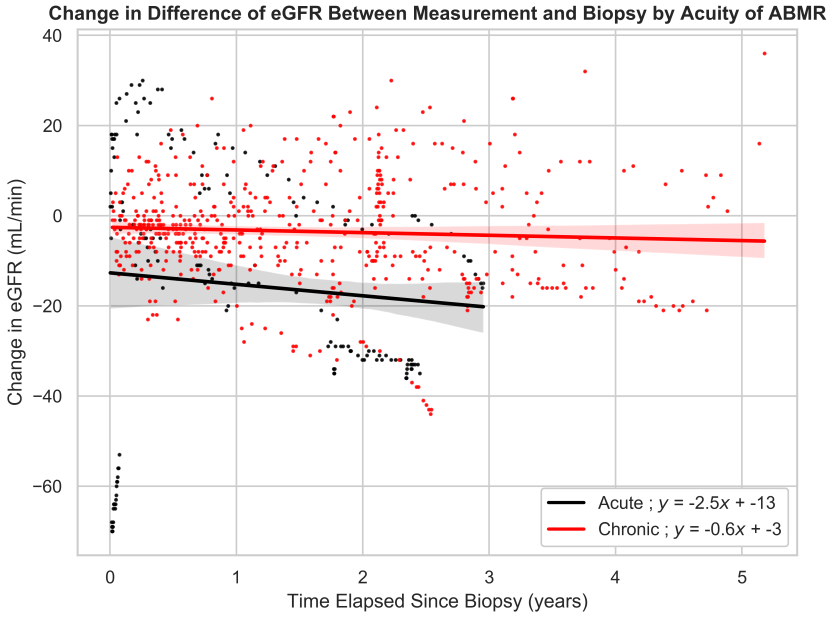


Figure 3: Y-intercept was higher amongst patients with chronic ABMR (p = 0.001387). However, difference in slope did not reach statistical significance, likely due to low starting eGFRs (p =

0.365990). Outliers were not removed for this group, as many of them were patients with acute ABMR.

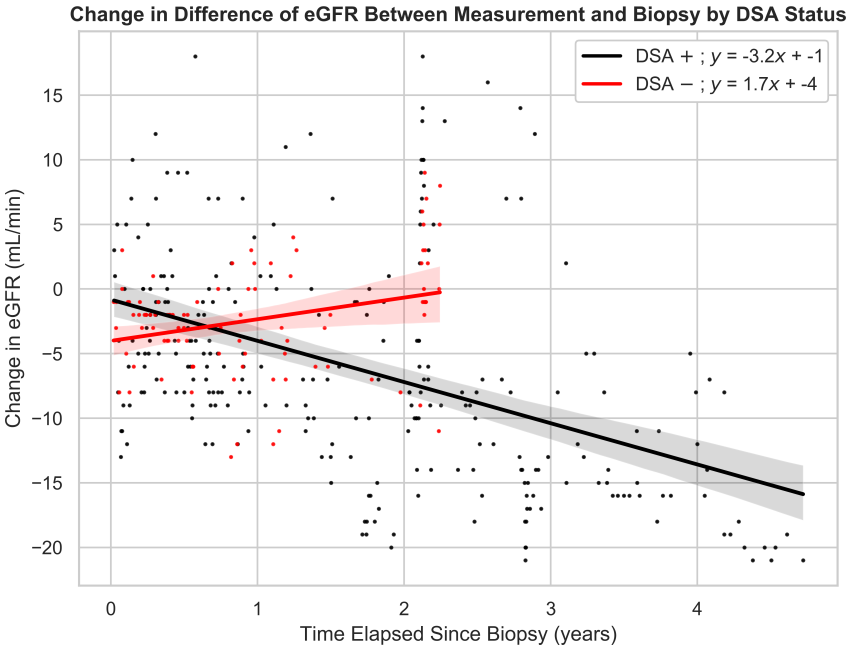


Figure 4: Y-intercept was higher amongst patients without DSAs (p = 0.003509). However, slope was higher amongst DSA + patients (p = 0.000000). Before outliers were removed, y-intercept was higher amongst patients with DSAs (p = 0.024363); there was no statistical difference in slopes between the two groups (p = 0.067416).

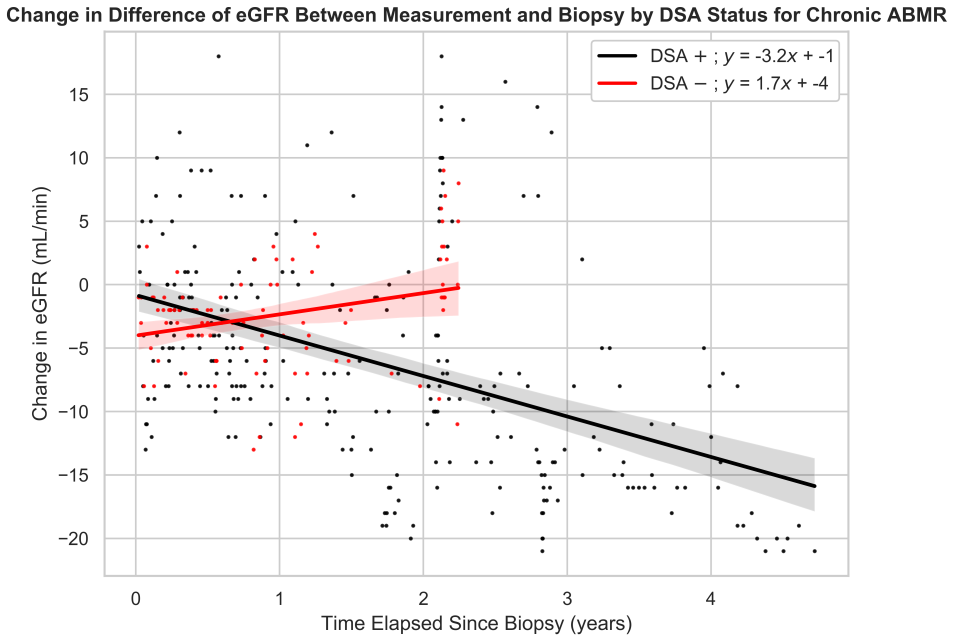


Figure 5: Y-intercept was higher amongst patients with DSAs (p = 0.003509). However, slope was higher amongst DSA + patients (p = 0.000000). Before outliers were removed, y-intercepts were not statistically different (p = 0.267006), though slope was higher amongst DSA + patients (p = 0.002579).

Table 1: Epidemiological Tables for PPV and NPV as Functions of Varying Prevalence Using the Single-Ag Bead Assay as a Gold Standard for Detecting anti-HLA Abs.

Prevalence of AMR	**0.48110465**		
	Dz+	Dz-	**PPV/NPV**
Test+	300	260	0.53571429
Test-	31	97	0.7578125
Sn/Sp	0.90634441	0.27170868	
LR+	1.24448059		
LR-	0.34469119		
Pre-Test Prob	0.48110465		
Pre-Test Odds	0.92717087		
Post-Test Odds	1.15384615	0.31958763	
Post-Test Prob	0.53571429	0.2421875	
Prevalence of AMR	**0.05**		
	Dz+	Dz-	**PPV/NPV**
Test+	31.1782477	476.011204	0.06147259

Test-	3.22175227	177.588796	0.98218161
Sn/Sp	0.90634441	0.27170868	
LR+	1.24448059		
LR-	0.34469119		
Pre-Test Prob	0.05		
Pre-Test Odds	0.05263158		
Post-Test Odds	0.06549898	0.01814164	
Post-Test Prob	0.06147259	0.01781839	
Prevalence of AMR	**0.1**		
	Dz+	Dz-	**PPV/NPV**
Test+	62.3564955	450.957983	0.12147815
Test-	6.44350453	168.242017	0.96311369
Sn/Sp	0.90634441	0.27170868	
LR+	1.24448059		
LR-	0.34469119		
Pre-Test Prob	0.1		
Pre-Test Odds	0.11111111		
Post-Test Odds	0.13827562	0.03829902	
Post-Test Prob	0.12147815	0.03688631	
Prevalence of AMR	**0.15**		
	Dz+	Dz-	**PPV/NPV**
Test+	93.5347432	425.904762	0.1800686
Test-	9.6652568	158.895238	0.94266001
Sn/Sp	0.90634441	0.27170868	
LR+	1.24448059		
LR-	0.34469119		
Pre-Test Prob	0.15		
Pre-Test Odds	0.17647059		
Post-Test Odds	0.21961422	0.06082786	
Post-Test Prob	0.1800686	0.05733999	
Prevalence of AMR	**0.2**		
	Dz+	Dz-	**PPV/NPV**
Test+	124.712991	400.851541	0.23729339
Test-	12.8870091	149.548459	0.92066382
Sn/Sp	0.90634441	0.27170868	
LR+	1.24448059		

LR-	0.34469119		
Pre-Test Prob	0.2		
Pre-Test Odds	0.25		
Post-Test Odds	0.31112015	0.0861728	
Post-Test Prob	0.23729339	0.07933618	
Prevalence of AMR	**0.25**		
	Dz+	Dz-	**PPV/NPV**
Test+	155.891239	375.798319	0.29319974
Test-	16.1087613	140.201681	0.89694379
Sn/Sp	0.90634441	0.27170868	
LR+	1.24448059		
LR-	0.34469119		
Pre-Test Prob	0.25		
Pre-Test Odds	0.33333333		
Post-Test Odds	0.41482686	0.11489706	
Post-Test Prob	0.29319974	0.10305621	
Prevalence of AMR	**0.3**		
	Dz+	Dz-	**PPV/NPV**
Test+	187.069486	350.745098	0.34783268
Test-	19.3305136	130.854902	0.87128901
Sn/Sp	0.90634441	0.27170868	
LR+	1.24448059		
LR-	0.34469119		
Pre-Test Prob	0.3		
Pre-Test Odds	0.42857143		
Post-Test Odds	0.53334883	0.1477248	
Post-Test Prob	0.34783268	0.12871099	
Prevalence of AMR	**0.35**		
	Dz+	Dz-	**PPV/NPV**
Test+	218.247734	325.691877	0.40123523
Test-	22.5522659	121.508123	0.84345269
Sn/Sp	0.90634441	0.27170868	
LR+	1.24448059		
LR-	0.34469119		
Pre-Test Prob	0.35		
Pre-Test Odds	0.53846154		

Post-Test Odds	0.67010494	0.18560295	
Post-Test Prob	0.40123523	0.15654731	
Prevalence of AMR	**0.4**		
	Dz+	Dz-	**PPV/NPV**
Test+	249.425982	300.638655	0.45344849
Test-	25.7740181	112.161345	0.81314423
Sn/Sp	0.90634441	0.27170868	
LR+	1.24448059		
LR-	0.34469119		
Pre-Test Prob	0.4		
Pre-Test Odds	0.66666667		
Post-Test Odds	0.82965373	0.22979413	
Post-Test Prob	0.45344849	0.18685577	
Prevalence of AMR	**0.45**		
	Dz+	Dz-	**PPV/NPV**
Test+	280.60423	275.585434	0.50451177
Test-	28.9957704	102.814566	0.78001899
Sn/Sp	0.90634441	0.27170868	
LR+	1.24448059		
LR-	0.34469119		
Pre-Test Prob	0.45		
Pre-Test Odds	0.81818182		
Post-Test Odds	1.0182114	0.28202006	
Post-Test Prob	0.50451177	0.21998101	
Prevalence of AMR	**0.5**		
	Dz+	Dz-	**PPV/NPV**
Test+	311.782477	250.532213	0.55446262
Test-	32.2175227	93.4677871	0.74366517
Sn/Sp	0.90634441	0.27170868	
LR+	1.24448059		
LR-	0.34469119		
Pre-Test Prob	0.5		
Pre-Test Odds	1		
Post-Test Odds	1.24448059	0.34469119	
Post-Test Prob	0.55446262	0.25633483	

Prevalence of AMR	**0.75**		
	Dz+	Dz-	**PPV/NPV**
Test+	467.673716	125.266106	0.78873723
Test-	48.326284	46.7338936	0.4916243
Sn/Sp	0.90634441	0.27170868	
LR+	1.24448059		
LR-	0.34469119		
Pre-Test Prob	0.75		
Pre-Test Odds	3		
Post-Test Odds	3.73344178	1.03407357	
Post-Test Prob	0.78873723	0.5083757	
Prevalence of AMR	**0.99**		
	Dz+	Dz-	**PPV/NPV**
Test+	617.329305	5.01064426	0.9919487
Test-	63.7906949	1.86935574	0.02847021
Sn/Sp	0.90634441	0.27170868	
LR+	1.24448059		
LR-	0.34469119		
Pre-Test Prob	0.99		
Pre-Test Odds	99		
Post-Test Odds	123.203579	34.1244277	
Post-Test Prob	0.9919487	0.97152979	

Table 2: Epidemiological Tables for PPV and NPV as Functions of Varying Prevalence Using the Single-Ag Bead Assay as a Gold Standard for Diagnosing ABMR, Defining True Positive with Positive Doublet and Biopsy.

Prevalence of AMR	**0.24210526**		
	Dz+	Dz-	**PPV/NPV**
Test+	21	63	0.25
Test-	2	9	0.81818182
Sn/Sp	0.91304348	0.125	
LR+	1.04347826		
LR-	0.69565217		
Pre-Test Prob	0.24210526		
Pre-Test Odds	0.31944444		
Post-Test Odds	0.33333333	0.22222222	
Post-Test Prob	0.25	0.18181818	

Prevalence of AMR	**0.05**		
	Dz+	Dz-	**PPV/NPV**
Test+	4.33695652	78.96875	0.05206074
Test-	0.41304348	11.28125	0.96467991
Sn/Sp	0.91304348	0.125	
LR+	1.04347826		
LR-	0.69565217		
Pre-Test Prob	0.05		
Pre-Test Odds	0.05263158		
Post-Test Odds	0.05491991	0.03661327	
Post-Test Prob	0.05206074	0.03532009	
Prevalence of AMR	**0.1**		
	Dz+	Dz-	**PPV/NPV**
Test+	8.67391304	74.8125	0.1038961
Test-	0.82608696	10.6875	0.92825112
Sn/Sp	0.91304348	0.125	
LR+	1.04347826		
LR-	0.69565217		
Pre-Test Prob	0.1		
Pre-Test Odds	0.11111111		
Post-Test Odds	0.11594203	0.07729469	
Post-Test Prob	0.1038961	0.07174888	
Prevalence of AMR	**0.15**		
	Dz+	Dz-	**PPV/NPV**
Test+	13.0108696	70.65625	0.15550756
Test-	1.23913043	10.09375	0.89066059
Sn/Sp	0.91304348	0.125	
LR+	1.04347826		
LR-	0.69565217		
Pre-Test Prob	0.15		
Pre-Test Odds	0.17647059		
Post-Test Odds	0.18414322	0.12276215	
Post-Test Prob	0.15550756	0.10933941	
Prevalence of AMR	**0.2**		
	Dz+	Dz-	**PPV/NPV**

Test+	17.3478261	66.5	0.20689655
Test-	1.65217391	9.5	0.85185185
Sn/Sp	0.91304348	0.125	
LR+	1.04347826		
LR-	0.69565217		
Pre-Test Prob	0.2		
Pre-Test Odds	0.25		
Post-Test Odds	0.26086957	0.17391304	
Post-Test Prob	0.20689655	0.14814815	
Prevalence of AMR	**0.25**		
	Dz+	Dz-	**PPV/NPV**
Test+	21.6847826	62.34375	0.25806452
Test-	2.06521739	8.90625	0.81176471
Sn/Sp	0.91304348	0.125	
LR+	1.04347826		
LR-	0.69565217		
Pre-Test Prob	0.25		
Pre-Test Odds	0.33333333		
Post-Test Odds	0.34782609	0.23188406	
Post-Test Prob	0.25806452	0.18823529	
Prevalence of AMR	**0.3**		
	Dz+	Dz-	**PPV/NPV**
Test+	26.0217391	58.1875	0.30901288
Test-	2.47826087	8.3125	0.77033493
Sn/Sp	0.91304348	0.125	
LR+	1.04347826		
LR-	0.69565217		
Pre-Test Prob	0.3		
Pre-Test Odds	0.42857143		
Post-Test Odds	0.44720497	0.29813665	
Post-Test Prob	0.30901288	0.22966507	
Prevalence of AMR	**0.35**		
	Dz+	Dz-	**PPV/NPV**
Test+	30.3586957	54.03125	0.35974304
Test-	2.89130435	7.71875	0.72749392
Sn/Sp	0.91304348	0.125	

LR+	1.04347826		
LR-	0.69565217		
Pre-Test Prob	0.35		
Pre-Test Odds	0.53846154		
Post-Test Odds	0.56187291	0.37458194	
Post-Test Prob	0.35974304	0.27250608	
Prevalence of AMR	**0.4**		
	Dz+	Dz-	**PPV/NPV**
Test+	34.6956522	49.875	0.41025641
Test-	3.30434783	7.125	0.68316832
Sn/Sp	0.91304348	0.125	
LR+	1.04347826		
LR-	0.69565217		
Pre-Test Prob	0.4		
Pre-Test Odds	0.66666667		
Post-Test Odds	0.69565217	0.46376812	
Post-Test Prob	0.41025641	0.31683168	
Prevalence of AMR	**0.45**		
	Dz+	Dz-	**PPV/NPV**
Test+	39.0326087	45.71875	0.46055437
Test-	3.7173913	6.53125	0.6372796
Sn/Sp	0.91304348	0.125	
LR+	1.04347826		
LR-	0.69565217		
Pre-Test Prob	0.45		
Pre-Test Odds	0.81818182		
Post-Test Odds	0.85375494	0.56916996	
Post-Test Prob	0.46055437	0.3627204	
Prevalence of AMR	**0.5**		
	Dz+	Dz-	**PPV/NPV**
Test+	43.3695652	41.5625	0.5106383
Test-	4.13043478	5.9375	0.58974359
Sn/Sp	0.91304348	0.125	
LR+	1.04347826		
LR-	0.69565217		
Pre-Test Prob	0.5		

Pre-Test Odds	1		
Post-Test Odds	1.04347826	0.69565217	
Post-Test Prob	0.5106383	0.41025641	
Prevalence of AMR	**0.75**		
	Dz+	Dz-	**PPV/NPV**
Test+	65.0543478	20.78125	0.75789474
Test-	6.19565217	2.96875	0.32394366
Sn/Sp	0.91304348	0.125	
LR+	1.04347826		
LR-	0.69565217		
Pre-Test Prob	0.75		
Pre-Test Odds	3		
Post-Test Odds	3.13043478	2.08695652	
Post-Test Prob	0.75789474	0.67605634	
Prevalence of AMR	**0.99**		
	Dz+	Dz-	**PPV/NPV**
Test+	85.8717391	0.83125	0.99041267
Test-	8.17826087	0.11875	0.01431238
Sn/Sp	0.91304348	0.125	
LR+	1.04347826		
LR-	0.69565217		
Pre-Test Prob	0.99		
Pre-Test Odds	99		
Post-Test Odds	103.304348	68.8695652	
Post-Test Prob	0.99041267	0.98568762	

Table 3: Epidemiological Tables for PPV and NPV as Functions of Varying Prevalence Using the Single-Ag Bead Assay as a Gold Standard for Diagnosing ABMR, Defining True Positive with Positive Doublet, Presence of DSAs and Positive Biopsy.

Prevalence of AMR	**0.452380952**		
	Dz+	Dz-	
Test+	18	15	0.54545455
Test-	1	8	0.88888889
Sn/Sp	0.947368421	0.34782609	
LR+	1.452631579		
LR-	0.151315789		

Pre-Test Prob	0.452380952		
Pre-Test Odds	0.826086957		
Post-Test Odds	1.2	0.125	
Post-Test Prob	0.545454545	0.11111111	
Prevalence of AMR	**0.05**		
	Dz+	Dz-	
Test+	1.989473684	26.0217391	0.07102419
Test-	0.110526316	13.8782609	0.99209894
Sn/Sp	0.947368421	0.34782609	
LR+	1.452631579		
LR-	0.151315789		
Pre-Test Prob	0.05		
Pre-Test Odds	0.052631579		
Post-Test Odds	0.076454294	0.00796399	
Post-Test Prob	0.071024189	0.00790106	
Prevalence of AMR	**0.1**		
	Dz+	Dz-	
Test+	3.978947368	24.6521739	0.13897281
Test-	0.221052632	13.1478261	0.98346513
Sn/Sp	0.947368421	0.34782609	
LR+	1.452631579		
LR-	0.151315789		
Pre-Test Prob	0.1		
Pre-Test Odds	0.111111111		
Post-Test Odds	0.161403509	0.01681287	
Post-Test Prob	0.13897281	0.01653487	
Prevalence of AMR	**0.15**		
	Dz+	Dz-	
Test+	5.968421053	23.2826087	0.2040414
Test-	0.331578947	12.4173913	0.97399171
Sn/Sp	0.947368421	0.34782609	
LR+	1.452631579		
LR-	0.151315789		
Pre-Test Prob	0.15		
Pre-Test Odds	0.176470588		
Post-Test Odds	0.256346749	0.02670279	

Post-Test Prob	0.2040414	0.02600829	
Prevalence of AMR	**0.2**		
	Dz+	Dz-	
Test+	7.957894737	21.9130435	0.26640927
Test-	0.442105263	11.6869565	0.96354992
Sn/Sp	0.947368421	0.34782609	
LR+	1.452631579		
LR-	0.151315789		
Pre-Test Prob	0.2		
Pre-Test Odds	0.25		
Post-Test Odds	0.363157895	0.03782895	
Post-Test Prob	0.266409266	0.03645008	
Prevalence of AMR	**0.25**		
	Dz+	Dz-	
Test+	9.947368421	20.5434783	0.32624113
Test-	0.552631579	10.9565217	0.9519833
Sn/Sp	0.947368421	0.34782609	
LR+	1.452631579		
LR-	0.151315789		
Pre-Test Prob	0.25		
Pre-Test Odds	0.333333333		
Post-Test Odds	0.484210526	0.0504386	
Post-Test Prob	0.326241135	0.0480167	
Prevalence of AMR	**0.3**		
	Dz+	Dz-	
Test+	11.93684211	19.173913	0.3836886
Test-	0.663157895	10.226087	0.93909974
Sn/Sp	0.947368421	0.34782609	
LR+	1.452631579		
LR-	0.151315789		
Pre-Test Prob	0.3		
Pre-Test Odds	0.428571429		
Post-Test Odds	0.622556391	0.06484962	
Post-Test Prob	0.383688601	0.06090026	
Prevalence of AMR	**0.35**		

	Dz+	Dz-	
Test+	13.92631579	17.8043478	0.43889141
Test-	0.773684211	9.49565217	0.92466074
Sn/Sp	0.947368421	0.34782609	
LR+	1.452631579		
LR-	0.151315789		
Pre-Test Prob	0.35		
Pre-Test Odds	0.538461538		
Post-Test Odds	0.782186235	0.08147773	
Post-Test Prob	0.438891413	0.07533926	
Prevalence of AMR	**0.4**		
	Dz+	Dz-	
Test+	15.91578947	16.4347826	0.49197861
Test-	0.884210526	8.76521739	0.90836653
Sn/Sp	0.947368421	0.34782609	
LR+	1.452631579		
LR-	0.151315789		
Pre-Test Prob	0.4		
Pre-Test Odds	0.666666667		
Post-Test Odds	0.968421053	0.10087719	
Post-Test Prob	0.49197861	0.09163347	
Prevalence of AMR	**0.45**		
	Dz+	Dz-	
Test+	17.90526316	15.0652174	0.54306952
Test-	0.994736842	8.03478261	0.88983502
Sn/Sp	0.947368421	0.34782609	
LR+	1.452631579		
LR-	0.151315789		
Pre-Test Prob	0.45		
Pre-Test Odds	0.818181818		
Post-Test Odds	1.188516746	0.12380383	
Post-Test Prob	0.543069523	0.11016498	
Prevalence of AMR	**0.5**		
	Dz+	Dz-	
Test+	19.89473684	13.6956522	0.59227468
Test-	1.105263158	7.30434783	0.86857143

Sn/Sp	0.947368421	0.34782609	
LR+	1.452631579		
LR-	0.151315789		
Pre-Test Prob	0.5		
Pre-Test Odds	1		
Post-Test Odds	1.452631579	0.15131579	
Post-Test Prob	0.592274678	0.13142857	
Prevalence of AMR	**0.75**		
	Dz+	Dz-	
Test+	29.84210526	6.84782609	0.81335953
Test-	1.657894737	3.65217391	0.68778281
Sn/Sp	0.947368421	0.34782609	
LR+	1.452631579		
LR-	0.151315789		
Pre-Test Prob	0.75		
Pre-Test Odds	3		
Post-Test Odds	4.357894737	0.45394737	
Post-Test Prob	0.813359528	0.31221719	
Prevalence of AMR	**0.99**		
	Dz+	Dz-	
Test+	39.39157895	0.27391304	0.99309442
Test-	2.188421053	0.14608696	0.06257719
Sn/Sp	0.947368421	0.34782609	
LR+	1.452631579		
LR-	0.151315789		
Pre-Test Prob	0.99		
Pre-Test Odds	99		
Post-Test Odds	143.8105263	14.9802632	
Post-Test Prob	0.993094425	0.93742281	

Figure 6a: ROC Curve for True Positives Defined by Positive Doublet and Biopsy

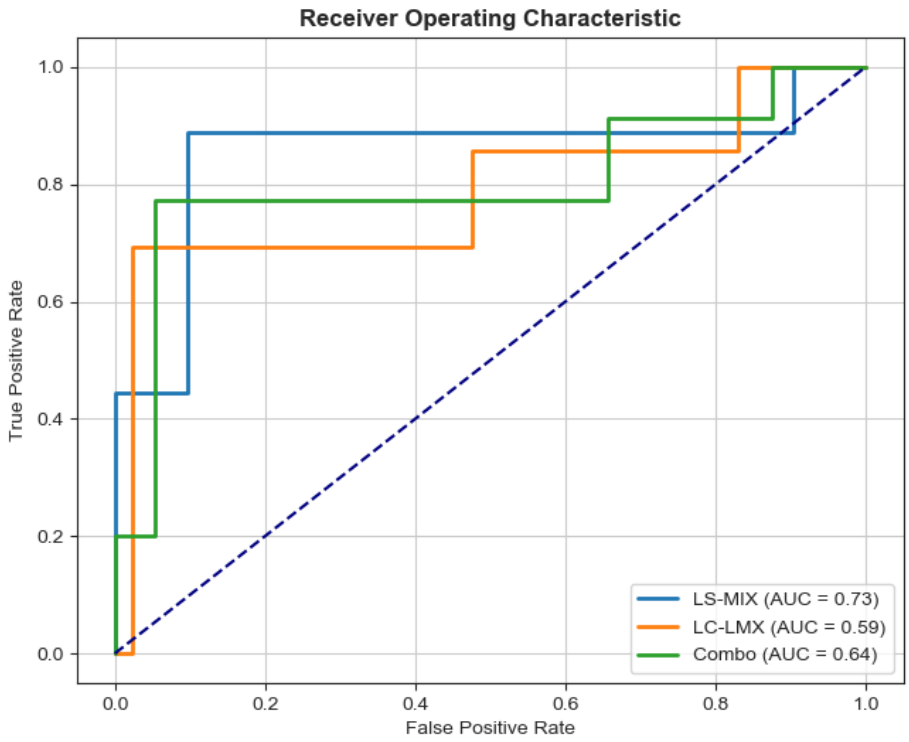
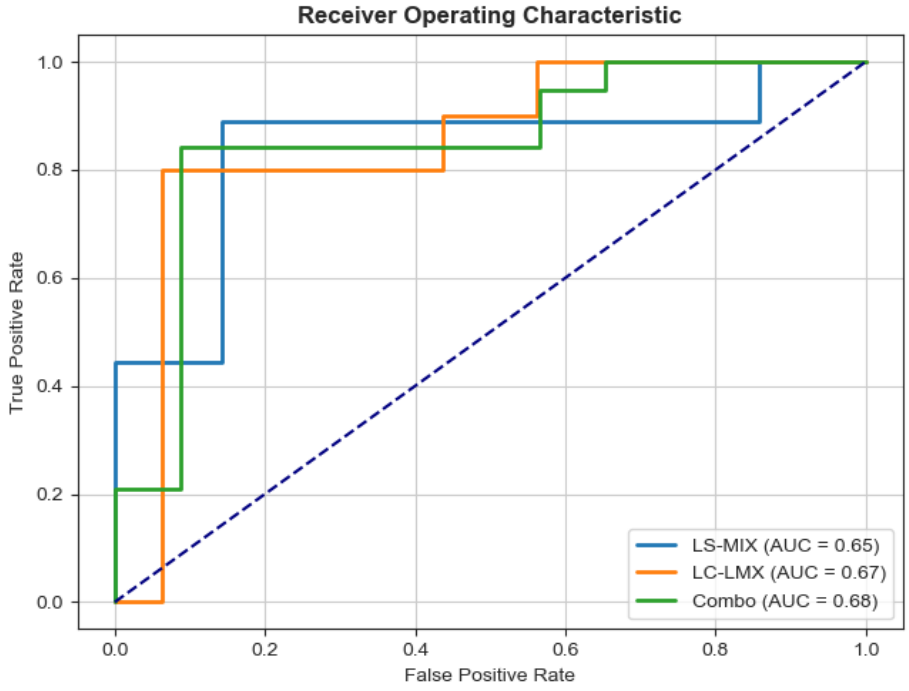


Figure 6b: ROC Curve for True Positives Defined by Positive Doublet, DSA and Biopsy



Figures 7-10: Variation in GFR According to Screen Result

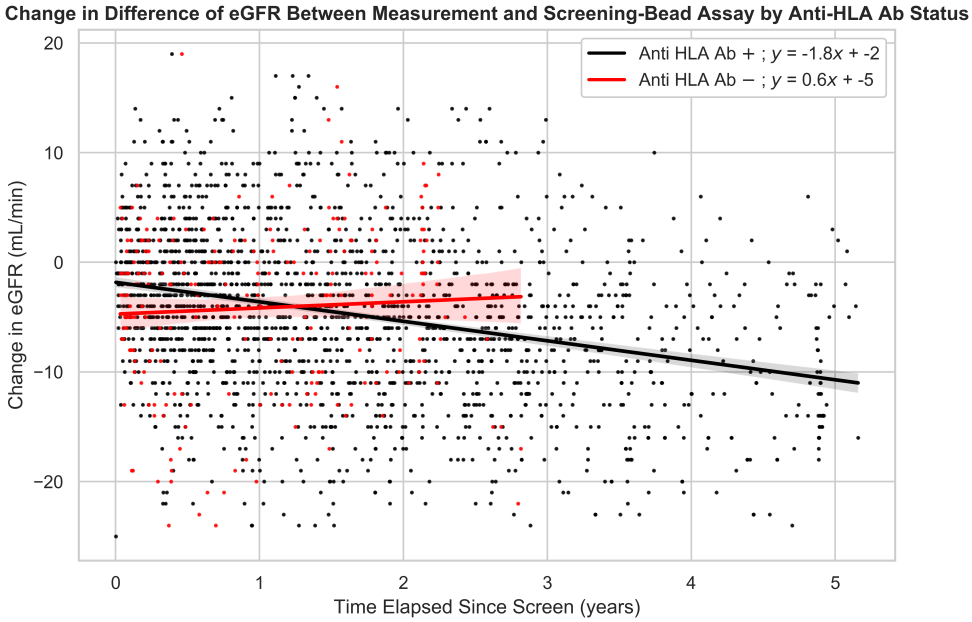


Figure 7: Y-intercept for patients with anti-HLA-Abs was significantly higher than those without ($p = 0.000525$) while slope was significantly lower ($p = 0.00243$). r^2 were 0.087 and 0.003 respectively. Before removing outliers, both y-intercept and slope were worse for those patients with anti-HLA-Abs ($p = 0.004163$ and $p = 0.007649$ respectively).

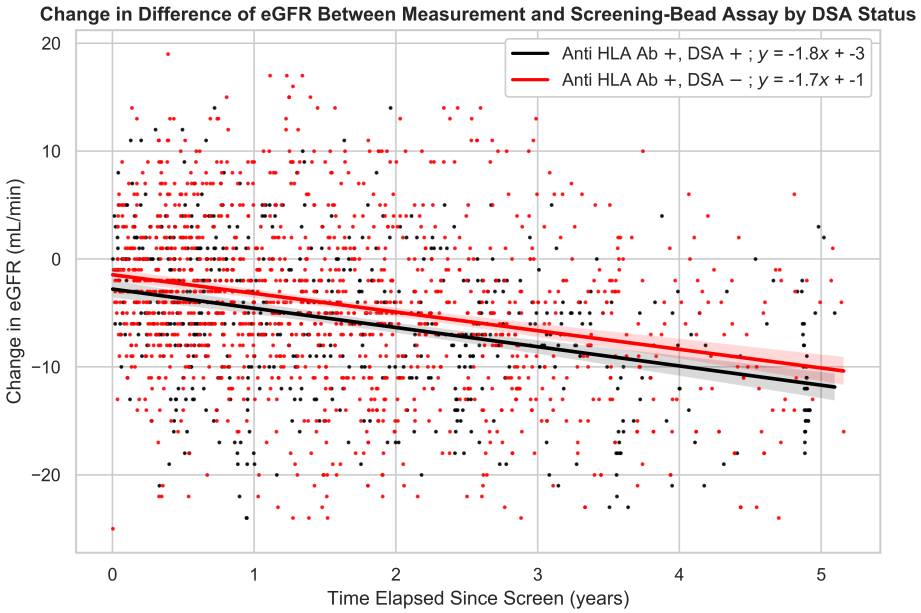


Figure 8: Y-intercept was higher for patients without DSAs than those with (p = 0.011678), though there was no significant difference between their slopes (p = 0.833792). r^2 values were 0.123 and 0.070 respectively. Before removing outliers, y-intercept was also higher for patients without DSAs (p = 0.001450) though there was no statistically significant difference in slopes (p = 0.055989).

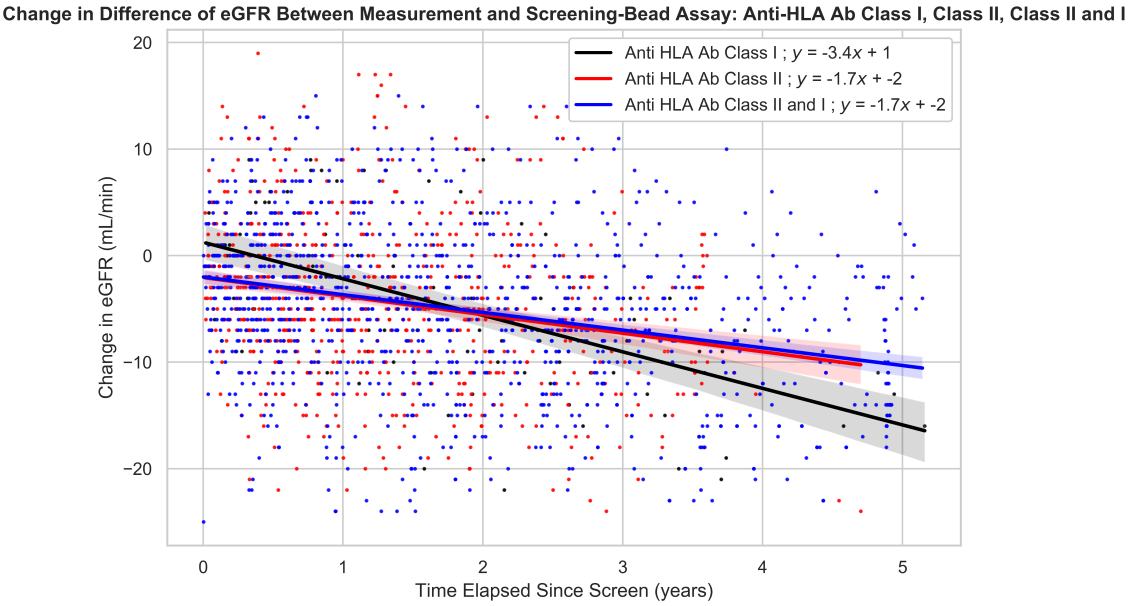


Figure 9: When comparing patients with Class II anti-HLA-Abs to those with Class I anti-HLA-Abs, y-intercept was higher in the latter (p = 0.001000). Slope was steeper for the latter (p = 0.001351). When comparing patients with Class II anti-HLA-Abs to those with Class II+I anti-HLA-Abs, y-intercept and slope were similar between groups (p = 0.925901 and 0.801724 respectively). r^2 values for patients with Class I, II and II+I anti-HLA-Abs were 0.391, 0.070 and 0.134 respectively.

Before removing outliers, when comparing patients with Class II anti-HLA-Abs to those with Class I anti-HLA-Abs, y-intercept and slope were higher in the latter (p = 0.000002 and 0.000093). When comparing patients with Class II anti-HLA-Abs to those with Class II+I anti-HLA-Abs, y-intercept and slope were also similar between groups (p = 0.764545 and p = 0.417490).

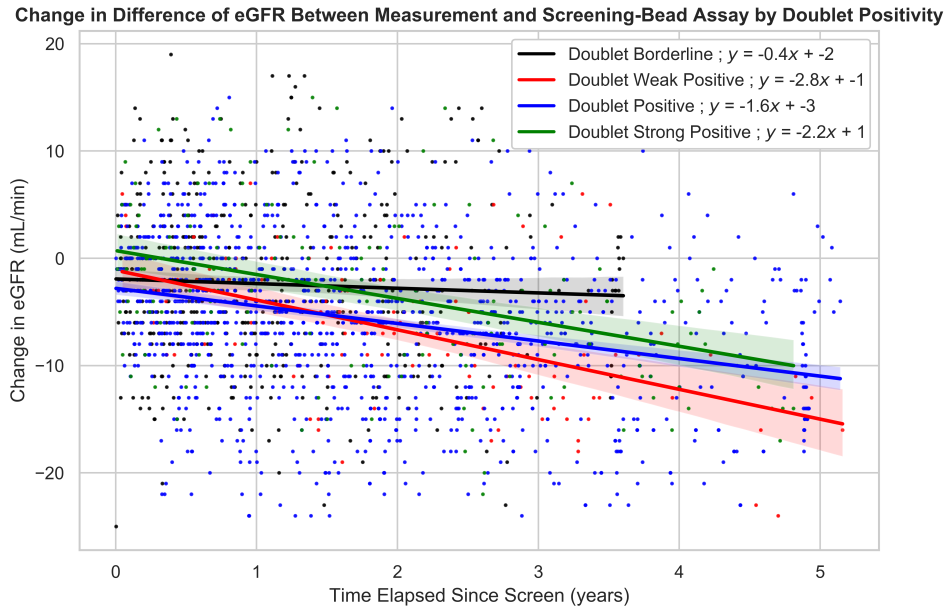


Figure 10: Each group was compared to 'Borderline' results. Y-intercept was not different when compared to 'Weak Positive' and 'Positive' results (p = 0.479562 and 0.156691 respectively), but was higher for 'Strong Positive' results (p = 0.014890). Slope was lower in 'Weak Positive', 'Positive' and 'Strong Positive' results (p = 0.000036, 0.002359 and 0.001255 respectively). 3-way ANOVA between 'Weak Positive', 'Positive' and 'Strong Positive' results showed p = 0.0009 for y-intercepts and p = 0.051 for slopes. r^2 values for patients with 'Borderline', 'Weak Positive', 'Positive' and 'Strong Positive' screens were 0.003, 0.272, 0.083 and 0.149 respectively.

Before removing outliers, when comparing y-intercepts to 'Borderline' results, there was no difference compared to 'Weak Positive' results (p = 0.598770), 'Positive' had a lower y-intercept (0.000007) and 'Strong Positive' results had a higher y-intercept (p = 0.016815). When comparing slopes to 'Borderline' results, there was no difference compared to 'Weak Positive' results (p = 0.29619), though slope was higher in patients with 'Positive' and 'Strong Positive' results (p = 0.008417 and 0.000063 respectively). 3-way ANOVA between 'Weak Positive', 'Positive' and 'Strong Positive' results showed p = 0.0000 for y-intercepts and p = 0.0437 for slopes.