RBC ALLOANTIBODIES IN TRANSPLANT PATIENTS AND THE HISTOLOGIC DISTRIBUTION OF RBC GROUP ANTIGENS IN DECEASED DONOR TISSUES

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ABSTRACT

The full extent of factors responsible for histocompatibility is not entirely understood. Despite continuous efforts, solid organ transplants with apparent ideal histocompatibility matching can still fail, and avenues outside of the human leukocyte antigen (HLA) system are being explored. Currently, there are only a few non-HLAs that have been studied and sought out for their contribution to solid organ transplantation, and the goal of this investigation was to add red blood cell (RBC) antigens to that growing list.

The influence of RBC antigen alloimmunization on solid organ transplantation outside of the ABO blood group system is not well known, but the clinical significance of transfusion-associated RBC alloimmunization is widely recognized and applied in standard transfusion medicine practice. RBC antigens that have been identified outside of RBCs are termed histoblood group antigens. Detection of these antigens in deceased donor tissues will designate additional non-HLA histocompatibility for transplant recipients, especially for those that have pre-formed non-ABO RBC alloantibodies in suspected cases of antibody-mediated rejection (AMR).

This investigation aimed to identify the (i) frequency of non-ABO RBC alloantibodies in transplant patients and (ii) detect histoblood group epitopes in deceased donor tissues to further characterize non-HLA expression. To do this, serum of transplant patients was tested for the presence and specificity of commonly encountered, non-ABO RBC antibodies, and immunohistochemistry (IHC) was used to identify select histoblood group epitopes in deceased donor tissues (heart, lung, kidney, and pancreas).

The total study population had 5% of patients testing positive for non-ABO RBC antibodies. Clinically significant antibodies identified were anti-K, and anti-Fy^a. A clinically insignificant, nonspecific cold reacting antibody was also detected. The results obtained for the reported clinically significant antibodies align within documented characteristics; these antibodies are commonly encountered, and target antigens that have higher immunogenicity.

Immunodetection used in this investigation further classified the location of histoblood group antigens from the MNS, Rh, Kell, Lewis, Duffy, Kidd, XG, Chido/Rodgers, and Knops blood group systems.

IHC data collected in this investigation reveals that the expression of histoblood group antigens in deceased donor tissues (heart, lung, kidney, and pancreas) is comparable to that of healthy individuals. Additionally, this investigation provides further details for the detection of histoblood group antigens in tissue substructures where comparable IHC data were unavailable, e.g., in intercalated discs (heart), bronchial epithelium (lung), and urothelium (kidney), and new data for the detection of both Ss (GYPB) and Kell antigens outside of RBCs.

This study offers new evidence for the frequency of non-ABO RBC antibodies in transplant patients, and for the distribution of histoblood group antigens on deceased donor tissues. These data facilitate consideration of non-ABO RBC antibodies, when present, in cases of suspected AMR when HLA donor-specific antibodies (DSAs) are absent.

To my late husband, Dr. Brian Wlosinski.
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LIST OF SYMBOLS

<	Less Than
≤	Less Than or Equal To
>	Greater Than
≥	Greater Than or Equal To
%	Percent
CH/RG	Chido/Rodgers Blood Group System
FY	Duffy Blood Group System
JK	Kidd Blood Group System
KEL	Kell Blood Group System
KN	Knops Blood Group System
L	Liter
LE	Lewis Blood Group System
M	Molar
MNS	MNS Blood Group System
mL	Milliliter
mg	Milligram
mm	Millimeter
mM	Millimolar
n	Sample Size
RH	Rh Blood Group System
µL	Microliter
µg	Microgram
µm	Micrometer
×g	Times Gravity

XG	Xg Blood Group System

LIST OF ABBREVIATIONS

AA	Amino Acid
AABB	Association for the Advancement of Blood & Biotherapies
ACKR1	Atypical Chemokine Receptor 1
AHG	Anti-Human Globulin
AMR	Antibody-Mediated Rejection
AP	Alkaline Phosphatase
APOL1	Apolipoprotein L1
AT1R	Angiotensin II Type 1 Receptor
Banff	Banff Foundation for Allograft Pathology
BD	Becton Dickinson
C4A	Complement C4A (Rodgers blood group antigen)
C4B	Complement C4B (Chido blood group antigen)
CD	Cluster of Differentiation
CDC	Complement-Dependent Cytotoxicity
cDNA	Complementary DNA
CH	Chido
CR1	Complement Receptor 1
DARC	Duffy Antigen Receptor for Chemokines
DAT	Direct Antiglobulin Test
DBD	Deceased Donors due to Brain Death
DCD	Deceased Donors due to Cardiac Death
DNA	Deoxyribonucleic Acid
DSA	Donor-Specific Antibody
DTT	Dithiothreitol

EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EST	Expressed Sequence Tags
ETAR	Endothelin-1 Type A Receptor
FFPE	Formalin-Fixed Paraffin-Embedded
Fy	Duffy blood group system antigens
GOLM	Gift of Life Michigan
GYPA	Glycophorin A
GYPB	Glycophorin B
HDFN	Hemolytic Disease of the Fetus and Newborn
HEA	Human Erythrocyte Antigen
HLA	Human Leukocyte Antigen
KLH	Keyhole Limpet Hemocyanin
HPA	Human Protein Atlas
HTR	Hemolytic Transfusion Reaction
IAT	Indirect Antiglobulin Test
Ig	Immunoglobulin
IHC	Immunohistochemistry
IHP	Investigative HistoPathology Laboratory
IL	Interleukin
IRB	Institutional Review Board
ISBT	International Society of Blood Transfusion
ISHLT	International Society for Heart and Lung Transplant
Jk	Kidd blood group system antigens
Kn	Knops blood group antigens

Le	Lewis blood group system antigens
MHC	Major Histocompatibility Complex
MICA	MHC Class I Chain-Related Antigen A
mRNA	Messenger RNA
MSU	Michigan State University
NaCl	Sodium Chloride
NBF	Neutral Buffered Formalin
OPO	Organ Procurement Organization
OPTN	Organ Procurement and Transplant Network
PRA	Panel Reactive Antibody
RBC	Red Blood Cell
RG	Rodgers
RNA	Ribonucleic Acid
Se	Secretor
Seq	Sequencing
SLE	Systemic Lupus Erythematosus
TBS	Tris Buffered Saline
TNF	Tumor Necrosis Factor
UTR	Untranslated Region
XM	Crossmatch

INTRODUCTION

The long-term success of solid organ transplantation depends on predicting all incompatibilities that can lead to graft failure. The ABO blood group compatibility between donor and recipient is the primary determination for the best engraftment outcome, and this precedes human leukocyte antigen (HLA) compatibility testing. Presently, the only antigens and antibodies evaluated in pre-transplant testing involve the ABO and HLA systems.[1]

The HLA system is considered the hallmark for tissue compatibility, yet engraftment can still fail with apparent ideal histocompatibility matching, and this has led to the pursuit of immunogenic markers beyond HLAs.[1-3] The influence of antigens outside of the HLA system is of increasing interest within transplant medicine, and the identification and development of testing for pathologic markers associated with non-HLA antibody-mediated rejection (AMR) has become more prevalent.[3-5] At this time, several non-HLAs have been studied for their contribution to routine histocompatibility testing, but the true extent of histocompatibility between donors and recipients is not fully understood and there are more uncharacterized non-HLA targets that have yet to be described.[3,4,6]

Red blood cell (RBC) transfusion is a common procedure, with over 11 million blood units transfused annually in the United States.[7] Every RBC transfusion will usually have varying degrees of antigen mismatch between donor and recipient, and this can lead to the development of RBC alloantibodies.[7] RBC antibodies outside of the ABO system are referred to as *unexpected*, meaning that their presence is due to alloexposure to RBC antigens, typically through transfusion, transplant, or pregnancy. It is estimated that 2-5 percent (%) of transfused individuals in the general population develop non-ABO RBC alloantibodies and are at risk of mounting an anamnestic response upon re-exposure to offending antigens.[7,8] Transplant patients are regularly transfused pre-, during, and post-transplant, but research is lacking on the influence of RBC alloimmunization on solid organ transplantation outside of the ABO blood group system.[7-9]

However, the clinical significance of transfusion-associated RBC alloimmunization is widely recognized and applied in standard transfusion medicine practice.[7,9]

The ABO blood group system is the only RBC antigen system evaluated in pre-transplant testing, but there are many more RBC antigen systems in existence, and research is showing that not all RBC antigens are exclusive to RBCs.[1,10-13] These RBC antigens are termed histoblood group antigens given that their presence extends beyond RBCs and into other tissues.[1,10,11] Using complementary deoxyribonucleic acid (cDNA) libraries and expressed sequence tags (ESTs), histoblood group antigens have been identified in both normal and malignant tissues.[1,10,11] However, this information is limited in its ability to provide details on how these antigens are expressed in organs and their various substructures, and presence/documentation of expression can vary between databases.[1] On the other hand, immunohistochemistry (IHC) has been used to catalog antigen expression in both normal and malignant tissues,[12,13] but it is unknown whether the expression patterns of histoblood group antigens differ in deceased donor allografts, as might occur from changes influenced by cause of death, or medical intervention.

In 2023, the leading mechanisms of death for deceased donors in Michigan are, cardiovascular (24%), intracranial hemorrhage/stroke (21%), blunt injury (20%), and drug intoxication (15%).[14] Deceased donor tissues used in solid organ transplantation are considered normal, but variations in histoblood group antigen expression following physiologic changes, or through deceased organ donor management protocols designed for the recovery of transplantable organs, is not fully understood.[15-17]

REVIEW OF THE LITERATURE

1. The HLA System

The immunologic matching required for donors and recipients prior to solid organ transplantation relies heavily on the major histocompatibility complex (MHC).[18] The MHC, called the HLA system in humans, is a gene complex that encodes cell surface proteins essential for the adaptive immune response.[18] The MHC is one of the most polymorphic sets of genes known in vertebrates, located on the short arm of chromosome six in humans and comprising around 224 genes.[9,18,19] Roughly 40% of the genes that make up the MHC encode molecules involved in both allo- and autoimmunity.[19] Of those gene products, HLAs coordinate both cellular and humoral immune responses through the recognition of self and non-self, and HLAs are a key target for allogenic transplant engraftment.[9,18,19]

The classical HLA proteins that are involved in the immune response are categorized as either class I or class II.[18] Class I HLA molecules (HLA-A, B, C) are expressed on all nucleated cells in the body, but the level of expression varies on different cells and tissues.[18] Class I molecules consist of a polymorphic alpha polypeptide chain, and a nonpolymorphic beta 2-microglobulin protein with a gene on chromosome 15.[18,20] Peptides presented on class I HLA molecules are endogenous in origin and recognized by cytotoxic cluster of differentiation (CD) 8+ T lymphocytes.[18] Class II HLA molecules (HLA-DR, DQ, DP) are expressed on antigen presenting cells such as B lymphocytes, macrophages, and dendritic cells.[18] Class II molecules consist of both an alpha and a beta polypeptide chain, and peptides presented on HLA class II molecules are exogenous in origin, recognized by CD4+ T helper lymphocytes.[18,20]

The main function of the HLA system is discriminating self versus non-self through the presentation of peptides to T lymphocytes via HLA class I or class II proteins.[9] Histocompatibility is an essential part of the transplant process because HLA molecules on the donor allograft are a primary target for the immune response of the recipient,[3,9] with up to 10% of recipient T cells recognizing foreign HLAs.[21-23] Pre-transplant histocompatibility testing involves the detection of

anti-HLA antibodies in the recipient and identifying the HLA type of both the donor and the recipient.

Matching for HLAs (determining the alleles that are present at each HLA locus), typically includes 11 genes from both HLA class I and class II, which currently make up over 35,000 alleles.[24] Tissue typing and HLA antibody identification are important because transplant incompatibility due to pre-formed anti-HLA antibodies or de novo antibody formation against HLA proteins can induce graft rejection and graft loss.[3] Having a well-matched organ is ideal, but the probability of finding a well-matched, unrelated donor is low due to the highly polymorphic nature of HLAs.[25] The likelihood of finding a deceased donor kidney with zero HLA-A, B, and DR mismatches is 21.4% in Whites, 14.3% in Hispanics, 7% in Blacks, and 6.6% in Asians.[25] Given this, transplants are not always an exact match, which can lead to sensitization and lower allograft survival (approximately 20% decreased survival for mismatched deceased donor kidney transplants, compared to zero mismatched).[25] The degree of sensitization is proportionate to the amount of mismatch between donor and recipient, and more than 70% of transplant patients become sensitized.[25] These recipients create anti-HLA antibodies to their allograft, which can also impact compatibility with any subsequent allografts.[25] Though the HLA system is highly polymorphic, matching for HLA is the gold standard for transplant compatibility.[9] But the ABO system is the primary antigen-antibody system that influences the survival of transplanted organs.[9]

2. The ABO System

The discovery of the ABO system in the early 1900s by Karl Landsteiner brought the science of immunohematology into existence.[26] The ABO system is composed of carbohydrate antigens with wide tissue distribution, and these antigens are not a primary gene product; they are built up through the addition of monosaccharides, a process catalyzed by specific glycosyltransferases.[26] The ABO blood group antigens are characterized by immunodominant carbohydrate epitopes on glycoproteins and glycolipids[9], where the A antigen is defined by the

presence of N-acetylgalactosamine, and the B antigen is defined by the presence of D-galactose.[9,26] Individuals with the O phenotype do not express A or B antigens, and individuals with the AB phenotype express both A and B antigens; these alleles are codominant.

ABO incompatibility is the primary immunologic concern regarding donor selection for solid organ transplantation.[9] Isohemagglutinins against A and B antigens occur in the body without RBC alloantigen exposure, and the transfusion of ABO-incompatible blood can result in acute intravascular hemolysis, which can be fatal.[9] This ABO incompatibility is the same for solid organ transplantation and results in hyperacute humoral rejection, via classical complement activation.[9] Despite the risks, in December 2014 the Kidney Allocation System policy 8.5.D. went into effect, involving the transplant of group A subtype (non-A1) donor kidneys into group B recipients.[27] Group A subtypes (20% of the blood group A population) differ qualitatively and quantitatively from the traditional A1 blood group (80% of the blood group A population), making them the safer option for major-ABO incompatible transplants.[9,20] Group A subtypes do not possess the A1 antigen, and only 1-8% of these individuals make anti-A1 antibodies upon alloexposure.[20,27] The single base substitution at nucleotide 467, and the single base deletion at nucleotide 1060 that characterizes group A subtypes leads to an altered specificity of the A glycosyltransferase, producing a non-A1 antigen with fewer antigen sites (240,000-290,000 antigens per RBC) compared to A1 individuals (810,000-1,170,000 antigens per RBC).[20] Even though protocols for the transplantation of deceased donor blood group A subtype kidneys to blood group B recipients has increased these transplants to 1.4% of all kidney transplants since 2014,[27] ABO compatibility remains foundational for safe pre-transfusion and pre-transplant testing.[9]

Transfusion medicine has shown that RBC antigens are a source of potential immune stimulation. It would be of benefit to learn how non-ABO RBC antigen incompatibility contributes to graft dysfunction, especially in the absence of detectable donor-specific antibodies (DSAs) or HLA incompatibilities.

3. Non-HLAs

Despite best efforts, transplants can still fail between donors and recipients that meet all the pre-transplant criteria used for determining a well-matched allograft: (i) compatible ABO blood types, (ii) a complete HLA haplotype match, and (iii) a negative lymphocyte crossmatch with the absence of DSAs. Although HLA and ABO are considered the most important histocompatibility systems in transplantation, the awareness of non-HLA compatibility is on the rise. Non-HLA influence on adverse allograft outcomes was first identified when HLA-identical siblings experienced allograft rejection,[9,28] and research is beginning to yield evidence that non-HLA antibodies play a role in successful transplantation.[2,3]

Non-HLAs are commonly referred to as minor histocompatibility antigens, which are non-MHC proteins that can induce an immune response through direct or indirect allorecognition.[29] Any antigenic protein can be thought of as a minor histocompatibility antigen, especially if that antigen can give rise to an alloimmune response post-transplant.[29]

Testing for non-HLA antibodies is warranted when there is histological evidence, post-transplant, suggesting AMR (i.e., microvascular invasion score, greater than or equal to (≥) 1 + C4d labeling, or microvascular invasion score ≥2 + C4d negative labeling), with no detectable HLA DSAs.[5,30] Though commercial non-HLA testing kits are becoming more available, they remain incomplete as the knowledge surrounding the identity of non-HLA targets continues to evolve.[6] Additionally, studies aimed at determining the clinical significance of non-HLA antibodies are highly variable, and have different immunosuppression regimes, measured outcomes, and reported incidences of antibodies.[3] Despite these shortcomings, it is still essential to develop diagnostic tests for non-HLA antibodies that are dependable and sensitive.[3]

Non-HLAs that have been studied include MHC class I chain-related antigen A (MICA), angiotensin II type 1 receptor (AT1R), endothelin-1 type A receptor (ETAR), vimentin, perlecan, tubulin, myosin, and collagens.[6,9,30] At this time, research aimed at developing assays that accurately detect non-HLA antibodies has produced two commercially available testing kits:

Immucor LIFECODES® non-HLA antibody kit can detect up to 60 non-HLA targets,[31] and One Lambda LABScreen Autoantibody kit can detect up to 33 non-HLA targets.[32] Though both the Immucor and One Lambda non-HLA testing kits contain some of the same non-HLA target antigens, neither of these kits contain RBC blood group system antigens.[4,31,32]

Most non-HLAs, except for MICA, were thought to be targets of autoantibody production, solely directed against the endothelium, but research has shown that this is not the case.[3,9] Non-HLA antibodies can target allo- or autoantigens, presenting pre-transplant, or de novo post-transplant.[3] Though most non-HLAs have been identified on the endothelium and epithelium, non-HLA expression overall has been found to be ubiquitous.[3] Endothelium is an important interface for transplantation because this is where the donor and recipient circulation first make contact; endothelium is a prime target for non-HLA antibodies.[3,6,29] Routine antibody screening and crossmatch methods that rely on the reactivity of lymphocytes limit the detection of non-HLA antibodies since endothelial cells express antigens that are not on lymphocytes.[6] Therefore, endothelial cells are ideal for identifying non-HLA antibodies.[6]

The immune response following primary alloexposure begins with the production of immunoglobulin M (IgM) antibodies, where antibody titers peak approximately one-week after exposure.[33] The IgM antibodies produced have lower affinity for antigens and a half-life of roughly five days.[33] Following the production of IgM antibodies, higher-affinity immunoglobulin G (IgG) antibodies are produced, and their titer will peak approximately three weeks following primary exposure (unless continuously exposed to the offending alloantigen).[33] The half-life of IgG1, the most abundant IgG antibody in circulation, is 21 days, and the total antibody titer (IgM + IgG) begins to decline roughly four weeks after primary exposure, eventually entering a low-level steady state (unless continually exposed to the offending alloantigen).[33] Upon secondary exposure, there is a memory response (anamnestic response) that produces exponentially higher titers of antibody, predominately higher-affinity IgG, over a shortened lag time (1-4 days)

compared to primary exposure (7-10 days).[33] Antibodies produced upon repeated exposure to an antigen are of higher-affinity and higher titer, that lasts longer compared to primary exposure.[33]

The inflammatory response induced by non-HLA antibodies can lead to the upregulation of non-HLA expression and modify antigen processing.[3] These changes could also increase HLA expression, further targeting allografts for an alloimmune response.[3] Additionally, incompatibilities with minor histocompatibility antigens may lead to chronic adverse outcomes in patients with heightened immunity.[2]

Sensitization to non-HLAs is thought to be like that of HLAs, considering that both responses produce inflammation and damage related to post-transplant injury and organ failure.[30] Another theory is that non-HLA antibodies frequently occur together with HLA antibodies, and cross-reactivity of HLA antibodies with epitopes on non-HLAs might induce graft rejection.[2]

There are limited studies on the pathogenic role of non-HLA antibodies in solid organ transplantation using animal and in vitro models.[3] Research on non-HLA antibodies in solid organ transplantation involving kidneys has documented the rate of graft loss from non-HLA influences, but study designs are inconsistent. In one study, 38% of patients had kidney allograft loss due to non-HLA mechanisms, compared to 18% from HLAs,[34] and in another study, looking specifically at anti-AT1R and anti-ETAR, graft loss was significantly higher in patients with these non-HLA antibodies (71%) compared to those without them (11%).[35] An additional study, looking at early AMR in kidney transplant patients, found that 2.3% of their population (10 out of 433) had early AMR due to non-HLA DSA.[36]

To evaluate the contribution of non-HLA immune responses to long-term kidney transplant outcomes, one study was designed to compare HLA panel-reactive antibody (PRA) testing results before and after transplant, but overall results are unclear.[2] The subset of transplants between HLA-identical siblings were reviewed for this investigation, including pre-transplant HLA PRA results and graft outcomes.[2] Sibling donors and recipients were HLA-matched at the HLA-A, B, and DR loci, but there may have been incompatibility at other HLA loci (the likelihood of such

incompatibility is less than (<) 3%).[2] Recipients of HLA-matched sibling kidneys also had their pre-transplant HLA antibodies recorded, even though these individuals should not have generated HLA antibodies to the donor graft that would have effected adverse graft outcomes.[2] Pre-transplant HLA PRA results were grouped into three categories: 0% HLA PRAs, less than or equal to (≤) 50% HLA PRAs, and ≥50% HLA PRAs.[2] Transplants from HLA-identical siblings showed no evidence of graft failure in the first-year post-transplant, but PRA testing showed additional antibodies developing throughout the following ten years (increase in % PRA) in all three PRA groups.[2] The antibodies identified in HLA-identical sibling transplants were IgG and suggested by the investigators of this study to be alloantibodies (IgM antibodies are suggested to be autoantibodies that did not influence graft survival), contributing to a decreased survival and delayed immune response for this cohort.[2] This study was unable to specifically determine whether the decline in graft survival was from the formation of non-HLA antibodies, from additional HLA antibodies (which could come from transfusion, transplant, pregnancy, or vaccination), or from DSAs (since the siblings were matched only at the HLA-A, B and DR loci).[2]

Non-HLA antibodies have been implicated in both acute and chronic injury following heart, lung, kidney, and liver transplantation, and therefore should be considered as biomarkers of injury both pre- and post-transplant.[30] Mitigating the humoral response directed against HLAs and non-HLAs expressed on allografts will improve long-term graft outcomes.[30] Additionally, further research for identifying and classifying additional non-HLAs and their influence on graft outcome will advance the field of transplantation and may lead to increased graft survival.

4. Public Databases

4.1. EST & cDNA Libraries

Recognition of the HLA system for tissue alloimmunity stimulated a major effort to explore the polymorphisms of this system in detail, and the same can be said for RBC antigens after discovering the influences of the ABO system.[11] Currently, clinical testing in solid organ transplantation ignores almost all antigens and antibodies outside of HLA and ABO for

determining compatibility.[11] Though RBC surface antigens were once considered to be confined only to RBCs,[1,10] research has shown that these antigens are located in other tissues. Histoblood group antigens are RBC antigens that have a wider tissue distribution than RBCs alone, and molecular methods have begun to provide a sensitive approach to identifying the tissue distribution of non-ABO RBC protein antigens.[1,11]

Most non-ABO blood group proteins are expressed on erythroid precursors during erythropoiesis, and polymorphisms between antithetical antigens do not alter the transcription of blood group genes.[1] The Lewis and Chido/Rodgers systems are exceptions to this rule because these protein antigens are passively adsorbed onto the RBC membrane from the plasma.[10] In contrast, carbohydrates are not gene products, and are instead built up through the addition of monosaccharides, catalyzed by specific glycosyltransferases,[26] which means that carbohydrate histoblood group antigens are synthesized sugars.[10] Since protein antigens are gene products, researchers can search DNA and/or protein databases for genetic sequences that share a relationship to a sequence of interest, but they cannot do so for carbohydrate antigens.[11]

Utilizing nucleotide databases with cDNA libraries and ESTs, one study was designed to identify histoblood group antigens on various cells and tissues.[1,11] ESTs are sequenced fragments of cDNA (the 3' untranslated region (UTR)) that represent messenger ribonucleic acid (mRNA) transcripts; cDNA is synthesized from mRNA transcripts.[1] EST sequences are characteristically short (about 400 base pairs), and were identified after selecting random cDNA clones and performing automated sequencing (seq).[37] Despite their inaccuracy, ESTs can be a valuable resource in the discovery of new genes and seq infrastructure for large-scale projects.[37,38]

The 3' UTR regulates mRNA localization and/or protein abundance, either through translation regulation or stability of mRNAs.[39] Compared to other areas of mRNA sequences, 3' UTRs rarely contain introns and they are not well conserved, making these coding sequences easier to differentiate when comparing individual genes from gene families.[38] The variance of 3' UTRs between isoforms suggests that 3' UTRs can also be used to distinguish the functions of

similar proteins.[39] The 3' UTR is cell-type specific, and an important regulator of subcellular mRNA localization which allows for regulation of protein production.[39] Expression ratios of 3' UTRs change during physiologic development and differentiation as well as during disease.[39]

The GenBank subset, UniGene, is a database that contains thousands of unique ESTs (3' UTR) to compare and classify new sequences.[38] UniGene retired in 2019, because reference genomes are more available now than they were when the database was created, but the data are still available through the National Center for Biotechnology Information. UniGene focused on protein-coding genes of the nuclear genome, and therefore, genes identified as ribosomal RNA or mitochondrial sequences are eliminated.[40] For an EST sequence to have been included in UniGene, the clone must have had at least 100 high-quality, non-repetitive base pairs since repetitive sequences can sometimes lead to false alignments.[40]

Complementary DNA libraries can be prepared from larger tissue samples, or from single cell lines.[11] Research has found that the mRNA transcripts and ESTs from protein-based RBC antigens exhibits wide distribution across different cell and tissue types.[1,11] The EST data available for the selected protein-based blood group antigen systems used for this study are noted in Table 1 for the heart, lung, kidney and pancreas.[1] Overall data (not shown) show that the predicted expression patterns of RBC group antigens on non-erythroid cells and tissues is very heterogeneous, apart from the Kell system for which no expression was found, and the MNS and Rh blood group systems, which are largely considered to be confined to erythroid cells.[1] There was no obvious association between RBC antigen function and tissue distribution of the antigen based on the information in these databases.[1]

Since many RBC antigens have an identified role in physiologic, or pathologic processes,[10] there is a need to go beyond EST data to precisely learn the tissue expression of histoblood group antigens. These antigens may be of clinical value in solid organ transplantation, or may be used for targeting immune responses and/or homing of infectious agents.[1]

Table 1. Select Protein-Based Blood Group System Antigen ESTs Detected in the Heart, Lung, Kidney, and Pancreas

Tissue Type	Blood Group System							
	002 MNS	**004 RH**	**006 KEL**	**008 FY**	**009 JK**	**012 XG**	**019 XK±**	**022 KN**
Heart	0	0	0	0	**+**	0	0	0
Pericardium	0	0	0	**+**	**+**	**+**	0	0
Aorta, Artery	0	0	0	**+**	**+**	0	0	**+**
Pulmonary Artery Endothelial Cells	0	0	0	0	0	0	0	0
UniGene Pool Heart	**+**	**+**	0	**+**	**+**	**+**	**+**	0
Lung	0	0	0	**+**	**+**	0	0	0
Epithelial Cells	0	0	0	0	0	0	0	0
Focal Fibrosis	0	0	0	**+**	0	0	0	0
UniGene Pool Lung	0	**+**	0	**+**	**+**	0	0	**+**
Kidney	0	0	0	**+**	**+**	0	0	**+**
UniGene Pool Kidney	**+**	**+**	0	**+**	**+**	0	0	0
Pancreas	0	0	0	0	0	0	0	0
Islets of Langerhans	0	0	0	**+**	0	0	0	0
UniGene Pool Pancreas	0	0	0	**+**	**+**	**+**	0	**+**

Adapted from: Rojewski, MT, et al. (2006) Additional File 1 p.10-15. https://doi.org/10.1016/j.transci.2006.05.008
The International Society of Blood Transfusion (ISBT) number precedes each blood group system.
Blood group system symbols: Kell (KEL), Duffy (FY), Kidd (JK), Knops (KN).
Tissues in **Bold** font does not report specified locations of EST detection.
UniGene Pool refers to data from a pool of different cDNA libraries in UniGene.
± XK is associated with the Kell blood group system.
+ Detection
0 No Detection

4.2. The Human Protein Atlas

To cultivate a greater understanding of human biology and disease, researchers strove to map the molecular details of protein expression throughout different tissues and organs in the human body.[12,13] The goal of the human protein atlas (HPA) is to “define structure, function, localization, expression, and interactions of all proteins” in the human body.[12]

At the time of publication, samples from all major tissues (32 tissues, 47 cell lines) in the human body have been analyzed (using antibodies and their corresponding protein-encoded genes) to generate more than 13 million IHC tissue images for the HPA database.[12] Quantitative mRNA expression from deep seq of tissue samples (RNA-Seq)[41] was also performed using a combination of cell types and the specific location of their related proteins identified after IHC.[12] Based on the data collected, there is concordance between mRNA transcripts and protein expression for a given gene across various tissues.[12]

The use of IHC and tissue microarrays allows for spatial recognition of protein expression, and when combined with mRNA expression data of the same tissue-type, this yields a more efficient histological and/or pathological classification.[12] The HPA data available for the selected histoblood group antigens used for this study are noted in Table 2 for the heart, lung, kidney and pancreas.[13]

Table 2. HPA Results for Selected IHC-Detected Histoblood Group Antigens in the Heart, Lung, Kidney, and Pancreas

Tissue and Cell Type		Blood Group Systems				
		MNS		RH ±	KEL	LE (FUT3)
		MN (GYPA)	Ss (GYPB)			
Heart	Cardiomyocytes	**Low ***	-	Not Detected	-	**Medium**
Lung	Alveolar Cells	**Low ***	-	Not Detected	-	Not Detected
	Macrophages	**Low ***	-	Not Detected	-	**Low**
Kidney	Bowman's Capsule	Not Detected	-	Not Detected	-	-
	Cells in Glomeruli	Not Detected	-	Not Detected	-	Not Detected
	Cells in Tubules	Not Detected	-	Not Detected	-	**Low**
	Collecting Ducts	Not Detected	-	Not Detected	-	-
	Distal Tubules	**Medium ***	-	Not Detected	-	-
	Proximal Tubules (Cell Body)	Not Detected	-	Not Detected	-	-
	Proximal Tubules (Microvilli)	Not Detected	-	Not Detected	-	-
Pancreas	Exocrine Glandular Cells	Not Detected	-	Not Detected	-	**Low**
	Endocrine Cells	Not Detected	-	Not Detected	-	**Low**

Adapted from The Human Protein Atlas (HPA) www. https://www.proteinatlas.org/; https://www.atlasantibodies.com/.
Blood group system symbols: Lewis (LE), Chido/Rodgers (CH/RG).
No IHC data in the HPA for Kell (KEL) and Ss (GYPB) blood group antigens.
* Highest IHC detection documented for all biopsies tested.
± Multiple antigens in this system were tested with the same polyclonal antibody.
- No IHC Data Available.

Table 2. (cont'd)

Tissue Types		Blood Group Systems				
		FY (ACKR1)	**JK (SLC14A1)**	**XG (CD99)**	**CH/RG (C4) ±**	**KN (CD35)**
Heart	Cardiomyocytes	**Low ***	**Medium**	Not Detected	**Low ***	Not Detected
Lung	Alveolar Cells	Not Detected	**Low**	Not Detected	Not Detected	**Low ***
	Macrophages	Not Detected	**Low**	**Low**	**Low ***	**Medium ***
Kidney	Bowman's Capsule	Not Detected	-	-	-	-
	Cells in Glomeruli	**Low ***	**Low**	**Medium ***	**Low ***	**High ***
	Cells in Tubules	Not Detected	**High**	Not Detected	**High ***	**Low ***
	Collecting Ducts	Not Detected	-	-	-	-
	Distal Tubules	Not Detected	-	-	-	-
	Proximal Tubules (Cell Body)	Not Detected	-	-	-	-
	Proximal Tubules (Microvilli)	**Medium ***	-	-	-	-
Pancreas	Exocrine Glandular Cells	**High ***	Not Detected	**Medium ***	Not Detected	**High ***
	Endocrine Cells	Not Detected	Not Detected	**High ***	Not Detected	**Low ***

As the number of completely sequenced genomes increases, so does the number of protein sequences in public databases. To facilitate a consolidation of resources, researchers from the HPA combined their antibody-based data with the public UniProt databases maintained by the UniProt consortium.[12,42] The UniProt consortium (uniprot.org) consists of groups from the European Bioinformatics Institute, the Swiss Institute of Bioinformatics, and the Protein Information Resource.[42] The UniProt knowledgebase is a central hub for protein information and provides protein sequences and functional information as the amount of available data increases (UniProt is updated every eight weeks).[42] Together, the HPA and UniProt have identified over 20 thousand protein-coding genes.[12,42]

The HPA has used IHC to catalog antigen expression in both normal and malignant tissues,[12,13] but the expression of histoblood group antigens in deceased donor tissues is unknown, and potentially different. Comparing the detection of histoblood group antigens in normal tissue (from the HPA) with that of deceased donor tissues is of benefit for identifying variation that may occur following the recovery of transplantable organs, for which these changes could lead to decreased allograft survival.

5. Non-ABO Histoblood Group Antigen Systems

5.1. MNS System

The MNS system is the second blood group system to be discovered after ABO and was assigned the ISBT number 002.[9,20,43] This blood group system consists of 50 antigens on two glycoproteins, and the complexity of this antigen system comes from recombination between closely linked homologous genes: *Glycophorin A* (*GYPA)* and *Glycophorin B* (*GYPB)*.[9] Of the 50 antigens within this system the M, N, S, and s antigens were the first to be discovered, and are the only polymorphic antigens within this system.[20] There is also linkage disequilibrium that exists with the expression of MN and Ss antigens.[20,44]

The M and N antigens reside on the GYPA protein, and the S and s antigens reside on the GYPB protein. GYPA (CD235a) and GYPB (CD235b) are type I single-pass membrane

sialoglycoproteins with an external N-terminal domain (containing numerous sialic acid-rich O-glycans) and a C-terminal cytosolic domain.[9,44] On the RBC surface, GYPA is more abundant, with about 1,000,000 copies per RBC, whereas GYPB has only about 200,000 copies per RBC.[9,20] There are approximately 1.5 times more GYPB on homozygous S (S+s-) RBCs than on homozygous s (S-s+) RBCs,[20,44] and the low number of GYPB on RBCs could be linked to the low incidence of detected anti-S and -s antibodies in routine blood bank testing.[9]

The MNS blood group antigens are well-developed at birth, and they also demonstrate dosage, (i.e., antibodies react stronger with RBCs that have homozygous antigen expression, compared to RBCs with heterozygous antigen expression).[20] The M and N antigens are antithetical, differing in their amino acid (AA) residues on GYPA (the M antigen possesses serine and glycine at AA position one and five, and the N antigen has leucine and glutamic acid at those positions),[9,20] and the S and s antigens are also antithetical, differing in their AA locations on GYPB (the S antigen has methionine at AA position 29, and the s antigen has threonine).[9,20] The location of the MNS system antigens on either GYPA or GYPB influence their susceptibility to routine blood bank enzymes, like ficin and papain, which can impact antibody identification in the presence of multiple RBC antibodies.[9] The location of the MN antigens near the N-terminus on GYPA makes them easily destroyed by enzyme treatment, thus promoting diminished or no antibody reactivity with these antigens.[9,20] The Ss antigens are positioned lower on GYPB, closer to the RBC membrane, making them less easily destroyed by routine blood bank enzymes.[9,20]

RBCs that do not express GYPA or GYPB are not related to disease or reduced RBC survival,[20] but, GYPA may act as a complement regulator, providing some protection to RBCs by inhibiting the formation of the membrane-attack complex.[26] Table 3 (page 44), at the end of this non-ABO histoblood group literature review, details the documented immunity-related function(s) of all non-ABO histoblood group antigen systems considered in this study.

GYPA was thought to be restricted to RBCs and is often referred to as an erythroid marker, but evidence suggests expression by other cells as well.[1,9] Both GYPA and GYPB appear to be

expressed on renal endothelium and epithelium[44] (this GYPA has been referred to as a "GYPA-like" molecule which may be a desialylated glycoprotein since only sialic acid-independent anti-M and anti-N have been documented to react with this structure).[9,20,44] Table 4 (page 47), at the end of this non-ABO histoblood group literature review, details the published locations of all non-ABO histoblood group antigen systems considered in this study. EST data shows expression of the MNS system antigens in heart and kidney, but not in lung or pancreas (Table 1),[1] and IHC shows detection of GYPA in the heart, lung, and kidney, but does not show detection in the pancreas (Table 2).[13] There are no HPA data available for GYPB assessed by IHC (Table 2).[13]

Of the GYPA antibodies, anti-M and -N are usually not clinically significant in transfusion medicine.[9] However, Anti-M has been associated with hemolytic transfusion reactions (HTRs), and if the antibody reacts at 37 °C and/or after the addition of anti-human globulin (AHG), antigen-negative RBCs need to be provided for transfusion.[9,20] Anti-M has been found in the serum of individuals who have not been exposed to the M antigen through transfusion, transplant, or pregnancy, and in these cases, the reactivity of anti-M occurs at temperatures below 37 °C.[20,44-46] Although anti-M has commonly indicated as IgM, 50-80% of anti-M reactivity is IgG or has an IgG component.[20] Antibodies to the M antigen bind little to no complement and are only considered clinically significant if there is reactivity at 37 °C and/or with addition of AHG.[20,26,47]

Of the GYPB antibodies, anti-S and -s are typically IgG with reactivity at 37 °C and/or with the addition of AHG.[20] Both antibodies are clinically significant, have been implicated in HTRs, and they sometimes bind complement.[9,20]

There are a few reports suggesting the involvement of MN antibodies in transplant situations (e.g., passenger lymphocyte syndrome,[48] pre-transplant testing,[49,50] post-transplant,[51] and hemodialysis),[52-54] but transplant-related complications directly related to antibodies of the MNS blood group system have not been documented.

5.2. Rh System

The Rh system is the most immunogenic of the minor blood group systems and is assigned the ISBT number 004.[9,43] This system involves two closely linked genes on chromosome 1, each encoding a polypeptide that expresses either the RhD or RhCE proteins.[9,20] The *RHD* and *RHCE* genes are 97% identical (their products differing by 32-35 AAs).[9] They each have 10 exons and are inherited as codominant alleles.[9,20] The *RHD* gene encodes the D antigen, and the *RHCE* gene encodes the C, c, E, and e antigens, which are inherited in four combinations: ce, cE, Ce, and CE.[9] Of the RhCE phenotypes, AA 226 is proline for the E antigen and alanine for the e antigen, while AA 103 is serine for the C antigen and proline for the c antigen.[9] The Rh proteins are unique in that they are neither glycosylated nor phosphorylated.[9,20] RhD and RhCE are multipass transmembrane proteins, for which there are 100,000-200,000 antigen sites per RBC, combined.[44] Rh proteins have twelve membrane-spanning domains with only small loops exposed on the RBC surface, but many conformational differences that lead to various Rh blood types.[20,44]

Of the 56 antigens that make up the Rh blood group system, it is the five major antigens D, C, c, E, e, that are responsible for most Rh incompatibilities.[9] The RhD antigen is the most immunogenic; exposure to just 10 microliters (µL) of RhD-positive blood can stimulate antibody production in a RhD-negative person.[55] The c antigen follows RhD in immunogenicity; as for anti-RhD antibodies, anti-c antibodies can cause severe hemolytic disease of the fetus and newborn (HDFN).[9] The remaining Rh antigens are less immunogenic but still may stimulate production of alloantibodies that can cause mild to moderate HDFN.[9]

It is reported that 8-15% of the population are negative for the RhD protein.[9] Most individuals of European decent that are RhD negative have a deletion of the *RHD* gene,[9,44,56] while in Asian and African populations, there is an intact *RHD* gene, but this gene is either inactive or silenced.[9,44] It is believed that the absence of the whole RhD protein (those with an *RHD* gene deletion) could explain why alloexposure of the RhD antigen results in such a robust alloimmune response.[9] Approximately 30% of hospitalized RhD-negative patients receive RhD-positive RBCs,

and 20-50% of this population create alloanti-RhD antibodies.[9,55] Studies have also shown that on average, 64% of healthy RhD-negative volunteers make alloanti-RhD antibodies 1-9 months following small-volume (0.01-10 milliliters (mL)) RhD-positive RBC infusions, and 84% do so following whole RBC unit transfusions (200-500 mL).[55]

Most Rh system antibodies are IgG, but some are IgM.[9,20] The Rh system antibodies are long-lasting, clinically significant, and can produce a strong anamnestic response.[20] Anti-Rh antibodies typically do not fix complement because the Rh antigens are not in close enough proximity to allow for multiple IgG antibodies to activate the classical cascade.[9,20] Therefore, RBC destruction due to anti-Rh antibodies is primarily extravascular.[9,20] Table 3 (page 44), at the end of this non-ABO histoblood group literature review, details the documented immunity-related function(s) of all non-ABO histoblood group antigen systems considered in this study.

During routine hemagglutination testing, detection of Rh antigens is enhanced by enzyme treatment, and Rh antigens can demonstrate dosage and react at 37 °C, which is useful during antibody identification.[9] The Rh antigens are thought of as erythroid-specific,[20,44] but their function is not completely known. Rh antigens are associated with the RBC membrane skeleton, and play a role in transport, particularly of carbon dioxide and/or ammonium.[20,44] Table 4 (page 47), at the end of this non-ABO histoblood group literature review, details the published locations of all non-ABO histoblood group antigen systems considered in this study. EST data identified Rh system antigens in the heart, lung, and kidney, but not in the pancreas (Table 1),[1] and IHC from the HPA were unable to identify the Rh antigens in the heart, lung, kidney, or pancreas (Table 2).[13]

Though Rh antigens are considered erythroid-specific, research has identified some clinical impact for Rh antigens and antibodies on solid organ transplant; however, retrospective analysis of both cadaveric and living donor transplants show conflicting results. One study of 1,500 living donor kidney transplants found that there was no statistically significant difference in the survival rates of RhD-identical (RhD-positive donor and recipient, or RhD-negative donor and recipient), and RhD-non-identical (RhD-negative recipient and RhD-positive donor, or RhD-

positive recipient and RhD-negative donor) transplants.[57] In an investigation of 786 primary cadaveric kidney transplants plus data for 11 thousand kidney transplants from the United Network for Organ Sharing, RhD-identical transplants had better outcomes than RhD-non-identical transplants.[58] Since around 80% of the population is RhD positive, there is a higher incidence of RhD-identical transplants, that promote better graft outcomes.[58]

The development of anti-Rh antibodies after transplantation is not a common occurrence, but there are a few reports suggesting their involvement in transplant situations, predominantly with passenger lymphocyte syndrome.[59-70] Despite these reports, transplant-related complications directly related to antibodies of the Rh blood group system have not been documented.

5.3. Kell System

The Kell blood group system, ISBT number 006, consists of 36 antigens, but the most-encountered antigens are the antithetical pair K and k.[9,20,43] The Kell system antigens are located on a single-pass, type II glycoprotein that is highly folded by disulfide bonds; the K antigen has methionine at AA 193, and threonine is at AA 193 for the k antigen.[9] The Kell glycoprotein (CD238) is covalently linked to the XK protein (ISBT number 019) by a single disulfide bond.[9,20,44] The XK protein is a multipass membrane protein, and the expression of Kell system antigens is dependent on its presence.[9,20]

The Kell glycoprotein shares homology with other members of the neprilysin family of zinc-dependent endopeptidases that catalyze formation of a variety of peptide hormones, notably the vasoconstrictor endothelin-3 from the biologically inactive peptide big-endothelin-3.[9,44] Though the enzymatic activity of the Kell glycoprotein has been discovered, its physiologic role is unclear.[20,44] The enzymatic activity of Kell antigens was found to be optimal at an acidic pH, suggesting an intracellular role, possibly related to transport and processing of endothelin precursors or to the endocytic pathway.[71] It is not known if the Kell-XK complex on non-erythroid tissues behaves the same way as it does on RBCs, but Kell and XK are also disulfide linked on non-erythroid cells; this could indicate complementary functions of these two proteins.[71,72] The

mRNA sequence identity between erythroid and non-erythroid Kell antigens also suggests similar enzymatic function.[71] Table 3 (page 44), at the end of this non-ABO histoblood group literature review, details the documented immunity-related function(s) of all non-ABO histoblood group antigen systems considered in this study.

The K antigen, the first antigen discovered in this system, has a 9% prevalence in people of European ancestry, and a 2% prevalence in those of African ancestry, making this a low-prevalence antigen.[9,20,44] The k antigen, the second antigen discovered in this system, has a 99% prevalence in the general population, making it a high-prevalence antigen.[20,44] The null Kell (K_o) phenotype is rare and there is no associated pathogenesis.[9] The absence of the XK protein is also very rare and is linked with the McLeod phenotype.[9]

The K antigen is well-developed at birth, and the number of antigen sites per RBC is fairly low (around 3,500-18,000) considering that the K antigen is very immunogenic.[20,44] In terms of immunogenicity within the minor blood group antigens, the K antigen is second to the RhD antigen.[9,20] During routine hemagglutination testing, Kell-system antigens are not destroyed by routine enzymes, but they are destroyed by thiol-reducing agents like 2-aminoethylisothiouronium bromide (AET) and 0.2M dithiothreitol (DTT).[9,20]

The Kell system antibodies are predominately IgG1, and one-third of all non-Rh antibodies investigated in the blood bank are anti-K.[9] Anti-K antibodies are the third most common antibodies seen in the blood bank, behind ABO and RhD.[9,20] On the other hand, Anti-k antibodies are rarely encountered, and only about 2 in 1,000 people whose RBCs lack the k antigen will make the corresponding antibody.[20] Anti-k antibodies have been implicated in HTRs and HDFN, but only rarely.[20]

Anti-K is mostly induced by transfusion and pregnancy, and anti-K antibodies have been implicated in mediating severe HTRs as well as HDFN.[9,20] There are rare examples of anti-K antibodies binding complement, but most in vivo hemolysis is extravascular by macrophages in the spleen.[20] HDFN from anti-K antibodies is believed to be more from the suppression of

erythropoiesis (in-vitro studies found that anti-Kell antibodies inhibit the growth of Kell-positive progenitor cells from cord blood)[73] than from hemolysis, and an estimated 10% of neonatal anemias related to HDFN are caused by anti-K antibodies, and 50% of these affected babies suffer from hydrops fetalis or require phototherapy or exchange transfusion.[73]

Studies have indicated that the Kell glycoprotein is primarily expressed in erythroid cells, with XK having wider tissue distribution, but recent studies have found that most tissues contain low-levels of Kell mRNA transcripts.[71,72] Kell-system antigens are not found on platelets, lymphocytes, granulocytes, or monocytes and are primarily found in testes, liver (fetal), and bone marrow.[20,44,71] Lower amounts of Kell antigens are also found in lymphoid organs (including spleen), the heart, colon, and skeletal muscle, and they may be present on myeloid progenitor cells.[9,44,72] The XK protein is found in lymphoid organs, the brain, heart, pancreas, placenta, and skeletal muscle.[9,20,71] Table 4 (page 47), at the end of this non-ABO histoblood group literature review, details the published locations of all non-ABO histoblood group antigen systems considered in this study. There are no EST data for the expression of Kell system antigens (Table 1), and HPA data are unavailable for the Kell glycoprotein distribution based on IHC (Table 2).[1,13]

Despite the well-documented role of the Kell system in transfusion medicine, to date, the Kell system has not been implicated in any adverse transplant outcomes.

5.4. Lewis System

The Lewis (Le) blood group system, ISBT number 007,[43] is among the most complex of the blood group systems in that phenotypic expression is influenced by multiple genes. The two primary antigens of this system, Le^a and Le^b, are not antithetical because they are not from different alleles of a solitary gene.[20] Le antigens are not a primary gene product; they are the product of interactions between two fucosyltransferases encoded by two genes, Lewis (*Le*) and secretor (*Se*).[9,20,44] The *Le* gene is required for the expression of the Le^a antigen, while both the *Le* and *Se* genes are required for the expression of the Le^b antigen.[9,20]

The Le antigens are not produced by RBCs but are instead created by endodermal epithelia (primarily exocrine gland epithelial cells).[20,74] These antigens are carbohydrates bound either to glycolipids or to glycoproteins built on type I precursor structures that are passively adsorbed onto the RBC membrane from soluble Le glycolipid in plasma.[9,20,44] Le antigens are not intrinsic to the RBC membrane and can be removed from transfused RBCs within a few days.[20,44] The soluble glycoprotein form of the Le antigen is found in all bodily fluids except cerebrospinal fluid, and the glycolipid form is adsorbed onto RBCs from the plasma.[44] Le antigens are resistant to routine blood bank enzyme treatment (markedly enhancing their reactivity); this should be taken into consideration during antibody identification assays.[20,44]

The null Le phenotype Le(a-,b-) is produced due to a lack of the *Le* gene (*lele*), allowing for the creation of Le system antibodies.[20] There are no apparent pathological consequences associated with this null phenotype.[44] Anti-Le antibodies do not require an RBC stimulus to be produced; however, they can be generated secondary to RBC exposure during pregnancy (due to a transient Le-null phenotype), in which case they are often IgM and do not cross the placenta.[9,20,44] Le antigens are also not expressed on cord blood RBCs and their expression is often reduced or removed from a mother's RBCs during pregnancy.[9,20] Expression of Le antigens may also be removed from RBCs following hemolytic mononucleosis infection, alcoholic cirrhosis, and/or alcoholic pancreatitis.[44]

Although anti-Le antibodies are typically considered clinically insignificant in transfusion medicine, since they are mostly IgM, they can activate complement and lead to hemolysis.[9,20,44,75] During routine hemagglutination testing, anti-Le antibodies are typically identified during room temperature testing (20-24 °C),[20] but any reactions at 37 °C should not be ignored, especially if in-vitro hemolysis is seen.[20] Typically, it is only persons with the null Le phenotype that can create Le antibodies, but in rare cases, people with the Le(a+b-) phenotype can create anti-Le^b antibodies.[20] Given this, RBC phenotyping of patient cells during antibody identification is important for proper Le antibody identification.[9,20]

Besides being on RBCs, Le antigens are adsorbed onto platelets, lymphocytes, monocytes, and endothelial cells, and they are expressed on the membranes of certain cells in the renal parenchyma, pancreas, skeletal muscle, adrenal glands, genitourinary tract, and gastrointestinal tract.[9,20,44] Multiple methods have been used to identify Le antigens unassociated with RBCs. One study using immunofluorescent labeling showed inconsistent results for Le antigens on kidney allografts, some distal tubules having strong labeling, and some having faint labeling.[76] All epithelial cells in Le-positive kidneys were strongly positive, and both the distal tubules and collecting ducts of Le-null kidneys were negative.[76] Vascular endothelia of both Le-positive and Le-null kidneys were negative.[76] IHC has also identified enhanced Le expression on the renal collecting ducts and urothelium of secretors compared to non-secretors.[77] Table 4 (page 47), at the end of this non-ABO histoblood group literature review, details the published locations of all non-ABO histoblood group antigen systems considered in this study. Since Le antigens are carbohydrates, they were not identified using ESTs (Table 1), but the HPA shows that Le expression was detected in the heart, lung, kidney, and pancreas using IHC (Table 2).[1,13]

Le antigens may be capable of inciting both cytotoxic cell-mediated and humoral immune responses, but research is conflicting. Le-specific lymphocytotoxic antibodies have been detected in the sera of Le null patients, even in the event of a well-matched HLA graft that happened to be Le positive. This has made the role of pre-formed Le antibodies in the presence of a Le positive graft a controversial topic.[78] Table 3 (page 44), at the end of this non-ABO histoblood group literature review, details the documented immunity-related function(s) of all non-ABO histoblood group antigen systems considered in this study.

Recipient anti-Le antibodies can be neutralized by Le blood group substance present in transfused plasma, which is why hemolysis is rare and reversible in the presence of anti-Le^a or anti-Le^b antibodies.[9,20] It has been shown in some cases that after being transfused with Le incompatible blood products, transfused RBCs assume the recipient's Le phenotype by

adsorption or loss of antigens within two to seven days.[75] However, this is not the case for bone marrow transplants, as recipients are found to maintain their Le phenotype.[75]

Patients undergoing hemodialysis have demonstrated decreased Le expression on RBCs, while Le substance in the plasma remains.[79] This can lead to false RBC phenotyping and misrepresentation of the survival data for allografts with Le incompatibility.[79] Routine immunohematologic methods to detect Le antibodies have detection limitations, therefore, Le phenotyping alone is not a completely reliable predictor of an individual's ability to make Le alloantibodies. An individual's Le phenotype may appear to be null, but this may not be because of an inactive *Le* gene; therapies such as transfusion and dialysis need to be taken into consideration when assessing Le phenotype (whether the recipient, living donor, or deceased donor) in relation to transplant outcome.[80,81]

In one study, Le-null prevalence was reportedly 12% (higher the expected prevalence of 6%),[20] but these patients were on dialysis, so the Le phenotype for some of these patients may have been incorrect.[78] This kind of misclassification in phenotype can lead to erroneous outcome data; no difference was noted in the one-year survival rates for this cohort.[78] An additional study looking at cadaveric transplants did not find any correlation between Le compatibility and graft outcome, highlighting the importance of accurately identifying Le phenotype prior to organ donation, given the likelihood of donors being transfused prior to death.[81]

The Le-null phenotype is more prevalent in Black populations, suggesting race plays a role in allograft survival where Le incompatibility is concerned. In a study of 1,300 cadaveric kidney transplants, allograft survival was 8% lower in Le-null recipients then in Le-positive recipients.[82] This study also assessed age, race, and transplant center; the greatest difference in survival between Le-null and Le-positive patients was in those considered to be at high risk: non-White patients, and those at transplant centers with < 50% survival rates.[82] However, both Le status and race have been able to show a stronger correlation with Le incompatibility and graft survival, finding that Blacks transplanted with kidneys from Black donors did worse than Blacks

transplanted with kidneys from White donors.[82-84] Despite multiple studies on the Le system, it should be noted that not all studies of kidney transplants have shown a significant relationship between allograft function and Le phenotype, related to race.[81]

Even though the null Le phenotype is more common in Blacks, and previous studies have considered there to be a relationship between Le phenotype and race, more recent investigations and reports could be influencing these outcomes. Kidneys transplanted from Black deceased donors fail sooner than those from White deceased donors,[85] recipients of kidney allografts from Black deceased donors with variants in the apolipoprotein L1 gene (*APOL1*) are at increased risk for allograft failure.[85] Having two variants of *APOL1* poses the greatest risk to allograft failure, and 13% of Blacks have two *APOL1* variants.[85] The discovery of the importance of *APOL1* in Black kidney donors and recipients, has led to a national observational study called APOLLO (theapollonetwork.org), that tests donors and recipients for *APOL1* variants to improve kidney donation and transplant outcomes.

The type of solid organ being transplanted can influence the presence or absence of Le antigens. There is evidence that transplanted kidneys possessing Le antigens can independently synthesize those antigens regardless of the Le phenotype of the recipient.[76] After analysis of urine samples from Le-null transplant recipients, any Le substance identified was found to be synthesized by the Le genes of the donor allograft.[76] In that study, Le enzyme was found only in Le-positive kidneys, showing that this organ can produce its own Le substance.[76] Interestingly, the liver is a plentiful source of RBC Le glycolipids given that liver transplant recipients maintain their Le phenotype after receiving Le-incompatible organs.[75] In liver transplant patients, Le incompatibility does not affect overall graft survival; this could be because liver transplants are more tolerant overall to ABO and HLA incompatibility compared to kidney transplants.[75]

Despite conflicting research, the Le antigen system is considered by some to be influential in histocompatibility and allograft survival.[81] One report presented three case studies involving Le antibodies that bound complement. In these cases, two patients had pre-formed Le antibodies,

and one did not. All patients had negative DSAs, and allografts were ABO compatible (the Le phenotype of the donor was unknown but was presumed to be Le(a-,b+)).[80] In the two cases with an anamnestic response, biopsies showed signs of AMR with C4d deposition. After follow-up treatment and plasmapheresis, both patients successfully retained their allografts. The third case did not have pre-existing Le antibodies, but after seven months, this patient developed end-stage renal failure due to non-compliance.[80] This patient's post-transplant biopsy showed signs of AMR with C4d deposition. An anti-Le^b antibody was also detected in the patient's plasma, which was still measurable two years after transplant.[80] In these cases anti-Le antibodies were suspected to have contributed to AMR; there were no detectible DSAs, and crossmatches were compatible.[80]

In a study assessing anti-Le antibodies in the sera of kidney transplant patients (sample size (*n*)=36) using hemagglutination testing and enzyme-linked immunosorbent assay (ELISA), anti-Le antibodies were detected in all eight Le-null recipients who received Le-incompatible grafts.[86] All eight incompatible grafts were rejected, but the graft in the one compatible Le-null recipient was not rejected and no anti-Le antibody was created.[86] In this study of the 12 patients with anti-Le antibodies detected by ELISA, only 4 had anti-Le antibodies detected by hemagglutination.[86] This suggests that hemagglutination methods may be too insensitive to reliably detect anti-Le antibodies, and the study is evidence that anti-Le antibodies may be produced by an immune response to Le antigens on renal epithelial cells.[86]

Retrospective studies that have looked at the correlation between Le phenotype and HLA-haplotype show conflicting evidence. It is not clear if HLA-haplotype mismatch plays a direct role in Le-null patients being at an increased risk for kidney rejection.[78] In a study of 255 first-time kidney transplant recipients, transplants that had both HLA and Le compatibility demonstrated significantly higher survival rates.[87] However, in a study of 161 kidney transplant patients, HLA matching had no statistical impact on transplant outcomes, regardless of the recipient's Le phenotype.[88]

The overall influence of Le-system compatibility on kidney transplantation has not been completely identified despite numerous investigative studies, but the balance of evidence suggests that compatibility of both HLA (mainly HLA-A, B, and DR) and Le yield the best allograft results based on one-year survival rates.[78] Even though Le system has a minor impact on transplant incompatibility overall, it should still be considered important, especially if Le antibodies are detected pre- and post-transplant, and when coupled with other incompatibilities such as HLAs.[82] Anti-Le antibodies are less deleterious than ABO and HLA antibodies, but they should be considered relevant to transplantation.[78] It is estimated that approximately 95% of Le-null patients receive a Le positive graft, and therefore, anti-Le antibodies should also be considered in the re-transplant of patients with pre-existing Le-system antibodies.[78,80,83] Upon second transplant, patients with pre-existing anti-Le antibodies (presumably produced from the first allograft) had their one-year survival rate cut almost in half compared to those without pre-existing anti-Le antibodies.[83] Anti-Le antibodies that are present both before and after transplant seem to be responsible for the recurrence and treatment resistance of kidney allograft dysfunction, and they may injure these allografts via AMR.[80]

5.5. Duffy System

The commonly encountered antigens of the Duffy (Fy) blood group system, ISBT number 008, are the antithetical pair Fy^a and Fy^b.[20,43] Both antigens are well-developed at birth (approximately 6,000-13,000 antigen sites per RBC),[20,44] and are located on a multipass glycoprotein (with AA 42 being glycine for Fy^a and asparagine for Fy^b).[9] During routine hemagglutination testing, the Fy antigens are destroyed by common blood bank testing enzymes, apart from trypsin.[9,20] The Fy antibodies are usually IgG1; they demonstrate dosage and rarely bind complement, though they have been associated with both acute and delayed HTRs.[9,20] Anti-Fy^a antibodies are somewhat common and twenty times as common as anti-Fy^b antibodies, which usually occurs in conjunction with other antibodies.[9,20,44]

The null phenotype for the Fy-antigen system Fy(a-b-) is found in 68% of Blacks and the RBCs associated with this null phenotype are shown to withstand infection by *Plasmodium vivax* merozoites, an organisms that causes malaria in humans.[9,20] RBCs that do not express Fy antigens are of benefit in areas where *P. vivax* is endemic, which is an explanation for why this phenotype is common in persons from Asia and West Africa.[9,20] Individuals with the null phenotype do not express Fy^a or Fy^b on their RBCs due to an erythroid-specific GATA-1 (promoter region) mutation,[9,20,44,89] but these individuals still express the Fy^b antigen on other tissues and will not create anti-Fy^b antibodies if exposed to the Fy^b antigen. [9,20,44,89]

The Fy antigens are not found on granulocytes, lymphocytes, monocytes, or platelets,[44] but have been located on renal epithelial cells and endothelial cells of capillary and postcapillary venules, and in the brain, colon, lung alveoli, spleen, thyroid, and thymus.[9,20,44] Table 4 (page 47), at the end of this non-ABO histoblood group literature review, details the published locations of all non-ABO histoblood group antigen systems considered in this study. EST data shows expression in the heart, lung, kidney, and pancreas (Table 1), and the HPA documents detection of Fy antigens in the heart, kidney and pancreas (Table 2).[1,13]

The Fy antigens reside on glycoprotein D, which is a member of the immunoglobulin superfamily of chemokine receptors.[20,26] The Fy system is also known as the Duffy antigen receptor for chemokines (DARC), or more recently, atypical chemokine receptor 1 (ACKR1).[9] This transmembrane glycoprotein has an arrangement within the cell membrane that is characteristic of the G-protein-coupled superfamily of receptors.[9,26]

Chemokines are cytokines that function by attracting cells to the site of inflammation or infection,[20] and cytokines are soluble proteins or peptides that influence the immune response by binding to specific receptors on cells.[20] Cytokines can regulate differentiation, growth, and mobility of leukocytes. Cytokines include interleukins (IL), interferons, tumor necrosis factors (TNF), and colony stimulating factors.[20] Chemokines mediate cell migration; they bind to receptors on cells and play a role in pathogen destruction.[20] ACKR1 binds an assortment of proinflammatory

cytokines, but in contrast to classic G-coupled receptors, there is no signal associated with binding.[90,91] Therefore, ACKR1 is a chemokine-binding protein but not a chemokine receptor.[90] Despite what is known about the function of ACKR1 on RBCs, the function of ACKR1 on endothelial cells and other tissues is not completely understood.[9,20,91] Table 3 (page 44), at the end of this non-ABO histoblood group literature review, details the documented immunity-related function(s) of all non-ABO histoblood group antigen systems considered in this study.

ACKR1 is a promiscuous chemokine binding protein, that can bind both CXC chemokines (distinguished by a cysteine-AA-cysteine motif near the amino terminus) and CC chemokines (having two adjacent cysteines near the amino terminus); these chemokines have various functions within the immune system.[44] One suggested function of ACKR1 is aiding in the clearance of inflammatory mediators, including chemokines.[9] Studies of ACKR1 chemokine binding showed that CXCL8/IL-8 can bind to the Fy blood protein and be adsorbed to RBCs, thus decreasing measurable levels in the plasma.[92] RBCs have been shown to rapidly bind greater than (>) 80% of soluble IL-8 when introduced at concentrations within its biologically active range.[92] The sequestering of IL-8 by RBCs (acting like a “sink”) may limit neutrophil interaction with IL-8 in circulation, thus providing a line of defense against systemic neutrophil activation.[92] Studies have suggested that there are pro-inflammatory effects of Fy antigen expression on RBCs related to their ability to facilitate transcytosis and leukocyte migration, but these processes appear to be of limited importance considering the increased Black populations that lack the Fy antigens on their RBCs.[9,44,93]

In renal allograft rejection, Fy antigens may be considered minor histocompatibility antigens.[44,94] Using IHC to study the influence of ACKR1 on AMR, ACKR1 expression was identified on peritubular capillaries in areas of interstitial fibrosis and/or inflammation.[93] An upregulation of ACKR1 was noted in biopsy samples of suspected AMR, but there was also an abundance of ACKR1-positive biopsy samples that did not show signs of rejection.[93] This investigation revealed that upregulation of ACKR1 is not specific for AMR, but in that study

population, the incidence of AMR was higher in ACKR1-positive biopsies.[93] IHC performed in this study showed labeling in the peritubular capillaries and occasional signaling in small venules and arterioles.[93] The ACKR1 IHC results aligned well with the results of gene expression from microarray analysis.[93]

The function and action of ACKR1 during inflammation is still unknown, but ACKR1 upregulation may occur before tissue remodeling and fibrosis, making it a marker for more-severe alloimmune responses.[93] However, at this time the upregulation of ACKR1 has not been specifically associated with AMR or an overall worse graft outcome.[93] Studies of IHC labeling for ACKR1 in early rejection without fibrosis is required to determine the prognostic value of ACKR1 for allograft rejection.[93]

In the kidney, ACKR1 is expressed at sites where diapedesis is thought to occur.[91] ACKR1 expression on endothelial cells may bind chemokines that promote leukocyte adhesion and transendothelial migration of cells that are positive for CCR5 (a chemokine receptor on inflammatory cells that can induce chemotaxis) at the site of renal injury.[91] CCR5 positive leukocytes (i.e., T cells and macrophages) account for most interstitial infiltrates during transplant rejection.[91] IHC has been used to identify CCR5-positive infiltrating lymphocytes and ACKR1 expression in renal biopsy samples.[91] ACKR1 expression on normal renal tissues is low and is found to increase during acute transplant rejection.[91] In addition to an increase in ACKR1, CCR5-positive leukocytes were detected in tubules, endothelia, and glomeruli of samples representing acute transplant rejection.[91] Despite these findings, ACKR1 upregulation was still not found to be specific for allograft rejection.[91]

The influence of ACKR1 in kidney transplant rejection was investigated, and results showed that ACKR1-null patents had a decrease in allograft survival compared to ACKR1-positive patients,[95] leading investigators to the idea that ACKR1 may reduce the inflammatory effects of chemokines by inactivating them.[95] The Black population has the highest prevalence (68%) of Fy-null phenotypes, and research has focused on the influence of Fy-null phenotypes and renal

allograft outcomes. One theory is that Fy-null RBCs are unable to effectively remove chemokines from the allograft which can lead to increased exposure of chemokine-mediated injury and shortened allograft survival.[96] Blacks demonstrate higher rates of acute allograft failure, with a 30-40% decreased renal graft survival rate compared to non-Black patients.[95] It has also been reported that Fy-positive Black patients have better allograft function, leading to increased overall graft survival compared to Fy-null Black patients.[95,96]

Outside of the null phenotype, one case report highlighted the potential influence of anti-Fy^a antibodies on renal allograft dysfunction.[97] A patient with a history of anti-Fy^a antibodies received a deceased donor kidney (Fy phenotype unknown) after pre-transplant testing revealed a negative RBC antibody screen and the absence of HLA DSAs.[97] Complications arose during the first week post-transplant, and biopsies showed signs of acute rejection. One month post-transplant, testing for RBC antibodies was repeated and anti-Fy^a antibodies were identified in the recipient's plasma.[97] Post-transplant antibody screening did not identify any DSAs or HLA incompatibilities between the donor and recipient, and given the recipient's history of anti-Fy^a antibodies, all RBC transfusions had been with Fy^a-negative blood.[97] This case suggests the involvement of the donor allograft in producing an anamnestic response in the recipient, leading to graft dysfunction.[97]

Since ACKR1 has been identified as a "chemokine sink" and can influence an anti-inflammatory response, ACKR1 has been investigated for its role in transplantation.[95] The CXC chemokines – IL-8/CXCL8, CXCL1, CXCL4, CXCL7 – and the CC chemokines – MCP-1(monocyte chemotactic peptide)/CCL2 and RANTES (regulated on activation, normal T cell expressed and secreted)/CCL5 – all bind to ACKR1.[90] During allograft rejection, there is a localized increase in CCL2, CCL5, and CXCL8 chemokines, and in inflammatory cells with correlating receptors.[90]

The induction of ACKR1 can be evaluated in both cellular and humoral renal allograft rejection.[90] During cellular allograft rejection, the expression of ACKR1 on peritubular capillaries

increases, and during humoral graft rejection, ACKR1 is expressed at sites of C4d deposition.[90] C4d deposition is associated with decreased renal allograft survival and immunological graft loss, and since the detection of C4d in peritubular capillaries is a well-known indicator of acute humoral allograft rejection, ACKR1 is looked at as a secondary mediator of these effects due to its involvement in recruiting inflammatory cells.[90]

ACKR1 can regulate angiogenesis by clearing CXC chemokines from the tissue; evidence found that overexpression of ACKR1 on vascular endothelium of mice produced lower angiogenic responses to CXC chemokines.[95] Additional animal studies involving transfusion showed that mice that received ACKR1-null RBCs had increased neutrophil migration and microvascular permeability of the lungs, and increased cytokine production compared to mice that received ACKR1-positive RBC transfusions.[98] Increased pulmonary inflammation in these mice was induced by the reduction of RBC chemokine scavenging (found after increased storage time of RBCs), which could translate to increased lung inflammation in ACKR1-null patients.[98]

Despite the inability of ACKR1-null individuals to bind CXCL8, there are no developmental abnormalities associated with lacking this gene.[95] Individuals that lack ACKR1 on their RBCs have normal immune function, and ACKR1 does not produce any biological signal through chemokine binding. This is because ACKR1 lacks the necessary sequences found in G-protein coupled receptors that allow for a response to ligand-binding.[9,91] However, ACKR1 may play a role in leukocyte trafficking due to its increased presence on the endothelial cells that line the post-capillary venules of individuals with either the Fy-positive or Fy-null RBC phenotypes.[99] ACKR1 transports chemokines across endothelial cells, and interestingly, the retention of chemokines by ACKR1 does not lead to their degradation – it instead functions by means of immobilizing chemokines and influencing leukocyte trafficking in circulation.[99]

In addition to chemokine involvement, Fy blood group antigens are of interest for transplant immunology because of their expression on endothelial cells at sites of inflammatory cell recruitment, their increase on vessels during injury, and upregulation during AMR.[93]

5.6. Kidd System

The Kidd (Jk) antigen system, ISBT number 009,[43] is the product of a single, polymorphic gene encoding a transmembrane RBC urea transporter.[9,20,100] There are three antigens within the Jk blood group system, but only two of them are assessed for during routine blood bank testing, the antithetical antigens Jk^a and Jk^b.[20] The Jk antigens are well developed at birth (approximately 14,000 per RBC) and located on a multipass glycoprotein (Jk^a is defined by aspartic acid at AA 280, and Jk^b has asparagine at AA 280).[920,44]

The Jk antigen is identified as a RBC urea transporter[9,20,44] The rapid uptake of urea by this transporter prevents RBCs from shrinking after entering the hypertonic environment of the renal medulla.[26] To prevent swelling of RBCs, and the removal of urea from the kidney, urea is transported out of the RBCs as they leave the renal medulla.[26] The null phenotype of this blood group system is rare, and there are no associated clinical abnormalities with this phenotype, however, there may be a reduction in the ability to concentrate urine by about one-third.[9,20,44] Table 3 (page 44), at the end of this non-ABO histoblood group literature review, details the documented immunity-related function(s) of all non-ABO histoblood group antigen systems considered in this study.

Jk-system antibody titers fluctuate.[20,44] The antibodies commonly become undetectable in the weeks or months following alloexposure, and this rapid decline and associated failure to identify their presence can lead to delayed and sometimes severe HTRs.[9,20] Given that anti-Jk antibodies are characteristically difficult to detect during routine hemagglutination testing, these antibodies can be dangerous. Low levels of pre-formed anti-Jk antibodies that evade detection can provoke a strong anamnestic response with hyperacute AMR.[20,101]

Anti-Jk antibodies are usually IgG1 and IgG3 but sometimes include IgM; they are often found among other anti-RBC antibodies, and approximately 50% of them can bind complement.[9,20,44] Jk antigens are not denatured by typical blood bank enzymes, and their

reactivity is usually enhanced.[20,44] Compared to other non-ABO RBC antigens, Jk^a is rather immunogenic and is only exceeded in immunogenicity by the K and RhD antigens.[9]

Jk antigens are not found on platelets, lymphocytes, granulocytes, or monocytes, but they are found on renal endothelium and renal epithelial cells.[20,44] Table 4 (page 47), at the end of this non-ABO histoblood group literature review, details the documented locations of all non-ABO histoblood group antigens systems considered in this study. Jk antigens have been identified in the heart, lung, kidney, and pancreas using EST (Table 1).[1] The HPA documents reports of Jk antigen detection by IHC in the heart, lung, and kidney (Table 2).[13]

Given the role of Jk-expressing proteins as RBC urea transporters, and their location on both renal tubular epithelial cells and the endothelium of the vasa recta, transplant research involving the Jk blood group system has largely focused on mechanisms surrounding renal allografts.[44] A possible role for anti-Jk antibodies in renal allograft rejection is binding to their respective antigens on endothelial cells, thereby mediating destruction of the capillary endothelium.[102] Despite this theory, Jk-induced graft rejection is a poorly understood phenomenon. There is enough phenotypic variation among races that many donor and recipient pairs are likely mismatched at the Jk locus, but this does not explain why Jk incompatibility is pathological in some situations, but not in others.[101] Identification of a primary or secondary exposure in suspected cases of Jk-induced graft dysfunction is one key to understanding the pathophysiologic role for the Jk system in transplantation.[102]

A retrospective study of kidney transplant patients with a seven-year follow-up showed that Jk-incompatible deceased-donor transplants (37% of the cohort) had more interstitial inflammation compared to Jk-compatible transplants.[94] However, the lack of C4d deposition and pre-transplant anti-Jk antibodies in this study suggests that Jk incompatibility has little influence on kidney allograft survival.[94] But these data could also point to an inability to determine the influence of Jk antibodies on renal allografts since anti-Jk antibodies can sometimes be undetectable until after an anamnestic response.[94]

A case involving an anamnestic response following a living-donor kidney transplant between siblings ended in acute graft failure and a positive direct antiglobulin test (DAT) (IgG+C3d) around day 12 with the formation of anti-Jk^a antibodies.[103] The recipient was Jk^a negative and had no detectible RBC antibodies prior to transplant, but the anti-Jk^a antibodies included both IgG and IgM, which suggests a secondary response.[103] This hypothesis was supported when it was found that the recipient had received a paternal Jk^a-positive RBC transfusion years earlier.[103] Post-transplant transfusion and the Jk^a-positive donor kidney could have stimulated a secondary response in the recipient, leading to graft failure.[103]

Secondary exposure was also noted in a kidney transplant patient with known anti-Jk^a antibodies prior to transplant.[101] The Jk phenotype of the first donor organ was unknown prior to transplant, but the graft was quickly rejected, and a second deceased donor transplant was confirmed as Jk^a positive after post-transplant complications arose.[101] The anti-Jk^a antibodies were suspected to be DSAs that led to C4d-negative hyperacute rejection.[101] The Jk phenotypes of the first and second donors were eventually identified as homozygous Jk(a+) and heterozygous Jk(a+b-).[101]

Lastly, a case report regarding a liver transplant documented the formation of anti-Jk^b antibodies post-transplant in a patient who had received only one autologous unit of blood and one random unit of apheresis platelets.[104] This patient had no history of alloimmunization (apart from previous pregnancy) prior to transplant, yet the developed alloantibodies matched the phenotypes of both the allograft and the platelet donor.[104] The RBC content of platelet concentrates is very low, but it has been suggested that highly immunogenic RBC-derived microparticles may initiate or boost immune responses, thus posing the question of the antigen dose required to mount an immune response.[104] This case highlights the importance of patient history for the determination of Jk-antigen exposure, and touches on the inability to establish RBC antibody production as primary or secondary in the case of immunization from pregnancy.[104] What also makes this case unique for the identification of post-transplant anti-Jk antibodies is that only

autologous blood was used, whereas most liver transplants require copious RBC transfusions, and rarely is any of the blood autologous.[104]

Overall, the JK antigen system has left many questions regarding its impact on allograft survival, and more information is needed to uncover any definitive answers. Unfortunately, minor histocompatibility antigens such as Jk^a and Jk^b are not assessed in the preliminary testing for allograft compatibility.[101]

5.7. XG System

The two antigens of the XG blood group system, ISBT number 012,[43] are Xg^a and CD99 (formerly 12E7).[9] The Xg^a antigen (encoded by the *XG* gene) and the CD99 antigen (encoded by the *CD99* gene, formally *MIC2*) are not antithetical, but the expression of both antigens (Xg(a+), Xg(a-) and CD99-Low, CD99-High) depends on the *XG* gene, presenting a unique phenotypic relationship between these antigens.[9,105,106] The *CD99* gene is located on the short arm pseudoautosomal regions of the X and Y chromosomes, as are exons 1-3 of the *XG* gene, while exons 4-10 of *XG* are located exclusively on the X chromosome.[9,105] The relationship between Xg^a and CD99 is believed to be from transcriptional coregulation of the *XG* and *CD99* genes.[106]

The XG system was established in 1962, and the naming of XG came from it being "X-linked" and discovered in Grand Rapids ("G"), Michigan.[20,44] The Xg^a antigen is polymorphic and has a higher prevalence in females (89%) than in males (66%).[9,20] Females that are Xg(a+) have high expression of CD99 (CD99H) on their RBCs whereas Xg(a-) females have low expression of CD99 (CD99L) on their RBCs.[20] The phenotype for males differs; 68% of Xg(a-) males express CD99H on their RBCs, and 32% express CD99L.[20,106]

The Xg^a antigen is on a single-pass, type I membrane glycoprotein (up to 9,000 antigen sites per RBC), and is sensitive to routine blood bank enzyme treatment.[20,44] The Xg^a antigen is a poor immunogen, and individuals that possess the Xg^a-negative phenotype typically do not produce anti-Xg^a if exposed to the antigen.[107] Anti-Xg^a antibodies are rare and mostly found in men.[107] They are usually IgG and are not clinically significant in transfusion medicine.[9,20,44] CD99

is also associated with a single-pass, type I membrane glycoprotein, but little is known about the function of the CD99 antigen on RBCs.[9,44]

Unrelated to RBCs, the CD99 antigen is involved in critical biological processes such as inflammation, immune responses, apoptosis, cancer metastasis, leukocyte migration, T cell adhesion, and transmembrane protein transport.[44,107,108] It is essential for immune cell infiltration; monoclonal antibodies blocking CD99 can halt immune cell migration within the endothelium.[109] Cleavage of CD99 by the metalloprotease meprin-β increases immune cell migration in animal models.[107,109] Currently, more studies are needed to understand the impact of CD99 on human disease, and little is known about the role of Xg^a in health and disease.[107,109] Table 3 (page 44), at the end of this non-ABO histoblood group literature review, details the documented immunity-related function(s) of all non-ABO histoblood group antigen systems considered in this study.

Xg^a mRNA transcripts have been detected, using Northern Blot analysis, in the thymus, bone marrow, liver (fetal), heart, placenta, skeletal muscle, prostate, thyroid, spinal cord, and trachea.[44] The CD99 antigen is ubiquitously expressed at low levels on almost all human cell types,[108] but is most notably expressed on lymphocytes and platelets.[44] Higher expression of the CD99 antigen also occurs in the liver (fetal), spleen, lymph nodes, thymus, adrenal gland (fetal), bone marrow, and on fibroblasts, pancreatic islet cells, ovarian granulosa cells, and Sertoli cells.[44,108] Table 4 (page 47), at the end of this non-ABO histoblood group literature review, details the documented location of all non-ABO histoblood group antigen systems considered in this study. EST data show that XG system antigens are present in the heart and pancreas (Table 1).[1] While Xg^a expression was thought to be restricted to RBCs, the Xg^a protein has been detected elsewhere; it was detected by IHC in the myometrium and seminal vesicles,[107] and the HPA documents CD99 expression in the lung, pancreas, and kidney (Table 2).[13]

5.8. Chido/Rodgers System

The Chido/Rodgers (CH/RG) antigens, ISBT number 017,[43] are considered blood group antigens, but they are not made by RBCs.[9] Like the Lewis system, these antigens are not intrinsic

to the RBC membrane. The CH/RG antigens are on the fourth component of complement, C4, and is adsorbed to the RBC membrane from plasma.[9,20,44] The C4 protein consists of two isoforms, C4A (Rodgers) and C4B (Chido), which are located on the MHC class III region of chromosome 6.[110] Both C4 isoforms are glycoproteins; C4A preferentially binds to protein and C4B preferentially binds to carbohydrates.[44,110] C4A is active in immune regulation through solubilizing immune complexes, while C4B has greater specificity for binding to the RBC surface, making it more effective at promoting hemolysis through complement activation.[44,110] Table 3 (page 44), at the end of this non-ABO histoblood group literature review, details the documented immunity-related function(s) of all non-ABO histoblood group antigen systems considered in this study.

The antigens of the CH/RG system are all high prevalence (>90%),[9,20] and most RBCs have trace amounts of C4 on their surface.[9] During routine blood-bank testing, CH/RG antigens can be destroyed by proteolytic enzymes,[9] and they demonstrate variation in reaction strength among different RBC samples, thus impacting their detection.[20] The anti-CH/RG antibodies can directly agglutinate RBCs that have been artificially coated with C4d, and they can be neutralized (bound) by CH/RG-positive plasma.[9] Anti-CH/RG antibodies are usually IgG and are clinically insignificant for transfusions.[9,20]

The CH/RG antigens are soluble and bind to the RBC membrane.[9,44] Table 4 (page 47), at the end of this non-ABO histoblood group literature review, details the documented locations of all non-ABO histoblood group antigen systems considered in this study. This blood group system has not been evaluated by EST (Table 1);[1] IHC has documented expression of CH/RG in the heart, lung, and kidney (Table 2).[13]

The deposition of C4d, a tryptic fragment of both C4A and C4B, can be found throughout various body tissues, signifying the involvement of the classical or lectin complement pathways.[44,110] Given this, C4d is also a common indicator for AMR.[44,110] The copy number of C4A genes varies from zero to five, and the number of C4B genes varies from zero to four.[110] Inherited low-levels of C4 gene copy numbers (either C4A or C4B) may influence the development of

insulin-dependent diabetes and autoimmune chronic hepatitis.[44] A lack of C4A is associated with a predisposition to systemic lupus erythematosus (SLE), and a lack of C4B can predispose children to bacterial meningitis.[44]

The relationship between C4 serum concentrations and C4 genetic variation may also impact renal allograft rejection.[110] In one study, kidney transplant patients who had fewer than four total copies of C4 genes had better allograft survival after five years when transplanted with a deceased donor kidney than when transplanted with living donor kidneys.[110] The clinical impact of complement activation in the graft can be influenced by the degree of ischemia and microenvironment of the transplanted organ.[110] Given the role of C4A in immune regulation, its absence has greater clinical significance in allograft outcome compared to C4B.[110] Overall, patients with partial C4 deficiency, and therefore lower C4 expression (particularly of C4A), may have an advantage for increased graft survival after deceased donor kidney transplantation.[110]

5.9. Knops System

The Knops (Kn) antigen, ISBT number 022,[43] is located on complement receptor 1 (CR1), a complement-regulatory glycoprotein, and has around 90% prevalence in most populations.[9,20,44] The Kn antigen has around 20-1,500 sites per RBC;[44] expression is weakened during RBC storage, reactivity between RBC samples is variable.[20] This inconsistent reactivity is due to the varied expression of CR1 on RBCs from person-to-person.[20,44]

During routine blood bank testing, Kn antigens are resistant to ficin and papain, but are damaged or destroyed by trypsin, AET, and DTT; this can be useful for identification of multiple RBC antibodies.[20] Adsorption and elution of antibodies of this system are challenging, and are not neutralized by normal pooled serum.[20] Kn antibodies are usually IgG and are considered clinically insignificant for transfusions.[20]

The Kn antigen has been identified on granulocytes, B lymphocytes, T lymphocytes, monocytes, macrophages, neutrophils, eosinophils, glomerular podocytes, and follicular dendritic cells, and in the spleen, lymph nodes, and peripheral nerve fibers.[44] Table 4 (page 47), at the end

of this non-ABO histoblood group literature review, details the documented locations of all non-ABO histoblood group antigen systems considered in this study. EST data show the presence of Kn antigens in the heart, lung, kidney, and pancreas (Table 1),[1] and the HPA documents detection by IHC in the lung, kidney, and pancreas (Table 2).[13] Table 3 (page 44), at the end of this non-ABO histoblood group literature review, details the documented immunity-related function(s) of all non-ABO histoblood group antigen systems considered in this study.

RBC CR1 is closely studied for its influence on autoimmune disorders.[111] CR1 protects RBCs from autohemolysis by binding C3b and C4b, producing an inhibitory effect on the classical pathway C3 (C4b2a) and C5 (C4b2a3b) convertases, and on the alternate pathway C3 (C3bBb) and C5 (C3bBb3b) convertases.[44,111] Particles and immunoglobulins coated with C3b and C4b produce complexes that are bound by CR1 on RBCs and transported to the liver and spleen for subsequent removal from the circulation.[20,44] This process mediates phagocytosis of RBCs by neutrophils and monocytes and protects against complement-mediated tissue damage.[44,111]

The CR1 protein has been studied for its role in tissue damage, resulting from immune complex deposition and complement activation.[111] Studies have shown that CR1 in the bone marrow helps maintain B cell tolerance, given this, the complement-regulatory functions of CR1 may help lessen autoimmune tissue destruction.[111]

Disease processes that involve the Kn antigen system usually result in a decrease in expression of CR1 and therefore of Kn antigens; this is most noted in autoimmune diseases.[44] Kn antigens are depressed in conditions involving the removal of immune complexes; these include SLE, hemolytic anemia, diabetes mellitus, acquired immunodeficiency syndrome, and some malignant tumors.[44] Decreased expression of CR1 on RBCs may cause the accumulation of immune complexes on the walls of blood vessels, ultimately leading to vascular damage.[44]

Soluble CR1, formed by removal of the transmembrane portion of the CR1 protein,[112] has been studied for its anti-inflammatory and complement regulatory properties.[111] Ischemia-reperfusion injury is an important problem after lung transfusion, occurring in 10-20% of patients,

and soluble CR1 (TP-10) has been used to ameliorate this injury and improve graft outcomes.[112] Clinical trials involving soluble CR1 have also been formed to treat acute respiratory distress syndrome and to decrease tissue injury after myocardial infarction.[113] Animal models have shown that soluble CR1 (TP-10) prevents complement-mediated tissue injury.[113]

5.10. Overview of Non-ABO Histoblood Group Antigen System Functions

Table 3. Overview of Select Histoblood Group Antigen Function, Immune Processes

ISBT Number	Blood Group System		CD Marker/Gene	Immune Function
002	MNS	MN	CD235a/*GYPA*	Can activate complement (M, S, s). GYPA may function as a regulator of complement by inhibiting the formation of the membrane attack complex.
		Ss	CD235b/*GYPB*	
004	Rh (RH)	RhD	CD240D/*RHD*	No documented immune system involvement.
		RhCE	CD240CE/*RHCE*	
006	Kell (KEL)		CD238/*KEL*	May play a role in vascular tone. Enzymatic cleavage of big-endothelin-3 to endothelin-3 (biologically active vasoconstrictor).
007	Lewis (LE)		*FUT3*	Can activate complement.

Adapted from: Cohn CS, et al. Technical Manual. AABB (2023)[9]; Harmening DM, Modern Blood Banking & Transfusion Practices (2018)[20]; Daniels G, Human Blood Groups (2013)[26]; Reid ME, et al. The Blood Group Antigen Facts Book (2012)[44]; Darbonne WC, et al. doi: 10.1172/jci115442.[92]; Kläger J, et al. (2021) doi: 10.1111/tri.13904.[93]; Hamilton MS, et al. (2008) doi: 10.1111/j.1399-3046.2008.00954.x.[102]; Lee YQ, et al. (2020) PMID: 32324038[107]; Pasello M, et al. (2018) doi: 10.1007/s12079-017-0445-z.[108]; Bay JT, et al. (2013) doi: 10.1038/ki.2013.195[110]; Pernick, N (2023) https://www.pathologyoutlines.com/stains.html[114]

Table 3. (cont'd)

ISBT Number	Blood Group System		CD Marker/Gene	Immune Function
008	Duffy (FY)		CD234/*ACKR1*	Can activate complement. Proinflammatory chemokine receptor. Plays a role in inflammation and the immune response through immune cell migration (neutrophils), clearance of inflammatory mediators, and the removal of unwanted chemokines (CXCL8/IL-8).
009	Kidd (JK)		*SLC14A1*	Can activate complement. May induce renal allograft rejection by destruction of the capillary endothelium after anti-Jk antibody binding to antigen on endothelial cells.
012	XG		*XG/CD99(MIC2)*	(Xg^a) no documented immune function. (CD99) Role in inflammation and the immune response through immune cell migration, apoptosis of T cells via caspase independent pathway.
017	Chido/ Rodgers (CH/RG)	RG	*C4A*	Complement regulator. Clearance of immune complexes (C4A) and promoting hemolysis through complement activation (C4B).
		CH	*C4B*	
022	Knops (KN)		CD35/*CR1*	Complement regulator (inhibition of classical and alternative pathway), clearance of immune complexes, mediator of phagocytosis, inhibitor of hemolysis and tissue damage. Activation of B lymphocytes.

Based on the findings from the non-ABO histoblood group literature review, the histoblood group antigens selected for this study (apart from the Rh system) have documented functions that may influence the immune system. These functions may affect the inflammatory processes that impact allograft rejection.

5.11. Overview of Non-ABO Histoblood Group Antigen System Locations

Table 4. Overview of Select Histoblood Group Antigen Location

ISBT Number	Blood Group System		CD Marker/Gene	Location *
002	MNS	MN	CD235a/*GYPA*	Renal endothelium and epithelium.
		Ss	CD235b/*GYPB*	
004	Rh (RH)	RhD	CD240D/*RHD*	Erythroid cells.
		RhCE	CD240CE/*RHCE*	
006	Kell (KEL)		CD238/*KEL*	(KEL) Brain, heart, colon, myeloid progenitor cells (maybe), skeletal muscles, fetal liver, testes, lymphoid organs (including spleen and bone marrow). (Xk) Brain, lymphoid organs, heart, pancreas, placenta, and skeletal muscle.
007	Lewis (LE)		*FUT3*	Soluble glycoprotein is found in all bodily fluids except CSF, glycolipid form is adsorbed onto RBCs from plasma. Cell surfaces of the renal parenchyma, pancreas, skeletal muscle, adrenal glands, on platelets, lymphocytes and monocytes, endothelium, and on the genitourinary and gastrointestinal epithelium.
008	Duffy (FY)		CD234/*ACKR1*	Endothelial cells of capillary and post-capillary venules, epithelial cells of kidney, lung alveoli, Purkinje cells of the cerebellum, colon, spleen, thyroid, and thymus.

*These data do not include EST (Table 1) or HPA IHC (Table 2) information.
Adapted from: Cohn CS, et al. Technical Manual. AABB (2023)[9]; Nydegger UE, et al. (2005) doi: 10.1196/annals.1313.006[10]; Harmening DM, Modern Blood Banking & Transfusion Practices (2018)[20]; Reid ME, et al. The Blood Group Antigen Facts Book (2012)[44], Russo D, et al (2018) PubMed PMID: 10891471[71]; Camara-Clayette V, et al (2001) doi: 10.1042/0264-6021:3560171[72]; Pasello M, et al (2018) doi: 10.1007/s12079-017-0445-z[108]; Pernick, N (2023) https://www.pathologyoutlines.com/stains.html[114]

Table 4. (cont'd)

ISBT Number	Blood Group System		CD Marker/Gene	Location
009	Kidd (JK)		*SLC14A1*	Renal endothelium of the vasa recta (medulla), renal tubular epithelial cells.
012	XG		*XG/CD99(MIC2)*	(Xg^a) Erythroid cells. (CD99) Endothelial cells, ependymal cells, lymphocytes, platelets, thymus, bone marrow, fetal liver, heart, placenta, skeletal muscle, prostate, thyroid, spinal cord, trachea, fibroblasts, lymph nodes, spleen, pancreatic islet cells, ovarian granulosa cells, testicular Sertoli cells.
017	Chido/ Rodgers (CH/RG)	RG	*C4A*	No solid tissue histological localization. Soluble glycoprotein (C4) found in plasma, synthesized primarily by liver monocytes/macrophages.
		CH	*C4B*	
022	Knops (KN)		CD35/*CR1*	Granulocytes, B cells, T cells (10%), NK cells (some), monocytes, macrophages (some), neutrophils, eosinophils, basophils, glomerular podocytes, peripheral nerve fibers, follicular dendritic cells in spleen and lymph nodes.

6. Deceased Organ Donors

Organ procurement is the only hope for thousands of patients waiting for life-saving transplant procedures, and there are approximately 120,000 individuals waiting for organ transplantation in the United States at any given time.[16] The primary sources of transplanted organs are deceased donors due to brain death (DBD) and deceased donors due to cardiac death (DCD), but there are institutional, personal, and societal barriers to organ procurement.[16] The overall process of organ donation is highly structured, complex, and includes both public and healthcare education[16] with clinical expertise and logistical factors for organ recovery, distribution, and transplantation.[16]

Around the world, the supply of available organs does not meet the demand.[115] At present, little weight given to the evaluation of donor risk factors and the role of donor management before organ retrieval.[15] The main goal for deceased organ donor management is to find a universal, systematic approach to facilitate and optimize the increased need for donation.[116] To do this, there needs to be proper management of the donor from the time of death to the time of surgical recovery of organs.[115]

Donor organs need to be kept under optimal physiologic conditions until the time of retrieval.[17] Unfortunately, many countries that lack suitable procedures for the management of potential organ donors.[17] Communication throughout the donation process is critical to the success of donor management, and time is also of the essence. Coordinating with the local organ procurement organization (OPO) regarding the needs of potential organ donors must occur in a timely fashion.[16]

The duration between authorization and the donor's transport to the operating room can be up to 24-36 hours.[16] Given this waiting period, deceased donor management protocols allow for the protection of organs prior to donation.[16] Donor management protocols include correcting hypovolemia, maintaining organ perfusion, treating diabetes insipidus, providing corticosteroids, and administering lung ventilation as means of protection.[15]

Research has focused on how donor management impacts the quality of potential allografts. Understanding the fragility of deceased donors may help alert clinicians to procure organs sooner, or provide a more realistic timeline for medical intervention (to reduce damage to organs) prior to donation.[15]

Brain death involves permanent loss of all brain function, including metabolic, hemodynamic, and hormonal changes that result in a loss of 10-20% of donor tissue.[15,17] Increased attention to the impact that brain death has on organ donation, and developing appropriate donor management protocols can lead to increased viability of grafts and more organ donations.[15]

Organ procurement results in blood loss, hypothermia, hemodynamic instability, ischemic reperfusion injury, hypertension, and increased catecholamines.[15] Systemically, there is also complement activation, cytokine release, mitochondrial injury, and metabolic changes that have been associated with organ donation.[15] As mentioned in the histoblood group antigen review above, RBC antigens have identified functions, roles, and capabilities that can influence a systemic immune response. Histoblood group antigens that are capable of binding complement are M, S, s, Le, Fy, and Jk.[9,20,26] GYPA (MN antigens) may function as a complement regulator by inhibiting the formation of the membrane attack complex.[26] Complement is also regulated by both the Chido/Rodgers (C4B/C4A) and Knops (CR1) blood group systems through the clearance of immune complexes and promotion of hemolysis through complement activation.[44,110] Lastly, CD99 and ACKR1 (Fy) also play a role in inflammation and the immune response through effects on immune cell migration.[9,44,92,93,107,108]

The myriad pathophysiological problems related to organ donation after brain death include cardiovascular, respiratory, renal, endocrine, and systemic inflammatory responses.[15-17] Table 5 highlights pathophysiological changes that can occur following brain death.[117]

Table 5. Pathophysiological Changes after Brain Death

Change	Cause	Prevalence
Hypothermia	Hypothalamic dysfunction, vasoplegia	100%
Hypotension	Vasoplegia, hypovolemia, myocardial dysfunction	80-97%
Diabetes insipidus	Hypothalamic/pituitary dysfunction	65-90%
Arrhythmias	Catecholamine release, myocardial injury	25-32%
Pulmonary edema	Injury to vascular endothelium	15-20%
Cardiac Arrest	Prolonged hypotension, arrhythmia	5-10%

Adapted from: Hahnenkamp K, et al. (2016) doi: 10.3238/arztebl.2016.0552.[117]

After brain death, hypothalamic-pituitary-adrenocortical regulation in the donor is disrupted.[17] This causes an increase of both proinflammatory and anti-inflammatory mediators by the damaged brain, which include IL-1, IL-6, IL-8, C-reactive protein, procalcitonin, and TNF-α.[15,17,117] Increases in IL-6 and IL-8 are associated with poor graft outcome.[15,17]

The cytokine storm that is induced by brain injury and brainstem compression leads to an increase in systemic and left atrial blood pressure, cardiac afterload, and pulmonary capillary pressure.[15] Peripheral vasoconstriction leads to organ ischemia, with metabolism changes from aerobic to anaerobic respiration.[118] Loss of sympathetic tone after brainstem infarction can lead to increased vasoplegia and hypotension, and this can lead to reduced blood flow to all solid organs in the donor.[15,17,118] Myocardial dysfunction has been reported in 40-50% of potential DBD.[15]

Changes in the endocrine system are also common after brain death. Brain death results in pituitary failures, and 80% of brain-dead donors lose function in their posterior pituitary, resulting in diabetes insipidus.[17,118] The loss of free water due to diabetes insipidus leads to electrolyte imbalance, hypovolemia, and decreased cardiac output.[17] Hyperglycemia is an endocrine change that influences the inflammatory response, endothelial dysfunction, and

oxidative stress.[119] Hyperglycemia needs to be properly managed because decreased insulin concentrations can cause cell damage that may affect both pancreatic and renal allografts.[15,17,119]

Respiratory changes after brain death mostly include neurogenic pulmonary edema and inflammation due to ventilator-induced acute lung injury.[15,17] Neuropulmonary edema is related to a catecholamine storm (a dramatic increase in epinephrine (adrenaline) and norepinephrine brought on by increasing intracranial pressure after brain death);[120] leading to widespread vasoconstriction and an increase in systolic pressure, pulmonary edema and structural damage to capillary endothelium.[15,17] As mentioned earlier, endothelium is an important defense barrier for transplantation because it is the primary site of contact between donor and recipient circulation.[3,29] Endothelial cells also express antigens that are not present on lymphocytes, making these cells a primary target for non-HLA antibodies.[6] Donor management is very impactful for lung transplantation since these organs are typically the most unsuitable for transplant; only 10-20% of lungs become eligible.[17]

Changes to renal function between deceased and living donors is variable. One study using IHC found an increase in E-selectin and interstitial leukocyte invasion in deceased donor biopsies compared to living donor biopsies, indicating an early stage of inflammation prior to transplant.[121] DBD also produced a stress response that upregulated heat shock proteins, though the protective mechanisms of these proteins may not be as effective in deceased donors compared to living donors.[121] Animal studies have also found that organs from living donors had superior graft outcomes compared to both DBD and DCD grafts.[122] Though the incidence and severity of acute rejection was comparable for both DCD and DBD kidneys, this study indicated that DCD kidneys had a 73% probability of delayed graft function and DBD had a 27%.[122]

There is reduced survival of grafts from deceased donors compared to those from living donors, and this may be due to the increased inflammatory response produced after death.[15] The proinflammatory environment in deceased donors leads to the classical activation of complement, which is related to the acute rejection of kidney transplants from both DBD and DCD.[15] Living

donors are expected to be healthy during procurement, and their kidneys are subjected to very little warm and cold ischemia times prior to transplant; these factors contribute to the increased survival rates seen among living donor transplants compared to deceased donor transplants.[17,121,122]

7. Banff Criteria for Antibody-Mediated Rejection

The criteria for AMR for the solid organs investigated in this study are defined by the Banff Foundation for Allograft Pathology (Banff) (www.banfffoundation.org). Banff has become the chief classification system worldwide for allograft pathology, and is a major force in setting standards in transplant pathology.[123,124] The first consensus meeting on renal allograft pathology was held in Banff, Canada in 1991 and since then, the continuation of bi-annual meetings and expert working groups have strengthened the evidence and guidelines for the clinical diagnosis of graft failure.[123] The clinical validity in Banff criteria began after 1995, when criteria was expanded to other organs beyond the kidney.[124] The main goal of Banff is to set global standards in pathology, but there are limitations to this endeavor.[124] Global pathological classifications present limitations correlating to sampling error, reproducibility issues, and a lack of standard approach to morphometry and molecular testing.[124] Despite limitations, Banff working groups still aim at uncovering the best approach for classifying graft dysfunction and rejection. Banff working groups specific for kidney, pancreas, heart, and lung allografts have been formed, and the details highlighting antibody-mediated rejection will be described in the following text, with a synopsis in Table 6 (page 58).

The first Banff classification meeting focused on kidney allografts, and therefore, more comprehensive guidelines have been established for this organ compared to others. With the 2013 Banff meeting report came strong support for the diagnosis of AMR with negative or minimal C4d deposition within peritubular capillaries.[125] Accepting the diagnosis of C4d positive/negative AMR allowed for a better classification of either acute active or chronic active AMR.[125] The 2013

classification of C4d-negative AMR led to a higher sensitivity for the diagnosis of AMR, and improved graft outcomes.[5]

An additional recommendation from the 2015 Banff meeting was that prompt DSA testing should be done when biopsies meet the histologic criteria for AMR.[126] The 2015 meeting report highlighted a knowledge gap in the comparison of biopsies with suspected AMR from patients with HLA versus non-HLA antibodies,[126] and in identification of the molecular and histologic characteristics for non-HLA AMR.[126]

The 2017 Banff meeting highlighted some of the shortcomings of DSA testing and looked for possible alternative criteria in the absence of detectable DSAs for the diagnosis of AMR.[5] It was concluded that new criteria needed to be determined for AMR without detectable DSAs, and that the C4d-negative AMR classification from 2013 did not fit this situation, nor were either of these classifications considered “antibody-negative AMR”.[5] When DSA testing is not available to account for false-negative results, C4d positivity is now an alternative criteria for AMR.[5] At this time, there is recognition that present DSA testing methods do not detect all antibodies, including non-HLAs, that could lead to allograft failure.[5]

The guidelines for AMR vary by organ type. Pancreatic AMR is defined by both serological and immunohistopathological findings.[127] Recently, there has been an increase in the number of cases of pancreatic allograft dysfunction attributed to AMR.[127] Though the mechanisms of acute rejection compare with other solid organ transplants, AMR is still poorly characterized in pancreas transplants.[128] This disparity is presumed to be due to challenges integral to the graft itself.[128]

AMR may be unrecognizable in pancreatic biopsies when there is an absence of C4d labeling,[128] and suspicion of hyperacute rejection typically goes unrecognized due to early graft thrombosis that may not even be related to graft rejection.[127] In cases of well-functioning grafts with AMR, C4d labeling is typically absent, which is why microvascular inflammation/injury with concurrent detection of circulating DSAs can identify AMR independently of C4d labeling.[127]

Detailed histologic examination along with DSAs and C4d IHC are still foundational for the diagnosis of pancreatic AMR, and proper specimen collection is paramount.[127]

To diagnosis of pancreatic allograft rejection, needle core biopsies procure adequate tissue samples nearly 90% of the time.[128] The increase in quality biopsy samples yields broader recognition of pathological changes in pancreatic allografts, and better classification of allograft dysfunction.[128] Increased availability of acceptable biopsy material, and the increase in reported cases of AMR should lead to the development of reproducible, clinically relevant, and accepted grading criteria.[128] The development of standardized criteria for pancreatic AMR, including guidelines for the detection of non-HLA antibodies, should improve diagnostic accuracy and lead to an improved standard of care for pancreatic transplant patients.[128]

The consensus for heart and lung allograft outcomes is facilitated by the International Society for Heart and Lung Transplantation (ISHLT).[129] Currently, the long-term survival for lung allografts remains disappointing, with a median survival of six years.[129] Pulmonary AMR has become increasingly reported for allograft rejection, but the true incidence is unclear because the concept of pulmonary AMR has only been described in the literature within the last 10 years, and there are no published standardized criteria for diagnosis.[129]

The diagnosis of pulmonary AMR requires a multidisciplinary approach involving histopathologic changes, C4d labeling, and detection of DSAs.[125,129] Acute pulmonary AMR can present with a wide range of features and severity varying from asymptomatic with circulating DSAs to severe respiratory failure.[129] Difficulty in the diagnosis of AMR is because most findings show nonspecific patterns of acute lung injury.[125,129] Given these nonspecific patterns of injury, it was hoped that C4d labeling for pulmonary AMR would be as consistent as it is for renal and cardiac AMR, but the diagnostic sensitivity and specificity of C4d in the pulmonary allograft are much lower, and published results are conflicting.[129] Despite there not being formal, standardized criteria for pulmonary AMR (including guidelines for non-HLA antibody testing), early detection

and systematic monitoring of DSAs following transplantation, and reporting the relevance of C4d labeling for final diagnosis are recommended.[5,129]

The diagnosis and management of AMR in heart transplant recipients has evolved over the last two decades, but there is still more work that needs to be done to uncover diagnostic criteria for cardiac AMR.[129] Currently, the primary diagnostic tool for diagnosing cardiac AMR is the endomyocardial biopsy,[130] and the ISHLT grading system for cardiac AMR is constructed solely on evidence of pathology; grading does not require other indicators of clinical graft dysfunction and/or the detection of HLA or non-HLA DSAs.[130] Despite the lack of standardized criteria surrounding DSAs for cardiac patients, individuals with suspected cardiac AMR still undergo evaluation that includes testing for DSAs.[130] There is a lot of work still to do to unravel the effect of AMR on cardiac vasculature,[130] and the Banff cardiac working group is addressing a multidisciplinary approach to cardiac allograft outcomes by reviewing documented diagnostic criteria (clinical, serologic, and pathologic) of AMR from other transplant working groups and recording information that overlaps.[130] Utilizing overlapping diagnostic criteria allows for the development of standards to diagnose cardiac AMR.[130] Currently, molecular findings in kidney AMR, including C4d-negative AMR, are being assessed and findings from both kidney and liver AMR are being used to formally develop criteria for “chronic” cardiac AMR.[130]

Allograft rejection confirmed by histology is the cornerstone of transplant pathology.[5] The combination of histologic and serologic data provides an even better picture for proper diagnosis and treatment of allograft rejection. Banff strongly recommends DSA testing for all suspected cases of AMR where morphologic criteria have been met, but the 2013 and 2015 Banff classifications did not address what should happen when there are no detectable DSAs and the biopsy specimen meets all other requirements for AMR.[5] The 2017 Banff meeting addressed this by strongly advising that testing for non-HLA antibodies should be performed when DSAs are absent.[5] Even with this recommendation, Banff acknowledged that at this time, testing for non-HLA antibodies is not routinely performed, and the clinical effects of these antibodies are not

entirely understood.[5] Testing for non-HLA antibodies falls under the limitations of DSA testing highlighted in the 2017 meeting report; those limitations include variability in testing methods, diagnostic thresholds, and understanding of the clinical significance of antibodies.[5] These limitations hamper the ability of DSAs to be considered as a sole endpoint in the diagnosis of AMR.[5]

Table 6. Banff/ISHLT Criteria for Classifying Antibody-Mediated Rejection

Banff Classification of Antibody-Mediated Rejection in **Kidney** Allografts:
Active AMR
1. Histologic evidence of acute tissue injury*
2. Evidence of current/recent antibody interaction with vascular endothelium*
3. Serologic evidence of donor-specific antibodies (DSA to HLA or other antigens) *
C4d staining or expression of validated transcripts/classifiers as noted above in criterion 2 may substitute for DSA; however thorough DSA testing, including testing for non-HLA antibodies if HLA antibody testing is negative, is strongly advised whenever criteria 1 and 2 are met
Chronic Active AMR
1. Morphologic evidence of chronic tissue injury*
2. Identical to criterion 2 for active AMR*
3. Identical to criterion 3 for active AMR; including strong recommendation for DSA testing whenever criteria 1 and 2 are met*
Banff Classification of Antibody-Mediated Rejection in **Pancreas** Allografts:
Active AMR
1. Confirmed circulating donor-specific antibody
2. Morphological evidence of tissue injury
3. C4d positivity in interacinar capillaries
Chronic Active AMR
1. Identical criteria for acute AMR in combination with criteria for chronic allograft rejection/graft fibrosis**
2. No evidence of acute T cell-mediated rejection**
Banff/ISHLT Classification of Antibody-Mediated Rejection in **Heart** Allografts:
Current ISHLT grading system for cardiac AMR is based exclusively on pathologic evidence
Features of clinical graft dysfunction and/or presence of donor-specific HLA or non-HLA antibodies are not required^
Banff/ISHLT Classification of Antibody-Mediated Rejection in **Lung** Allografts:
Currently, there is no published or standard approach to classify pulmonary AMR regarding severity and management ^^

Full criteria not listed. Adapted from: *Haas M, et. al (2018) doi: 10.1111/ajt.14625[5]; **Drachenberg CB, et. al. (2011) doi: 10.1111/j.1600-6143.2011.03670.x[128]; ^Bruneval P, et. al. (2017) doi: 10.1111/ajt.14112[130]; ^^Roux A, et. al. (2019) doi: 10.1111/ajt.14990[129]; ^^Haas M, et. al. (2014) doi: 10.1111/ajt.12590[125]

MATERIALS AND METHODS

1. Experiment One: Unexpected RBC Antibodies in Transplant Patients

1.1. Aim

Test for the presence and specificity of commonly encountered, non-ABO RBC antibodies within the serum of transplant patients using standard hemagglutination screening and panel identification methods.

1.2. Introduction

RBC antibodies other than anti-A and anti-B are considered “unexpected” because these antibodies are created through alloexposure, typically via transfusion or pregnancy.[9] Incidence of alloimmunization is extremely variable among patient populations, as patients with recurrent transfusions (e.g., sickle cell disease) may present with alloantibodies 14-50% of the time, and the prevalence of alloantibodies in the general population can be as low as 0.5-1.5%.[9] Studies have shown that overall, approximately 2-5% of the transfused population develop RBC alloantibodies.[7,8] Unexpected RBC antibodies may be detected at lower rates in transplant patients compared to other patient populations due to immunosuppression, or transplant patients may present with unexpected RBC antibodies at similar or increased rates compared to the overall transfused population if these patients have been chronically transfused. Transplant patient being defined as someone who has already received a transplant, or is a candidate waiting for transplant. This study sought to determine if unexpected RBC antibodies in the transplant patient population are detected at the same frequency (2-5%) as the overall transfused population.[7,8]

Antibody identification depends on serum or plasma reacting with red cells of known phenotype (typically obtained from commercial suppliers), under optimal testing conditions.[9] The indirect antiglobulin test (IAT) is used to assess in-vitro reactions between RBCs and antibodies. This test is routinely used for antibody detection (screening), and antibody identification.[9]

Detecting the presence of unexpected RBC antibodies begins with a test that is called the antibody screen. The antibody screen utilizes either two or three commercially prepared reagent RBC samples of known antigen composition.[9] The FDA requires that all reagent RBCs collectively express all the following blood group antigens: D, C, c, E, e, M, N, S, s, P1, Le^a, Le^b, K, k, Fy^a, Fy^b, Jk^a, and Jk^b.[9] Additionally, RBCs selected as screening cells have more homozygous (double dose) antigen expression on each cell.[9] Figure 1 provides details of an antibody screen, as used in this study.

If an antibody screen is positive, the patient specimen is tested against an antibody identification panel. Antibody identification panels typically consist of 8-20 reagent RBC samples, which are commercially obtained, and each reagent RBC sample used for an antibody panel comes from a different donor with known antigen composition.[9] The reagent RBCs selected for an antibody identification panel allow for distinct patterns of positive and negative reactions to detect RBC antibodies.[9] RBC antibody identification panels must make it possible to confidently identify clinically significant antibodies.[9] Figure 2 provides an example of an antibody identification panel, as was used in this study.

System			Rh - Hr						Kell						Duffy		Kidd		Lewis		P1	MNS				Lutheran		Xg		Results				
Donor			D	C	c	E	e	C^W	K	k	Kp^a	Kp^b	Js^a	Js^b	Fy^a	Fy^b	Jk^a	Jk^b	Le^a	Le^b	P_1	M	N	S	s	Lu^a	Lu^b	Xg^a		IS	RT	37°C	AHG	v
1	R1R1	D1079	+	+	0	0	+	0	0	+	0	+	0	+	+	0	+	0	0	+	+	+	0	+	+	0	+	+	1					
2	R2R2	D6356	+	0	+	+	0	0	+	+	0	+	0	+	+	+	+	+	0	+	+	0	+	0	+	0	+	+	2					
3	rr	D4272	0	0	+	0	+	0	0	+	0	+	0	+	0	+	0	+	+	0	+	+	+	+	0	0	+	0	3					

Figure 1. Example of a Reagent RBC Antibody Screen Anagram

Donor identification is indicated with “D” followed by a unique identification number. Wiener haplotype information is provided next to the donor identification where R1R1 = DCe/Dce, R2R2 = DcE/DcE, rr = ce/ce. “+” indicates the presence of antigen, and “0“ indicates the absence of antigen on that donor cell. Testing reactions are recorded on the right side of the anagram, under their respective testing phases (i.e., Immediate Spin (IS), Room Temperature (RT), 37 °C, Anti-Human Globulin (AHG)). “√” indicates the use of Check Cells (Coombs control cells). Positive sample reactivity indicates the presence of an unexpected RBC antibody. Positive antibody screening samples are reflexed to the antibody identification panel.

System		Rh - Hr						Kell						Duffy		Kidd		Lewis		P1	MNS				Lutheran		Xg		Results				
Donor		D	C	c	E	e	C^W	K	k	Kp^a	Kp^b	Js^a	Js^b	Fy^a	Fy^b	Jk^a	Jk^b	Le^a	Le^b	P_1	M	N	S	s	Lu^a	Lu^b	Xg^a		IS	RT	37°C	AHG	√
1	R1wR1 D1080	+	+	0	0	+	+	0	+	0	+	0	+	0	+	+	+	+	0	+	0	+	0	+	0	+	+	**1**					
2	R1R1 D9985	+	+	0	0	+	0	+	+	0	+	0	+	+	+	+	0	0	+	+	+	+	0	+	0	+	+	**2**					
3	R1R1 D9516	+	+	0	0	+	0	0	+	0	+	0	+	+	+	0	+	0	+	+	+	0	+	0	0	+	+	**3**					
4	R1R1 D9233	+	+	0	0	+	0	0	+	0	+	0	+	+	0	+	+	0	+	0	0	+	0	+	0	+	0	+	0	**4**					
5	RzR1 D1408	+	+	0	+	+	0	0	+	0	+	0	+	0	+	0	+	0	+	+	+	0	+	+	0	+	0	**5**					
6	R2R2 D4607	+	0	+	+	0	0	0	+	0	+	0	+	+	0	+	+	+	0	+	+	+	+	+	0	+	+	**6**					
7	R2R2 D6562	+	0	+	+	0	0	0	+	0	+	0	+	0	+	+	0	0	+	+	+	0	+	+	0	+	0	**7**					
8	R2R2 D5674	+	0	+	+	0	0	+	0	0	+	0	+	0	+	0	+	0	+	+	+	0	+	+	0	+	+	**8**					
9	R2R2 D7082	+	0	+	+	0	0	0	+	0	+	0	+	+	+	+	+	0	+	0	0	+	0	+	0	+	+	**9**					
10	R1r D3349	+	+	+	0	+	0	0	+	0	+	0	+	0	0	+	+	0	+	+	+	+	0	0	0	+	+	**10**					
		D	C	c	E	e	C^W	K	k	Kp^a	Kp^b	Js^a	Js^b	Fya	Fyb	Jka	Jkb	Lea	Leb	P1	M	N	S	s	Lu^a	Lu^b	Xga						
11	r'r D1170	0	+	+	0	+	0	0	+	0	+	0	+	0	+	0	+	0	+	+	+	+	+	+	0	+	+	**11**					
12	r"r D9590	0	0	+	+	+	0	0	+	0	+	0	+	+	+	+	+	+	0	0	+	+	0	+	0	+	+	**12**					
13	rr D0821	0	0	+	0	+	0	+	+	0	+	0	+	0	+	+	0	0	+	+	+	0	0	+	0	+	0	**13**					
14	rr D1267	0	0	+	0	+	0	0	+	0	+	0	+	+	0	0	+	0	+	0	0	+	0	+	0	+	+	**14**					
15	rr D2539	0	0	+	0	+	0	0	+	0	+	0	+	0	+	+	0	+	0	+	+	0	0	+	0	+	+	**15**					
16	rr D1776	0	0	+	0	+	0	0	+	0	+	0	+	+	0	0	+	0	+	+	+	+	0	+	0	+	+	**16**					
17	rr D3565	0	0	+	0	+	0	0	+	0	+	0	+	+	+	+	0	0	+	+	+	0	+	0	0	+	+	**17**					
18	rr D2008	0	0	+	0	+	0	+	+	0	+	0	+	+	+	+	0	0	0	+	0	+	0	+	0	+	+	**18**					
19	rr D1990	0	0	+	0	+	0	0	+	0	+	0	+	+	0	+	+	+	0	+	0	+	+	+	0	+	+	**19**					
20	Ror D2609	+	0	+	0	+	0	0	+	0	+	0	+	+	0	+	0	+	0	+	+	+	0	+	0	+	0	**20**					

Figure 2. Example of a Reagent RBC Antibody Identification Panel Anagram

Donor identification is indicated with “D” followed by a unique identification number. Wiener haplotype information is provided next to the donor identification where R1R1 = DCe/Dce, R2R2 = DcE/DcE, rr = ce/ce. “+” indicates the presence of antigen, and “0“ indicates the absence of antigen on that donor cell. Testing reactions are recorded on the right side of the anagram, under their respective testing phases (i.e., Immediate Spin (IS), Room Temperature (RT), 37 °C, Anti-Human Globulin (AHG)). “√” indicates the use of Check Cells (Coombs control cells). Patterns of positive sample reactivity on the antibody identification panel (along with the antibody screen) can be correlated with antigen positivity (+) on donor cells, allowing for the identification of RBC antibodies.

1.3. Methods

1.3.1. Sample Collection

Frozen, archived serum samples of heart, lung, and kidney transplant patients that were previously submitted to the Michigan State University (MSU) Tissue Typing Laboratory for routine histocompatibility testing were screened to detect the presence of unexpected RBC antibodies. All samples were collected after study approval (STUDY00006233) from the MSU Biomedical and Health Institutional Review Board (IRB).

This cohort was not randomized, and samples were selected based on transfusion history. Only patients with documented transfusion history were selected for RBC antibody screening. To determine the likelihood of antibody production among transplant patients, sample size was determined using a calculation for one sample (transfused patients), with dichotomous outcome (antibody vs. no antibody).

$$n = p(1-p)\left(\frac{Z}{E}\right)^2$$

For this calculation, a z-score (*Z*) of 1.96 for a confidence interval (α) of 95%, desired margin of error (*E*) of 0.05, and proportion of success (*p*) of 0.03 were used.[131] RBC antibody prevalence data (proportion of success) was obtained from a previous study which found around 3% of the general transfused population presents with unexpected RBC antibodies,[8] which is within the 2-5% range that has been documented in the literature.[7] To confirm that the 95% confidence interval estimate of the proportion of transfused transplant patients making RBC antibodies is within 5% of the true proportion,[131] this study required 45 samples (*n*) from each transplant population (heart, lung, and kidney) for a total population (N) of 135 patients.

Serum samples collected from January 2021 through December 2021 provided 66 heart, 63 lung, and 51 kidney transplant patients for this investigation, giving 180 transplant patients. A random number generator (https://www.random.org/) was utilized to select 45 patients from each

transplant population for a total of *n*=135 patient samples to be tested in this study, in compliance with the statistical equations (above) and the IRB.

Serum from pancreatic transplant patients was not included in this aim of the study because these patients are not serviced by the MSU Tissue Typing laboratory; however, pancreas biopsy samples were utilized for the second aim of the study at the request of Gift of Life Michigan (GOLM) (Ann Arbor, MI).

The development of new antibodies and the monitoring of previously formed histoblood group antibodies through use of both pre- and post- transplant testing would be ideal, but this was outside the scope of this investigation.

1.3.2. Hemagglutination Testing

The serum of transplant patients was screened for unexpected RBC antibodies using the IAT tube agglutination method outlined in the Association for the Advancement of Blood & Biotherapies (AABB) Technical Manual, Method 3-3,[9] as well as any additional manufacturer's directions. The IAT demonstrates in-vitro reactions between RBC antigens and antibodies.[9]

Antibody screens were performed first by taking one 10x75 millimeter (mm) disposable, borosilicate glass tube (VWR International, Radnor, PA), and combining two drops (approximately 100 µL) of patient serum with one drop (approximately 50 µL) of commercially obtained reagent RBCs, prepared in a 2-4% red cell suspension (Panoscreen III, Immucor, Norcross, GA). The samples were immediately centrifuged (immediate spin) for 15 seconds, at 1000 times gravity (×g) (BD Sero-Fuge 2002; Becton Dickinson (BD), Franklin Lakes NJ) to observe for any possible agglutination and/or hemolysis. Figure 3 provides examples of reaction grading criteria. Next, the same samples were incubated at room temperature (20-24°C) for 15 minutes, and centrifuged for 15 seconds, at 1000 ×g to observe for agglutination and/or hemolysis. Testing at the immediate spin and room temperature phases allows for the detection of IgM class antibodies.

To begin the detection of IgG class (clinically significant) antibodies, two drops (approximately 100 µL) of low ionic strength saline (Gamma N-Hance) (Immucor, Norcross, GA)

were added to each sample that had previously undergone both the immediate spin, and room temperature phase testing, and samples were incubated at 37 °C for 15 minutes. After incubation, the samples were immediately centrifuged for 15 seconds, at 1000 ×g, and observed for agglutination and/or hemolysis. After interpreting and recording these 37 °C reaction grades after, the samples were washed three times for 60 seconds, at 1000 ×g per wash, with room temperature (20-24˚C) isotonic saline, (0.85% Sodium Chloride (NaCl) (w/v) (85 grams NaCl (Catalog # BP358-212) (Fisher Scientific, Waltham, MA) + 10 Liters (L) distilled water). Osmolarity testing was performed to confirm the reagent would not lyse RBCs during testing), and this was followed by the addition of two drops (approximately 100 µL) of polyspecific AHG (Ortho Clinical Diagnostics, Raritan, NJ), centrifugation for 15 seconds at 1000 ×g and observation for agglutination and/or hemolysis. After interpreting and recording reaction grades after the addition of AHG, samples that were found to have a negative reaction (no agglutination and/or hemolysis) were mixed with one drop (approximately 50 µL) of Coombs control cells (commercially prepared, pooled O RBCs coated with an IgG antibody, in a 4-6% cell suspension), also referred to as Check Cells (Ortho Clinical Diagnostics, Raritan, NJ), centrifuged for 15 seconds at 1000 ×g and assessed for agglutination to assure that the AHG reagent was functional and there were no false negative results due to improper testing. Lack of agglutination after the addition of Coombs control cells indicates an invalid test, and the procedure must be repeated.

The presence of agglutination and/or hemolysis with one or more screening cells after immediate spin, room temperature, 37 °C incubation, and/or the addition of AHG is a positive result.[9] Positive results denote the presence of antigen-antibody interaction between the antibodies in the patient's serum and antigens on commercially obtained RBCs. Positive screening results were reflexed to standard antibody identification practices using an antibody identification panel (Panocell-20, Immucor, Norcross, GA).

Antibody identification panel testing was also performed using the IAT tube agglutination method outlined in the AABB Technical Manual, Method 3-3,[9] along with additional manufacturer's

directions. The only modification from the screening procedure (see above) is that antibody identification panels utilize different commercially obtained donor RBCs.

One solid agglutinate, clear background.

Large separate agglutinates, clear background.

Medium sized agglutinates, clear background.

Small agglutinates, cloudy background.

No agglutinated RBCs are present. Homogeneous suspension of cells which flow, rather than "chip" from the cell button. There is a grainy, iridescent quality to negative reactions.

Figure 3. Hemagglutination Grading Reaction Examples; Tube Agglutination Method
After centrifugation, the packed RBCs within each 10x75 mm borosilicate glass tube are resuspended by gentle shaking and viewed using a tube agglutination viewer (not shown). Once all the centrifuged RBCs are no longer attached to the glass of the tube (completely resuspended), the strength of the reaction is graded on a 0-4+ scale using the above criteria, with “0” representing a negative reaction, and “4+” representing the strongest possible reaction.

1.4. Results

1.4.1. Antibody Screens

A total of seven patients (5%) had positive antibody screens. Of the positive results, one was from a heart patient (2%), and six were from kidney patients (13%). There were no lung patients (0%) with positive antibody screens (Table 7).

Table 7. Antibody Screen Results by Transplant Population

Antibody Screen	Heart	Lung	Kidney	Total (*n*=135)
Negative	44 (98%)	45 (100%)	39 (87%)	128 (95%)
Positive	1 (2%)	0	6 (13%)	7 (5%)

1.4.2. Antibody Identification Panels

Of the seven patients with positive antibody screens, the antibody identification panel identified six clinically significant antibodies (detection with the addition of AHG), and one nonspecific cold reacting antibody (panreactive detection at RT only) (Table 8). The heart transplant patient had an anti-Fy^{a} antibody, and four of the six kidney patients had an anti-K antibody, one of the kidney patients had an anti-Fy^{a} antibody, and the remaining kidney patient had a nonspecific cold reacting antibody.

Table 8. Antibody Identification Panel Results by Transplant Population

Antibody Identification	Heart	Lung	Kidney	Total (*n*=7)
Anti-K	0	Not Tested	4	4 (57%)
Anti-Fy^{a}	1	Not Tested	1	2 (29%)
Nonspecific Cold Antibody	0	Not Tested	1	1 (14%)

1.4.3. Patient Transplant Status and Documented Transfusion

The transplant status of each patient was assessed, and most of the samples tested in this study were collected post-transplant (Table 9). Of the patients who had positive antibody screens, four patient samples were collected pre-transplant; one heart, and three kidney, and three patient samples (all kidney) were collected post-transplant. Patients with positive antibody screens represent 24% of the pre-transplant patient samples, and 3% of the post-transplant patient samples.

Table 9. Transplant Status of Patient Samples by Transplant Population

Transplant Population	Heart	Lung	Kidney	Total (*n*=135)
Pre-Transplant Sample	12 (27%)	0	5 (11%)	17 (13%)
Post-Transplant Sample	33 (73%)	45 (100%)	40 (89%)	118 (87%)

The time between the last documented transfusion and sample collection was also evaluated. The longest duration between documented transfusion and sample collection was >120 months and most kidney patients fell into this category (18%), while the majority of both heart (40%) and lung (38%) patients fell within the shortest time duration of ≤10 months (data not shown). Of the patients with positive antibody screens, the time between documented transfusion and sample collection for the one heart patient was four months (male), three kidney patients had samples collected at 15 months (female), 18 months (male), and 30 months (male), and three kidney patients had collections >120 months (two females, one male) (Table 10).

There was high variability in the documentation of products transfused to the patients investigated in this study, as well as variability in the number of products transfused. For the seven patients with positive antibody screens, no details about blood products other than transfusion date were provided to the MSU Tissue Typing Laboratory. Given the inability to provide specific details on the transfusion of each patient, these data were not included in the results of this study.

Table 10. Time between Documented Transfusion and Sample Collection for Patients with Positive Antibody Screens, by Transplant Population

Time (months)	Heart	Lung	Kidney	Total (*n*=7)
≤10	1	N/A	0	1 (14%)
≥11 and ≤20	0	N/A	2	2 (29%)
≥21 and ≤30	0	N/A	1	1 (14%)
≥31 and ≤120	0	N/A	0	0 (0%)
>120	0	N/A	3	3 (43%)

N/A = Not Applicable

1.4.4. Patient Demographics

Many samples collected for this study were found to be from patients aged 46-75 years of age (Table 11), with most patients reporting as White (Table 12). Of the patients with positive antibody screens, two patients (one heart, one kidney) were reported as Hispanic and the remaining five kidney transplant patients were reported as White (data not shown). There were more reported males than females in both the heart and lung transplant patient populations, and more females than males in the kidney transplant patient population (Table 13). Of the patients with positive antibody screens, the one heart patient was male, and of the six kidney patients, three were male and three were female (data not shown). All three females from the positive kidney transplant patient group had documented histories of multiple pregnancies. Documented pregnancy history was found in 13 (93%) of the female heart transplant patients, in 12 (63%) of the female lung transplant patients, and in 20 (77%) of the female kidney transplant patients tested in this study (data not shown).

Table 11. Patient Age by Transplant Population

Age (years)	Heart	Lung	Kidney	Total (*n*=135)
≥18 and ≤25	2 (4%)	0	1 (2%)	3 (2%)
≥26 and ≤35	3 (7%)	1 (2%)	0	4 (3%)
≥36 and ≤45	2 (4%)	3 (7%)	6 (13%)	11 (8%)
≥46 and ≤55	9 (20%)	6 (13%)	7 (16%)	22 (16%)
≥56 and ≤65	14 (31%)	12 (27%)	16 (36%)	42 (31%)
≥66 and ≤75	15 (33%)	22 (49%)	13 (29%)	50 (37%)
>76	0	1 (2%)	2 (4%)	3 (2%)

Table 12. Patient Race by Transplant Population

Race	Heart	Lung	Kidney	Total (*n*=135)
White/Caucasian	37 (82%)	41 (91%)	31 (69%)	109 (81%)
Black/African American	5 (11%)	2 (4%)	8 (18%)	15 (11%)
Hispanic	3 (7%)	2 (4%)	4 (9%)	9 (7%)
Native American	0	0	1 (2%)	1 (1%)
Asian	0	0	1 (2%)	1 (1%)

Table 13. Patient Gender by Transplant Population

Gender	Heart	Lung	Kidney	Total (*n*=135)
Male	31 (69%)	26 (58%)	19 (42%)	76 (56%)
Female	14 (31%)	19 (42%)	26 (58%)	59 (44%)

2. Experiment Two: Histologic Distribution of RBC Group Antigens in Deceased Donor Tissues

2.1. Aim

Identify the distribution of select histoblood group epitopes in heart, lung, kidney, and pancreas from deceased donors using IHC, and compare findings to reported antigen expression found in tissues of healthy individuals.

2.2. Introduction

IHC can be used to detect and localize targeted antigens on cells and tissues through specific binding with antibodies.[132,133] The goal for IHC is to identify any antigen-positive components of a specimen of interest.[132] This requires: (i) specific detection of the antigen of interest, (ii) a relatively high signal-to-noise ratio (a relatively strong signal with little nonspecific labeling), and (iii) preservation of the histological details of the specimen.[132] There are many steps involved in achieving these goals, and the pre-analytic, analytic, and post-analytic phases of testing all play an important role.

Once specimens are obtained, they undergo a series of procedures that will alter the histological characteristics of cells and tissues to varying degrees.[132] Prior to IHC labeling, collected specimens typically undergo fixation, embedding, sectioning, adherence to glass microscope slides, and any necessary antigen retrieval processes. Regardless of steps taken to produce adequate specimens for IHC, samples will not be exactly the same as they were *in situ*.[132]

After collection, the loss of vascular support for the removed tissue forces cells to slowly undergo hypoxia.[132] If not stopped, immune mechanisms will generate cellular and tissue damage in the biopsy, secondary to hypoxia, creating an environment that is different from the tissue *in situ*.[132] Such changes include cell membrane injury and increased autolysis due to decreased pH, which activates lysosomal enzymes.[132] To stop metabolic reactions from occurring within the biopsy, the sample needs to be fixed as soon as possible.[132]

The main objective for fixation is to disable proteolytic enzymes that can digest or damage cells and tissues if activated.[132] In histopathology is 10% neutral buffered formalin (NBF), a cross-

linking fixative, is most commonly used.[132] It is the primary amino groups of cellular proteins that cross-linking fixatives covalently bond, doing so will anchor soluble proteins; increasing the stability of tissues.[132] One downside of fixation is that it can alter epitopes such that antibodies to them may not bind; this is also true with the use of paraffin wax.[132]

Paraffin wax offers support for formalin-fixed specimens that will eventually be viewed on microscope slides.[132] Prior to embedding with paraffin, specimens are first dehydrated, drawing out any interstitial water.[132] Dehydration (typically with ethanol) is performed in a series of increasing alcohol concentrations (the final concentration is 100%), ensuring the removal of water without causing substantial damage to the specimen.[132] After dehydration, there is a clearing step (typically with xylene) that dissolves lipids so they do not interfere with the paraffin.[132] Embedding with paraffin wax is the final step to adding support to specimens. All these processes alter the original structure and confirmation of proteins, therefore determining an appropriate method for antigen retrieval is usually necessary prior to IHC labeling.[132] But before antigen retrieval and IHC labeling can begin, formalin-fixed paraffin-embedded (FFPE) specimens need to be sectioned and adhered to glass microscope slides.[134]

During sectioning, FFPE specimens are cut, using a microtome, at a thickness of 3-5 micrometers (μm), which allows for adequate IHC labeling.[134] After the sections are cut, they float in a water bath until being picked up on to a glass microscope slide.[134] Sections need to lie flat against the slide, without bubbles, to ensure proper adherence to the microscope slide.[134] Slides are dried, typically at room temperature (20-24˚C) for 24 hours, or at 50-60°C for 1-24 hours,[135] followed by dewaxing and hydration steps.[134] Dewaxing is important because the paraffin wax embedded in the sections needs to be removed from the slide for reagents to penetrate and/or adhere to specimens during testing.[134] Dewaxing is typically performed by immersing slides in multiple washes of xylene (10 minutes per wash), followed by dipping the slides through multiple hydrating solutions (5 minutes per graded ethanol solution, beginning with 100% (absolute) ethanol, followed by 95% ethanol and sequentially decreasing concentrations through 70%

ethanol as needed) until the slides are finally submerged in distilled water (from 5 to 30 minutes).[133,134,136] After hydration, sections are ready to proceed with any necessary testing, e.g. antigen retrieval.

Antigen retrieval allows for IHC to be performed on FFPE tissues and cells.[133] Alteration of tissue and/or cell proteins after fixation and embedding can mask epitopes by having the protein fold upon itself, or by changing the distance between AAs that make up an epitope, such that an antibody cannot recognize the epitope because it lacks the protein's original three-dimensional structure.[132] Antigen retrieval methods use either heat or proteolytic enzymes to reverse the protein cross-linking and folding formed by the fixation and embedding processes.[132] Antigen retrieval methods are not suitable for all epitopes; therefore, the sample of interest must be considered when determining which antigen retrieval method is best for optimal antigen-antibody binding.[132]

After antigen retrieval, the selection of appropriate primary antibodies, dilutions, and blocking techniques will further optimize an IHC protocol, producing a reaction that is sensitive, specific, and reproducible.[132] Polyclonal antibodies were selected for this study because they recognize a broad range of epitopes for the same antigen, increasing the likelihood of detecting histoblood group antigens that are present on cells and tissues.[132] However, polyclonal antibodies may result in undesired cross-reactivity with other proteins.[132] To reduce the signal-to-noise ratio on FFPE sections, blocking techniques are typically employed.[132] Blocking for endogenous peroxidase, alkaline phosphatase (AP), and biotin is common, as well as blocking for nonspecific protein binding.[132] A non-biotin, AP detection system was selected for this study, so blocking for biotin was not needed, and blocking endogenous AP is not necessary for FFPE sections since processing of these specimens inactivates AP activity.[132] Nonspecific proteins are typically blocked using normal non-immune serum.[132] Normal serum applied for blocking must be identical to the species of the secondary antibody, which will prevent nonspecific binding of the secondary antibody to charged hydrophobic binding sites in the sample of interest.[132] Blocking of

endogenous or nonspecific activity will allow for increased sensitivity of secondary antibody binding, and enhanced immunodetection.[132]

The immunodetection method used in this investigation was indirect enzymatic IHC, which involves the detection of antigens using enzyme-labeled antibodies.[132] Details of this method, specific for this investigation, will be described in the following methods section. Generally, the indirect IHC method begins with the addition of a primary antibody, and after incubation and washing, an enzyme-labeled secondary antibody is added to the sample that will bind to the primary antibody after proper incubation.[132] After incubating with the secondary antibody and washing, the final step is the addition of a chromogen.[132] When a chromogen is cleaved by the enzyme-labeled secondary antibody, color is produced which can be observed by light microscopy.[132]

The AP system used for IHC labeling in this investigation was polymer-based, thus employing a secondary antibody integrated with a dextran polymer.[132,137] The dextran polymer contains an active enzyme, AP, that is concentrated to produce a greater reaction with chromogenic substrates, compared to other methods.[137] Polymer-based methods are known for being biotin-free, having increased sensitivity, and concise protocols for operation.[132,137]

As mentioned earlier, the function of a chromogen is to create a colored product that can be visualized by light microscopy.[132] Chromogens react in the presence of enzyme attached to a secondary antibody, and the intensity of the colored reaction product correlates to the amount of labeled secondary antibody bound to the sample.[132,138] In addition to the chromogen substrate, a counterstain is applied to the specimen that will aid interpretation. Counterstains will stain sections of the specimen that were not involved in the immunodetection steps of testing.[132] This provides a visual contrast from the areas of the sample that did react to immunodetection.[132] The type of counterstain selected for an IHC protocol depends on the type of chromogen selected. This investigation used a magenta substrate,[138] and the counterstain selected was hematoxylin

(producing a blue color), because this counterstain gives excellent contrast to the magenta chromogen substrate.[138]

In addition to labeling the specimen of interest, there must also be positive and negative control samples to ensure that testing performs as expected. Positive controls contain the target antigen of interest (primary antibody is expected to react), and negative controls do not contain the target of interest (primary antibody is not expected to react). If negative control samples cannot be obtained prior to testing antibodies with unknown tissue expression, an isotype control (in place of the primary antibody) is sufficient to produce a negative result.[133]

If using cells and/or tissues other than those selected for IHC labeling, all control samples need to be fixed, processed, and embedded the same manner as testing specimens used for IHC labeling.[132,139] In cases where there is not a well-documented control tissue for a particular antigen, cells can be the next reliable choice. FFPE cell pellets are commonly used for IHC when there are poorly characterized or ubiquitously expressed proteins.[139] FFPE cell pellets are valuable controls for the development of IHC protocols for FFPE tissue samples.[139] This study used human RBCs to detect primary antibodies (positive control), and an isotype control antibody served as the negative control. Details for the use of RBCs as control samples will be described in the methods section below.

2.3. Methods

2.3.1. Biopsy Procurement and Fixation

After study approval (STUDY00006102) from the MSU Biomedical and Health IRB, heart, lung, kidney, and pancreas biopsy samples from deceased donors ≥18 years old (whose family provided express consent for research and education of their organs) were obtained from GOLM. Donor demographics are detailed on Table 14. As mentioned previously, pancreas biopsies were added to this investigation at the request of GOLM. The sample size requested for this study was selected without the guidance of literature support, and included six heart, six lung, six kidney,

and six pancreas biopsies. One additional pancreas biopsy that became available from GOLM during the procurement period was accepted for this investigation (*n*=25).

Table 14. Deceased Donor Biopsy Demographics

Heart	Lung	Kidney	Pancreas
(H1) White, Male	(L1) White, Male	(K1) White, Female	(P1) Asian, Male
(H2) White, Male	(L2) White, Male	(K2) Hispanic, Female	(P2) White, Male
(H3) White, Male	(L3) White, Male	(K3) Hispanic, Female	(P3) White, Female
(H4) Black, Male	(L4) Black, Male	(K4) White, Male	(P4) White, Male
(H5) White, Female	(L5) White, Female	(K5) White, Male	(P5) White, Male
(H6) Hispanic, Male	(L6) White, Female	(K6) White, Male	(P6) White, Male
-	-	-	(P7) Black, Male

After procurement, biopsies were fixed in 10% NBF (catalog #NB0507) (StatLab Medical Products, McKinney, TX) at GOLM before being picked up by the investigators of this study. The amount of time that each biopsy was in fixative prior to pick up varied (Table 15).

Table 15. Biopsy Fixation Time

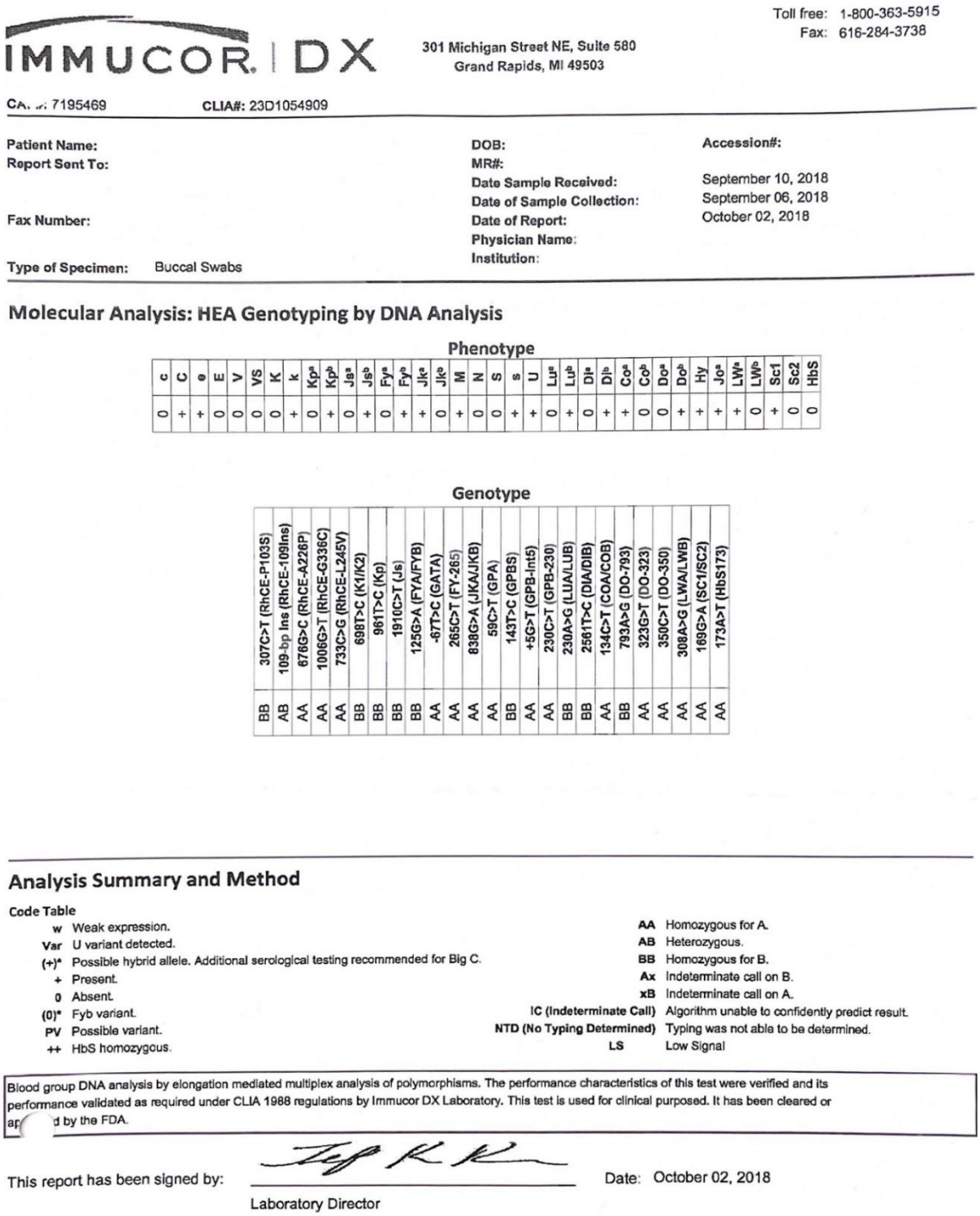
Heart	Lung	Kidney	Pancreas
(H1) 25 days	(L1) 19 days	(K1) 25 days	(P1) 27 days
(H2) 9 days	(L2) 19 days	(K2) 18 days	(P2) 12 days
(H3) 12 days	(L3) 12 days	(K3) 18 days	(P3) 9 days
(H4) 11 days	(L4) 11 days	(K4) 11 days	(P4) 19 days
(H5) 7 days	(L5) 7 days	(K5) 9 days	(P5) 14 days
(H6) 4 days	(L6) 7 days	(K6) 11 days	(P6) 12 days
-	-	-	(P7) 11 days

There are no standard guidelines for the time tissues must spend in fixative before processing, and instead, individual laboratories are to establish standard fixation times for all routine and specialized biopsies.[140] Therefore, each biopsy from this study was fixed for a minimum of four days, as recommended by the MSU Investigative HistoPathology (IHP) Laboratory (East Lansing, MI).

After fixation, biopsy samples were placed in 30% ethanol (v/v) (30 mL 200 Proof Pure Ethanol (Koptec) + 70 mL distilled water) by the investigator and transported to the IHP Laboratory for further processing.

2.3.2. RBC Control Samples: FFPE RBC Cell Pellet, and RBC Blood Smear Techniques

There are no well-documented control tissues that reliably contain (or do not contain) the antigens of interest for this investigation. Therefore, RBCs that underwent human RBC antigen (HEA) genotyping (Figure 4) were used to detect each primary antibody (positive control), and a rabbit IgG isotype control antibody served as a negative control antibody. Details for the selection and testing of primary antibodies and isotype control are detailed in section 2.3.4.3 below.



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Fax: 616-284-3738

CA. #: 7195469 CLIA#: 23D1054909

Patient Name:
Report Sent To:
Fax Number:
Type of Specimen: Buccal Swabs

DOB:
MR#:
Date Sample Received: September 10, 2018
Date of Sample Collection: September 06, 2018
Date of Report: October 02, 2018
Physician Name:
Institution:

Accession#:

Molecular Analysis: HEA Genotyping by DNA Analysis

Phenotype

c	C	e	E	V	VS	K	k	Kp^a	Kp^b	Js^a	Js^b	Fy^a	Fy^b	Jk^a	Jk^b	M	N	S	s	U	Lu^a	Lu^b	Di^a	Di^b	Co^a	Co^b	Do^a	Do^b	Hy	Jo^a	LW^a	LW^b	Sc1	Sc2	HbS
0	+	+	0	0	0	0	+	0	+	0	+	0	+	+	0	+	0	0	+	+	0	+	0	+	+	0	0	+	+	+	+	0	+	0	0

Genotype

	307C>T (RhCE-P103S)	109-bp Ins (RhCE-109Ins)	676G>C (RhCE-A226P)	1006G>T (RhCE-G336C)	733C>G (RhCE-L245V)	698T>C (K1/K2)	961T>C (Kp)	1910C>T (Js)	125G>A (FYA/FYB)	-67T>C (GATA)	265C>T (FY-265)	838G>A (JKA/JKB)	59C>T (GPA)	143T>C (GPBS)	+5G>T (GPB-Int5)	230C>T (GPB-230)	230A>G (LUA/LUB)	2561T>C (DIA/DIB)	134C>T (COA/COB)	793A>G (DO-793)	323G>T (DO-323)	350C>T (DO-350)	308A>G (LWA/LWB)	169G>A (SC1/SC2)	173A>T (HbS173)
BB																									
AB																									
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Analysis Summary and Method

Code Table

w	Weak expression.
Var	U variant detected.
(+)*	Possible hybrid allele. Additional serological testing recommended for Big C.
+	Present.
0	Absent.
(0)*	Fyb variant.
PV	Possible variant.
++	HbS homozygous.

AA	Homozygous for A.
AB	Heterozygous.
BB	Homozygous for B.
Ax	Indeterminate call on B.
xB	Indeterminate call on A.
IC (Indeterminate Call)	Algorithm unable to confidently predict result.
NTD (No Typing Determined)	Typing was not able to be determined.
LS	Low Signal

Blood group DNA analysis by elongation mediated multiplex analysis of polymorphisms. The performance characteristics of this test were verified and its performance validated as required under CLIA 1988 regulations by Immucor DX Laboratory. This test is used for clinical purposed. It has been cleared or ap[illegible]d by the FDA.

This report has been signed by: _____ Date: October 02, 2018

Laboratory Director

Figure 4. HEA Molecular Typing of IHC RBC Control Cells
Detailed report of genotype and phenotype results for RBC control cells. This RBC donor has no genetic mutations, and all RBC antigens are expected to be present. Note: This HEA typing did not test for LE, XG/CD99, CH/RG, or KN blood group systems. The LE phenotype was performed separately via serologic hemagglutination technique and found to be Le(a-,b+). It is noted that this RBC donor (White, Female) has an 11.3% chance of being Xg(a-) and CD99L, a 3.8% chance of a Chido (-1,-2,-3) negative phenotype, a 2% chance of a Rodgers (-11,-12) negative phenotype, and there are no known KN null phenotypes.[44]

Initially, RBCs were processed into a FFPE cell pellet to closely mimic tissue processing. To do this, one whole blood sample (blood group O, with full RBC genotyping (Figure 4)) was collected in a 10 mL, 18 milligram (mg) K2 Ethylenediaminetetraacetic Acid (EDTA) vacuum tube (catalog #366643) (Becton Dickinson, Franklin Lakes, NJ), and centrifuged at 1200 ×g for 10 minutes (Sorvall ST 8 Centrifuge; Thermo Scientific, Waltham, MA). Plasma was removed from the sample and discarded. The remaining packed RBCs were transferred to two 50 mL polypropylene conical tubes (VWR, Radnor, PA), with 2.5 mL RBCs per-tube. Each 50 mL conical tube containing 2.5 mL packed RBCs was filled with 25 mL 10% NBF, and fixation of each RBC sample occurred over four days, and four weeks respectively. These RBC control samples were fixed for two different time periods to detect any changes in IHC labeling from optimal fixation (four days), to increased fixation (four weeks), due to the variability in fixation time of biopsy samples from GOLM (Table 15).

After the desired fixation time (four days or four weeks), formalin-fixed RBC samples were centrifuged at 500 ×g for 20 minutes. The 10% NBF was removed from the RBC sample and replaced with 30% ethanol (v/v) before being transported to the IHP laboratory for further processing into a FFPE cell pellet. To make FFPE RBC cell pellet control specimens, the RBC samples were centrifuged at 1000 ×g for 1.5 minutes. The 30% ethanol supernatant was removed and replaced with 2 mL pre-warmed Histogel (catalog #HG4000) (Thermo Scientific, Waltham, MA). The sample was mixed with an applicator stick and incubated at 4 °C overnight (16-24 hours) to form a solidified sample plug. After incubation, the RBC sample plug was sliced into discs (2-5 mm in thickness) and prepared for tissue processing and embedding (the details of these processes can be found in section 2.3.3 below).

Once the FFPE RBCs were processed on to microscope slides, sections were tested with the same method used for biopsy tissues, beginning with the blocking step (details are described in the section 2.3.4.2 below); however, this method was found to be inadequate for producing positive labeling for all primary antibodies selected for this investigation. The FFPE RBC cell pellet

method could only produce positive results with MN (CD235a), Ss (GYPB), and Kell primary antibodies (antibody details are described in section 2.3.4.3 below). Because of this, a new method was utilized for the use of RBCs as a positive control.

The effects of fixation and processing of antigens on different cell types is not well understood.[141] Given the results from the FFPE RBC cell pellet samples, there were no specific histoblood group antigen structures that produced either positive or negative results with IHC testing (Table 16, Figure 5). Because of this, a simplified approach that utilized minimal fixation and a microscopic blood smear technique was applied to limit the effects of sample processing on RBCs.

To begin, one whole blood sample (Figure 4) was collected in a 10 mL, 18 mg K2 EDTA vacutainer tube (catalog #366643) (Becton Dickinson, Franklin Lakes, NJ), and centrifuged at 1200 ×g for 10 minutes (Sorvall ST 8 Centrifuge; Thermo Scientific, Waltham, MA). Plasma was removed from the sample and discarded. The remaining packed RBCs were transferred to one 50 mL polypropylene conical tube (VWR, Radnor, PA), containing approximately 5 mL RBCs. The RBCs in the 50 mL conical tube were washed, three times, by adding 45 mL of room temperature (20-24 °C) isotonic saline (0.85% NaCl (w/v)), mixed by gentle inversion for 1 minute, and centrifuged at 500 ×g for 20 minutes. After centrifugation, the supernatant was removed from the conical tube and discarded. After the third wash, 15 mL of isotonic saline was added to the conical tube (total sample volume 20 mL), creating a 25% RBC cell suspension. The packed RBCs were washed to remove unbound protein from the sample that could interfere with testing and were diluted into a cell suspension for ease of interpretation via the blood smear technique.

To create a thin blood smear, 10 µL of the prepared RBC cell suspension was placed on a clean, charged microscope slide (catalog #MER7200) (Mercedes Medical, Lakewood Ranch, FL) near its frosted edge. While this first slide lays on a flat surface, another clean microscope slide (the spreader slide) is brought up to the 10 µL drop of blood at a 45° angle, where the blood was allowed to spread along the length of the spreader slide. At this point the spreader slide

quickly pushes the blood forward on the adjoining slide, creating a blood smear, preferably with a feathered edge that reaches toward the unfrosted end of the slide. Blood smears were dried at room temperature for 30 minutes before being submerged in a room temperature (20-24 °C) 2% acetate formalin solution (w/v) (100mL 37% Formaldehyde (catalog #JT2106-1) (VWR, Radnor, PA) + 20g Sodium Acetate Anhydrous (catalog #S8750) (Sigma Aldrich, St. Louis, MO) + 900mL distilled water) for 10 minutes. After fixation, slides were rinsed in gentle running tap water (20-24˚C) for five minutes, and placed in 1x working solution of Tris Buffered Saline (TBS) +Tween 20 (500 mL TBS+Tween 20 (0.05 M tris, 0.15 M sodium chloride, 0.05% Tween 20) pH 7.4 (20x) buffer and 9.5 L distilled water) (ScyTek laboratories, Logan, UT) for five minutes before moving on to the blocking step for IHC (detailed below).

The blood smear technique was successful in producing positive labeling for all primary antibodies (positive control) and negative labeling for the rabbit IgG isotype control (negative control) (Table 17), in comparison to the FFPE RBC cell pellet technique that could only produce positive labeling with the MN (CD235a), Ss (GYPB), and Kell antibodies. Quality control results will be detailed for each primary antibody in section 2.4 below.

Table 16. Histoblood Group Antigen Structure

ISBT Number	System Name	System Symbol	Protein Structure
002	MNS	MN	Type I, single-pass membrane sialoglycoprotein (intracellular carboxyl terminus, extracellular amino terminus)
		Ss	Type I, single-pass membrane sialoglycoprotein (intracellular carboxyl terminus, extracellular amino terminus)
004	Rh	RHD	Multipass (12 domain) membrane protein (intracellular carboxyl and amino terminus)
		RHCE	Multipass (12 domain) membrane protein (intracellular carboxyl and amino terminus)
006	Kell	KEL	Type II, single-pass membrane glycoprotein (intracellular amino terminus, extracellular carboxyl terminus)
007	Lewis	LE	Carbohydrate moieties attached to lipid or protein, passively adsorbed onto RBC membrane from plasma.
008	Duffy	FY	Multipass (7 domain) membrane glycoprotein (intracellular carboxyl terminus, extracellular amino terminus)
009	Kidd	JK	Multipass (10 domain) membrane glycoprotein (intracellular carboxyl and amino terminus)
012	XG	XG	Type I, single-pass membrane glycoprotein (intracellular carboxyl terminus, extracellular amino terminus)
017	Chido/ Rodgers	CH/RG	Glycoprotein (C4) passively adsorbed onto RBC membrane from plasma.
022	Knops	KN	Type I, single-pass membrane glycoprotein (intracellular carboxyl terminus, extracellular amino terminus)

Adapted from: Cohn CS, et al. Technical Manual. AABB (2023)[9]; Reid ME, et al. The Blood Group Antigen Facts Book (2012)[44]

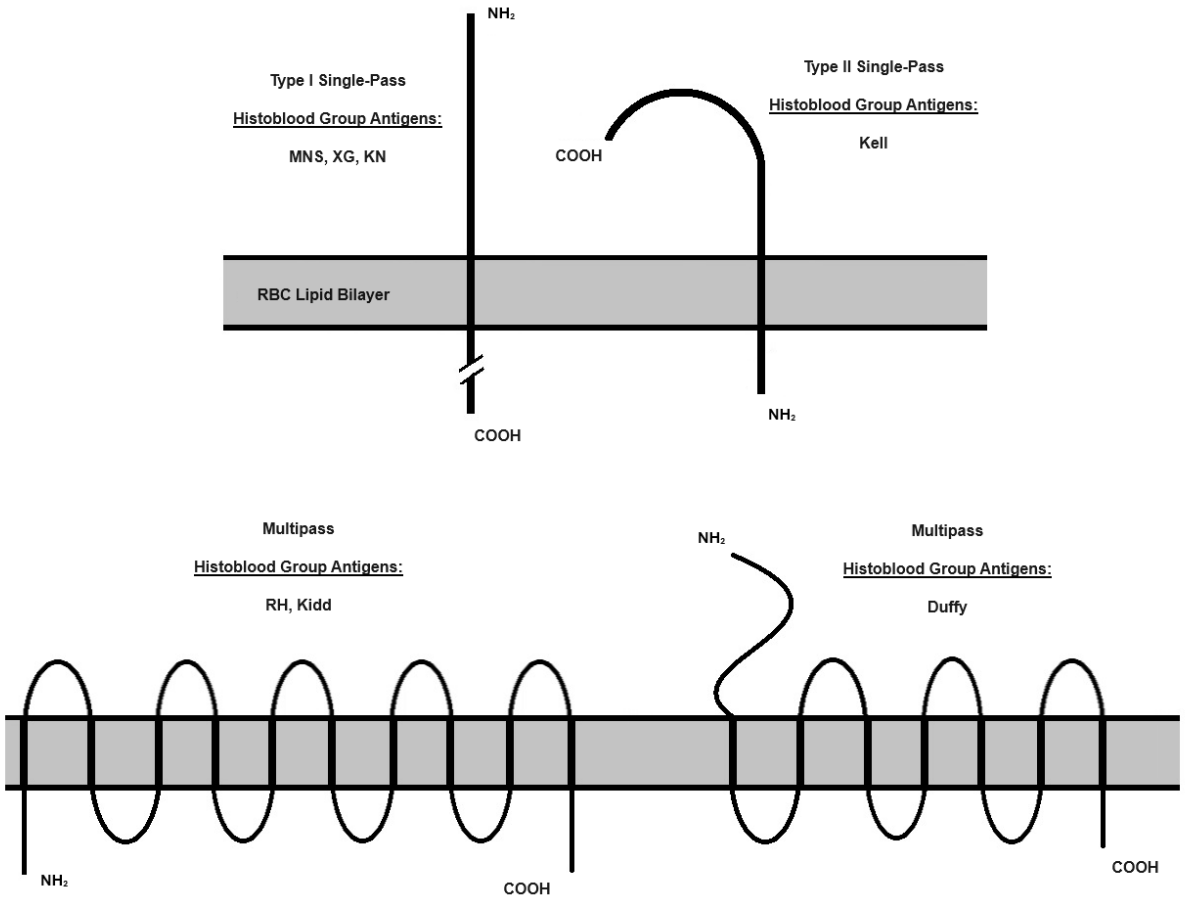

Figure 5. Histoblood Group Transmembrane Structures.
Simple diagram of RBC transmembrane proteins representing the histoblood group antigens in this study. Note: Lewis and Chido/Rodgers blood group antigens are not shown. Lewis and Chido/Rodgers antigens are passively adsorbed onto the RBC membrane from the plasma.
Adapted from: Cohn CS, et al. Technical Manual. AABB (2023)[9]; Reid ME, et al. The Blood Group Antigen Facts Book (2012)[44]

Table 17. Optimal Primary Antibody Dilutions and IHC Detection Methods for RBC Blood Smear Testing

System Name	Antibody	Antibody Dilution	Antibody Starting Concentration	Antibody Ending Concentration
MNS	CD235a	1/1,000	1,000 µg/mL	1 µg/mL
	GYPB	1/100	500 µg/mL	5 µg/mL
Rh	RHD	1/100	3,000 µg/mL	15 µg/mL
	RHCE	1/100	0.1 µg/mL	0.0005 µg/mL
Kell	KEL	1/100	1,500 µg/mL	15 µg/mL
Lewis	FUT3	1/100	1,000 µg/mL	5 µg/mL
Duffy	ACKR1	1/100	100 µg/mL	0.5 µg/mL
Kidd	SLC14A1	1/100	380 µg/mL	3.8 µg/mL
XG	CD99	1/100	57 µg/mL	0.285 µg/mL
Chido/ Rodgers	C4	1/100	520 µg/mL	2.6 µg/mL
Knops	CD35	1/100	920 µg/mL	4.6 µg/mL
Rabbit IgG Isotype Control		1/733	11,000 µg/mL	15 µg/mL *

Primary antibody information is detailed in section 2.3.4.3.
Additional protocol information: 4˚C overnight (16-24 hours) incubation of all primary antibodies; 45-minute polymer incubation; 20-minute substrate incubation; 15 second counterstain incubation.
*Isotype control is diluted to match the highest ending concentration of the primary antibodies.
µg = microgram

2.3.3. Infiltration, Embedding, and Sectioning

The IHP laboratory processed the RBC cell pellet samples and biopsies for this investigation to prepare the histology slides required for IHC testing. A two-step process of paraffin infiltration (PureAffin® 1, Cancer Diagnostics Inc., Durham, NC) and paraffin embedding (PureAffin® 2, Cancer Diagnostics Inc., Durham, NC) were performed on all formalin-fixed biopsies and formalin-fixed RBC cell pellet samples. After paraffin infiltration and embedding, sectioning was performed (4-5 µm in thickness) on all FFPE biopsies and FFPE RBC cell pellet samples. After sectioning, slides were dried (56 °C overnight (16-24 hours)), deparaffinized (slides placed in two fresh changes of xylene, 10 minutes each), and hydrated: slides were placed in two changes, 5 minutes each, of absolute alcohol (100% ethanol), followed by two changes, 5 minutes each, of 95% ethanol (v/v), rinsed in running tap water (20-24 °C) for 3 minutes, and finally rinsed in distilled water (20-24 °C) for 1 minute before being placed in 1x working solution of TBS (1 L TBS (0.05 molar (M) tris, 0.15 M sodium chloride) pH 7.4 (25x) buffer and 24 L distilled water) (ScyTek laboratories, Logan, UT)). Hydrated slides, submerged in 1x TBS, were picked up from the IHP laboratory by the investigators of this study and brought to the MSU Tissue Typing laboratory for testing.

2.3.4. Immunohistochemistry Testing Methods

2.3.4.1. Antigen Retrieval

FFPE sample slides were subject to heat-induced antigen retrieval using Vector Antigen Unmasking Solution, citrate pH 6.0 (catalog #H-3300-250) (Vector Labs, Burlingame, CA) in a vegetable steamer. Heat-induced antigen retrieval with pH 6.0 buffer was selected for this investigation because this method was primarily used on IHC samples reported in the HPA.[13,142]

For sections requiring antigen retrieval, the 100x (1 M) citrate pH 6.0 Vector Antigen Unmasking Solution was diluted to 1x (10 mM) with distilled water as needed (volume was determined by testing batch size). Slides were placed in polypropylene Coplin jar(s) prior to being placed in a vegetable steamer. Each polypropylene Coplin jar holds a maximum of 50 mL of

diluted antigen-unmasking solution, and five testing slides (maximum six Coplin jars per batch). Once the vegetable steamer reached a rolling boil (100 °C), the polypropylene Coplin jar(s) (with threaded screw cap) containing testing slides and diluted antigen-unmasking solution were placed into the steamer for 20 minutes. After incubation, the polypropylene Coplin jar(s) had their screw cap removed and were further incubated at room temperature (20-24 °C) for 10 minutes. After final incubation, slides were rinsed in 3-5 changes (30 seconds per change) of room temperature (20-24 °C) distilled water and placed in a 1x working solution of TBS+Tween 20 (20-24 °C) for 5 minutes to adjust pH. At this step, histology slides were ready for blocking.

2.3.4.2. Blocking

After removing slides from buffer, any excess liquid was carefully tipped off and blotted from the slide, without disturbing the sample section(s). Next, a hydrophobic waxy barrier was drawn on both sides of each section using the ImmEdge hydrophobic pen (catalog #H-1000) (Vector Labs, Burlingame, CA). Sections underwent a blocking step using diluted normal serum from the host species of the secondary antibody used in this study. Sections were each covered by 2-3 drops (approximately 50 µl per drop) of 2.5% normal horse serum (catalog #MP-5401) (Vector Labs, Burlingame, CA), followed by incubation at room temperature (20-24 °C) for 20 minutes in a dark humidity chamber. After incubation, excess serum was tipped off from each section and slides were ready for the addition of primary antibodies and isotype controls.

2.3.4.3. Primary Antibodies and Isotype Control

Nine blood group systems were selected for investigation using enzymatic IHC. Unconjugated polyclonal rabbit IgG primary antibodies were selected for each blood group system (Table 18). Blood group systems were selected based on antigen characteristics including: (i) phenotypic frequencies (likelihood of incompatibility between donors and recipients), (ii) ability to detect antibodies using standard hemagglutination methods, (iii) likelihood of alloantibody production, (iv) CD marker association, and (v) complement association.[44] Five of the selected histoblood group systems had associated CD markers (MNS, RH, Kell, Duffy, Knops); therefore,

primary antibodies were expected to detect common antigenic epitopes shared by alleles of the same gene, eliminating the need for RBC phenotyping or genotyping prior to IHC. The remaining four histoblood group system antigens that did not have an associated CD marker (Lewis, Kidd, XG, Chido/Rodgers) had primary antibodies selected based on the gene that encodes for synthesis of the main antigens of the blood group system,[44] also eliminating the need for RBC phenotyping or genotyping prior to IHC. See Appendix A for more information on IHC primary antibodies used in this study.

Table 18. Primary Antibodies for IHC

ISBT Number	System Name	System Symbol	CD Number/Gene	Antibody Catalog Number	Immunogen	Immunogen Amino Acid Sequence
002	MNS	MN	CD235a/*GYPA*	PA5-80679 *	Recombinant human GYPA	AA 1-91
		Ss	CD235b/*GYPB*	PA5-71844 *	KLH ^ conjugated synthetic peptide of GYPB	AA 22-51
004	Rh	RHD	CD240D/*RHD*	PA5-112694 *	Recombinant human RhD protein	AA 32-76
		RHCE	CD240CE/*RHDCE*	PA5-140007 *	Recombinant human RhCE protein	AA 388-417
006	Kell	KEL	CD238/*KEL*	PA5-97891 *	Recombinant human KEL glycoprotein	AA 201-500
007	Lewis	LE	*FUT3*	PA5-101596 *	Synthesized human FUT3 peptide	AA 94-144
008	Duffy	FY	CD234/*ACKR1*	PA5-82549 *	Recombinant human ACKR1 protein	AA 308-336
009	Kidd	JK	*SLC14A1*	PA5-110377 *	Synthetic human SCL14A1 peptide	AA 376-389
012	XG	XG	*XG/CD99(MIC2)*	PA5-32337 *	Recombinant full length human CD99 protein	AA 1-185
017	Chido/ Rodgers	CH/RG	*C4B/C4A*	PA5-29133 *	Recombinant fragment of human C4	AA 23-302
022	Knops	KN	CD35/*CR1*	PA5-98627 *	Recombinant human CR1 protein	AA 420-550

* Invitrogen (www.thermofisher.com)
^ Keyhole Limpet Hemocyanin (KLH)
See **Appendix A** for more information on IHC primary antibodies.

Well-documented negative control samples were unable to be obtained for this investigation, therefore, a rabbit IgG isotype control antibody (catalog #31235) (Thermo Fisher Scientific, Waltham, MA) was selected as the negative control for testing. A rabbit IgG isotype control was selected because this is a non-immune antibody from the same species that produced the primary antibodies selected for this investigation.

Primary antibodies and isotype controls need to be tested for their optimal dilution and incubation times prior to labeling unknown samples. Antibodies used in this investigation had the following information reviewed prior to the development of testing protocols: (i) antibody starting concentration, (ii) storage buffer, (iii) freeze-thaw recommendations, (iv) antigen retrieval, and (v) recommended dilution for use on paraffin-embedded samples for IHC.

To determine the optimal incubation for each antibody, a starting incubation of 4 °C overnight (16-24 hours) in a dark humidity chamber was performed, and adjustments were made as necessary. Antibody dilution was determined based on recommendations from each primary antibody (and isotype control) data sheet and titrated plus and minus 50% based on the recommended dilution. Table 19 provides an example of the primary antibody optimization protocol used in this study; additional dilutions were tested as needed.

Table 19. Primary Antibody Optimization Protocol, Two Sections per Slide

Slide	Primary Antibody	Isotype Control *	Antigen Retrieval
1	-50% Recommended Dilution	Same Ending Concentration as the Primary Antibody	Citrate, pH 6.0
2	Recommended Dilution	Same Ending Concentration as the Primary Antibody	Citrate, pH 6.0
3	+50% Recommended Dilution	Same Ending Concentration as the Primary Antibody	Citrate, pH 6.0

*Isotype control dilution is based on the ending concentrations (µg/mL) of each primary antibody. The isotype control dilution typically will not match the primary antibody dilution.

Isotype control dilutions were determined based on the concentration of the primary antibody (µg/mL) being tested, using the following equations:

$$SC\ (mg/mL)x\ 1{,}000 = SC(\mu g/mL) \div EC\ (\mu g/mL) = Dilution$$

$$SC\ (mg/mL)\ x\ 1000 = SC(\mu g/mL) \div Dilution = EC\ (\mu g/mL)$$

SC = Starting Concentration, *EC* = Ending Concentration

Primary antibodies and isotype controls were diluted with 2.5% normal horse serum (catalog #S-2012-50) (Vector Labs, Burlingame, CA) using the following equations:

$$(Final\ Volume)/Dilution = Volume\ of\ Concentrated\ Ab\ Needed$$

$$Final\ Volume - Volume\ of\ Concentrated\ Ab = Volume\ of\ Diluent\ Needed$$

For each antibody, IHC slides were reviewed, and the optimal antibody dilution and antigen retrieval method (or lack of antigen retrieval) were determined based on the intensity of labeling for positive control samples, a lack of labeling for negative controls, and low or no nonspecific background labeling for all samples. Optimized dilutions and incubations for the primary antibodies and isotype controls are provided in Table 20.

Approximately 100-200 µL (depending on the size of the section) of diluted primary antibody were added to each section and incubated for the time optimized for each antibody (Table 20). After incubation, sections were rinsed in several changes (30 seconds per change) of 1x working TBS+Tween 20 buffer and left to stand in their final change of buffer for five minutes, at room temperature (20-24 °C). Excess buffer was carefully blotted from each section before proceeding to the secondary antibody detection kit.

2.3.4.4. Secondary Antibody, AP-Polymer Detection Kit

The Vector ImmPRESS®-AP Horse Anti-Rabbit IgG Polymer Detection Kit (catalog #MP-5401) (Vector Labs, Burlingame, CA) was used in accordance with manufacturer's instructions. Depending on the size of each section, 2-3 drops (approximately 50 µl per drop) of the ImmPRESS®-AP polymer reagent were added to each section and incubated for 30 minutes (45 minutes for RBC blood smears) at room temperature (20-24 °C) in a dark humidity chamber. After

incubation, sections were rinsed in several changes (30 seconds per change) of 1x working TBS+Tween 20 buffer, followed by two, five-minute changes in 1x working TBS+Tween 20, at room temperature (20-24 °C), prior to the addition of an AP-chromogenic substrate.

2.3.4.5. Chromogenic Substrate

A substrate working solution was prepared using the ImmPACT® Vector Red Substrate Kit-AP (catalog #SK-5105) (Vector Labs, Burlingame, CA), in accordance with manufacturer's instructions. The AP substrate mixture contained: five mL of ImmPACT® Vector Red diluent, two drops (approximately 80 µL) of ImmPACT® Reagent 1, and two drops (approximately 60 µL) of ImmPACT® Reagent 2. Depending on the size of each section, 100-200 µL of the AP substrate working solution were added to each section and incubated at room temperature (20-24 °C) in a dark humidity chamber for 20-30 minutes (until the desired labeling developed). Substrate incubation was optimized for each sample type (Table 20). After incubation, sections were rinsed in three changes (30 seconds per change) of 1x working TBS+Tween 20 buffer and left to stand in their final change of buffer for five minutes, at room temperature (20-24 °C). Sections were rinsed in tap water for 30 seconds before counterstaining.

2.3.4.6. Counterstain and Mounting

Sections were covered with Hematoxylin QS counterstain (catalog #H-3404-100) (Vector Labs, Burlingame, CA), and incubated for 5-45 seconds (until desired staining was reached). Counterstaining incubation was optimized for each sample type (Table 20). After incubation, sections were rinsed with running tap water (20-24 °C) until rinse water ran colorless. Sections were allowed to dry for 10 minutes at room temperature (20-24 °C) before mounting with a permanent mounting medium.

Under a fume hood, 2-3 drops (approximately 50 µL per drop) of Permount (catalog #SP15-500) (Fisher Scientific, Hampton, NH) were added to each section, followed by the addition of a coverslip. After drying for 15 minutes inside the fume hood (20-24 °C), slides were ready to be viewed with a light microscope.

2.3.4.7. Optimized Primary Antibody Dilutions, Antigen Retrieval, and IHC Detection Methods

Table 20. Optimized Primary Antibody Dilutions, Antigen Retrieval, and IHC Detection Methods for FFPE Sample Testing

System Name	Antibody	Primary Antibody Dilution	Primary Antibody & Isotype Control * Ending Concentration
MNS	CD235a	1/5,000	0.2 (µg/mL)
	GYPB	1/1,000	0.5 (µg/mL)
Rh	RHD	1/2,000	1.5 (µg/mL)
	RHCE	1/200	0.0005 (µg/mL)
Kell	KEL	1/20,000	0.075 (µg/mL)
Lewis	FUT3	1/2,000	0.5 (µg/mL)
Duffy	ACKR1	1/200	0.5 (µg/mL)
Kidd	SLC14A1	1/100	3.8 (µg/mL)
XG	CD99	1/1,000	0.057 (µg/mL)
Chido/ Rodgers	C4	1/300	1.73 (µg/mL)
Knops	CD35	1/6,000	0.15 (µg/mL)

Additional protocol information: Heat-induced antigen retrieval with citrate pH 6.0 buffer; 4˚C overnight (16-24 hours) incubation of all primary antibodies; 30-minute polymer incubation; 10-minute substrate incubation; 15 second counterstain incubation.
* Isotype Control Starting Concentration = 11,000 µg/mL

2.4. Results

IHC detection is detailed below for each primary antibody. For comparison purposes, tissue substructures recognized for detection in this investigation align with substructures documented in the HPA (Table 2).

For heart tissue, detection is noted for cardiomyocytes, and this study also included intercalated discs. For lung tissue, detection is noted for alveolar cells and macrophages; this study also included bronchial epithelium. For kidney tissue, detection is noted in the glomeruli and in tubular epithelial cells of the cortex and medulla; this study also included the urothelium. For pancreas tissue, detection is noted in the exocrine glandular cells and endocrine cells (islets of Langerhans). Any detection noted outside of the substructures mentioned above was documented. A full summary, Table 21 is provided at the end of this section (page 140).

2.4.1. MNS System

2.4.1.1. MN

The MN (CD235a) protein was detected in the heart, lung, kidney, and pancreas. (Table 21). This antibody also produced positive labeling of RBCs in biopsy tissue.

In heart tissue, there was positive granular cytoplasmic labeling throughout the cardiomyocytes (*n*=6) (Figure 6). In lung tissue, positive cytoplasmic labeling was seen in the bronchial epithelium (*n*=6) (Figure 7). In kidney tissue, cytoplasmic labeling was seen in both the proximal and distal tubular epithelial cell of the cortex (*n*=6), and in the medullary tubules (mostly cuboidal epithelium) and urothelium, where included (biopsy K1 and K6) (Figure 8). In pancreas tissue, positive cytoplasmic labeling was noted in endocrine cells (islets of Langerhans) (*n*=7) (Figure 9).

Membranous labeling of RBCs within each organ served as the best positive control. In addition, the anti-MN (CD235a) antibody produced positive RBC labeling for both the FFPE cell pellet and the RBC blood smear technique (Figure 10).

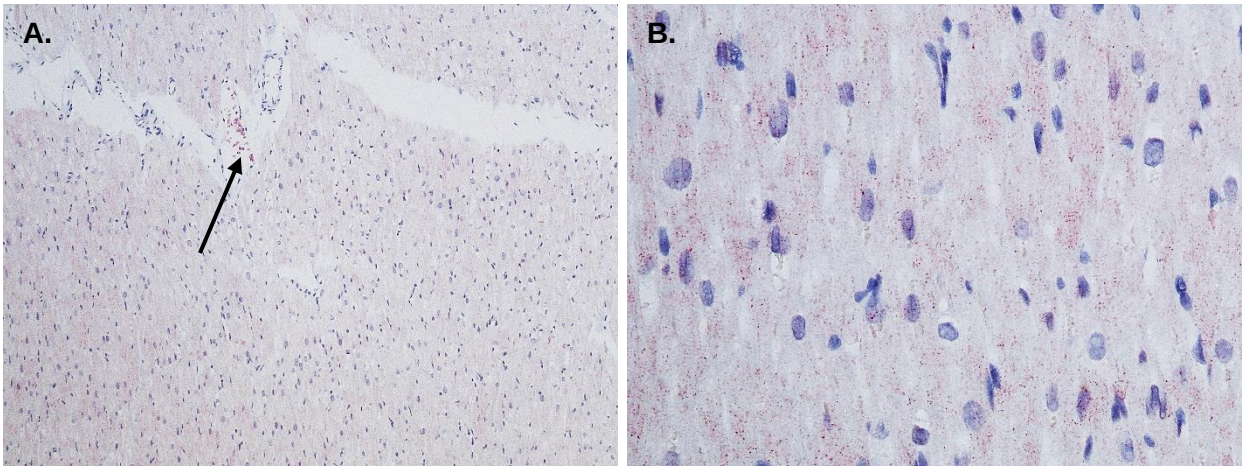

Figure 6. Microscopic Image of Heart Tissue with MN (CD235a) Polyclonal Antibody
Positive labeling of cardiomyocytes (A.) 10x, (B.) 50x. Positive labeling of RBCs is also present (arrow).

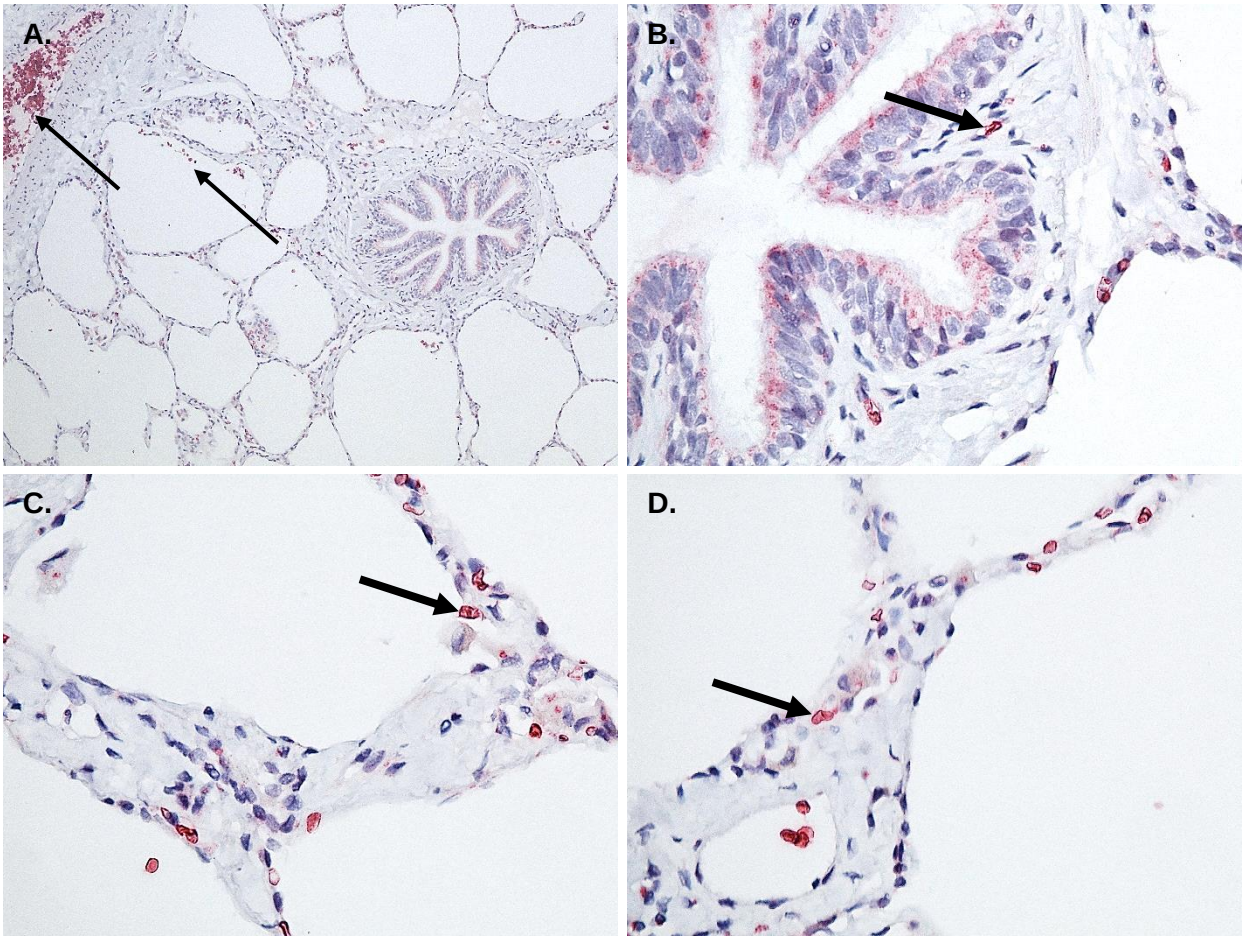

Figure 7. Microscopic Image of Lung Tissue with MN (CD235a) Polyclonal Antibody
Positive labeling of bronchial epithelium (A.) 10x, (B.) 50x. Negative labeling of alveolar cells and macrophages (C, D.) 50x. Positive labeling of RBCs is also present (arrow).

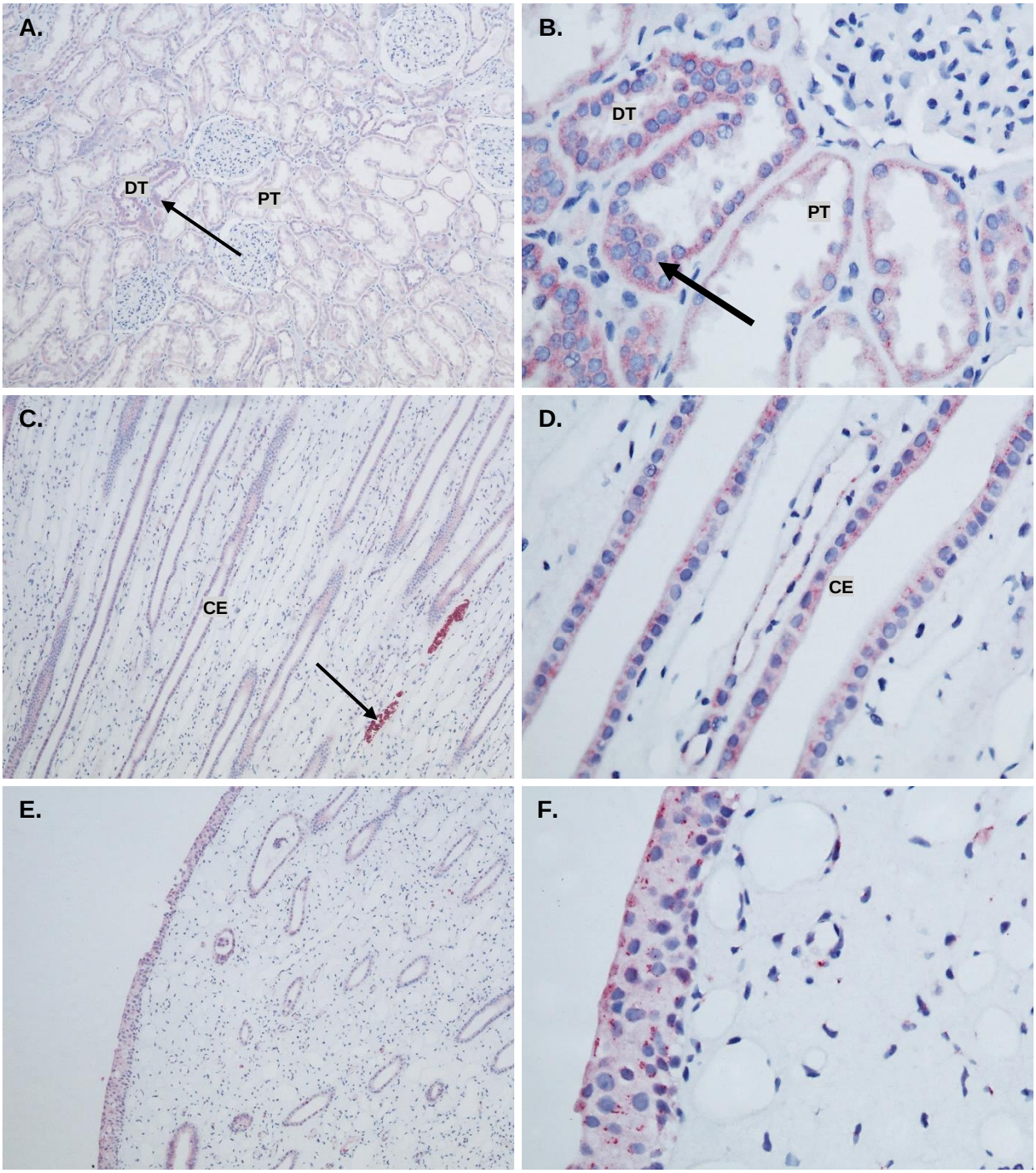

Figure 8. Microscopic Image of Kidney Tissue with MN (CD235a) Polyclonal Antibody Positive labeling of the proximal (PT) and distal (DT) (arrow) tubules of the cortex (A.) 10x, (B.) 50x. Positive labeling of the tubules (cuboidal epithelium (CE)) of the medulla (C.) 10x, (D.) 50x. Positive labeling of RBCs is also present (arrow). Positive labeling of urothelium (E.) 10x, (F.) 50x.

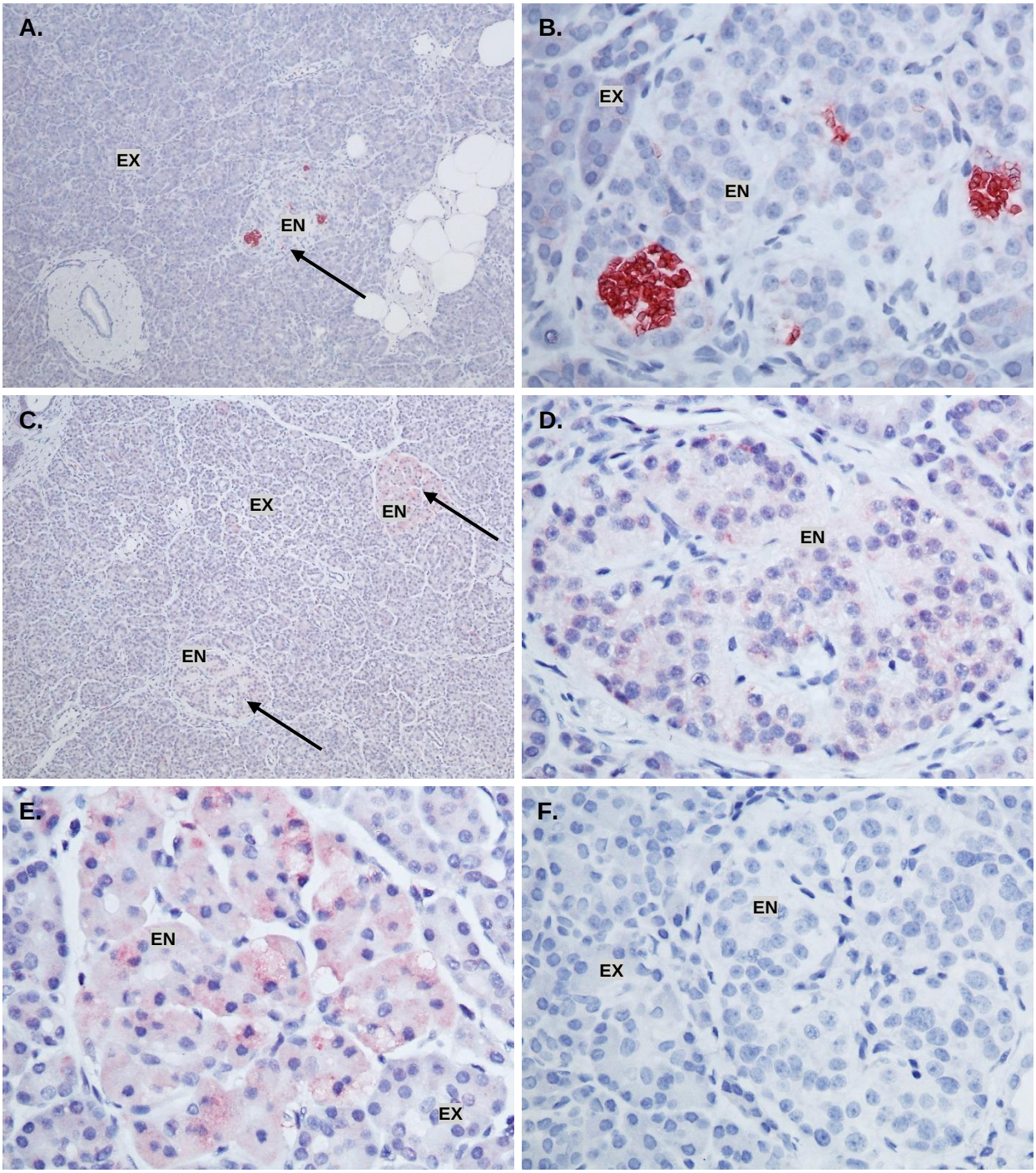

Figure 9. Microscopic Image of Pancreas Tissue with MN (CD235a) Polyclonal Antibody Positive labeling of endocrine cells (EN) (arrow), negative exocrine cells (EX), positive labeling of RBCs (A., C.) 10x, (B., D., E.) 50x. Negative control (F.) 50x.

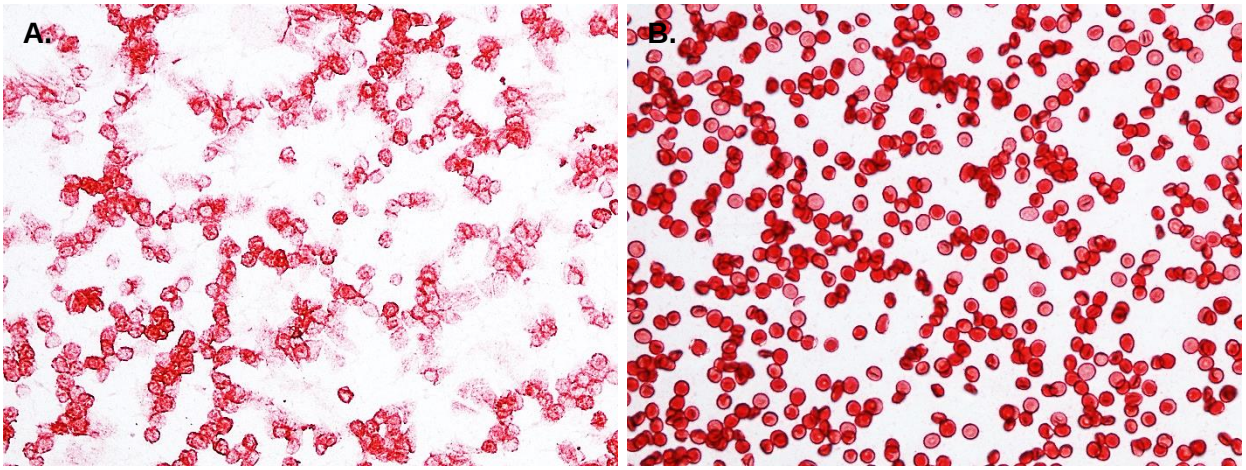

Figure 10. Microscopic Image of RBC Control with MN (CD235a) Polyclonal Antibody
Positive labeling of MN antigen-positive RBCs via blood smear technique (A.), and positive labeling of MN antigen-positive RBCs with FFPE RBC cell pellet (B.) 50x.

2.4.1.2. Ss

The Ss (GYPB) protein was detected in heart, kidney, and pancreas tissue (Table 21). This antibody also produced positive labeling of RBCs in biopsy tissue.

In heart tissue, there was positive cytoplasmic labeling throughout the cardiomyocytes (*n*=6) (Figure 11). Negative labeling was seen in lung tissue, but positive labeling of RBCs was detected (*n*=6) (Figure 12). In kidney tissue, positive cytoplasmic labeling was seen in both the proximal and distal tubular epithelial cells of the cortex (distal tubules had stronger labeling) (*n*=6), and in the medullary tubules (mostly cuboidal epithelium) and urothelium, where included in the sections (biopsy K1 and K6) (Figure 13). In pancreas tissue, positive cytoplasmic labeling was noted in endocrine cells. The intensity of endocrine cell (islets of Langerhans) labeling varied (low detection in biopsies P2, P3, P4, P5, and P6; high detection in biopsies P1 and P7) within and between biopsy samples (Figure 14).

Membranous labeling of RBCs within the heart, lung, and pancreas served as the best positive control. In addition, the anti-Ss (GYPB) antibody produced positive RBC labeling for both the FFPE cell pellet and the RBC blood smear technique (Figure 15).

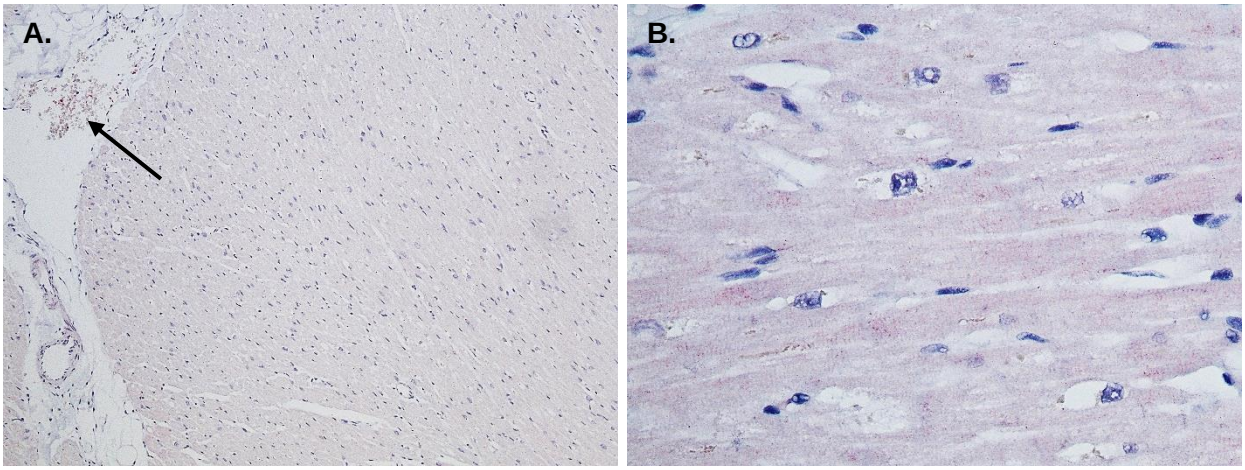

Figure 11. Microscopic Image of Heart Tissue with Ss (GYPB) Polyclonal Antibody
Positive labeling of cardiomyocytes (A.) 10x, (B.) 50x. Positive labeling of RBCs (arrow).

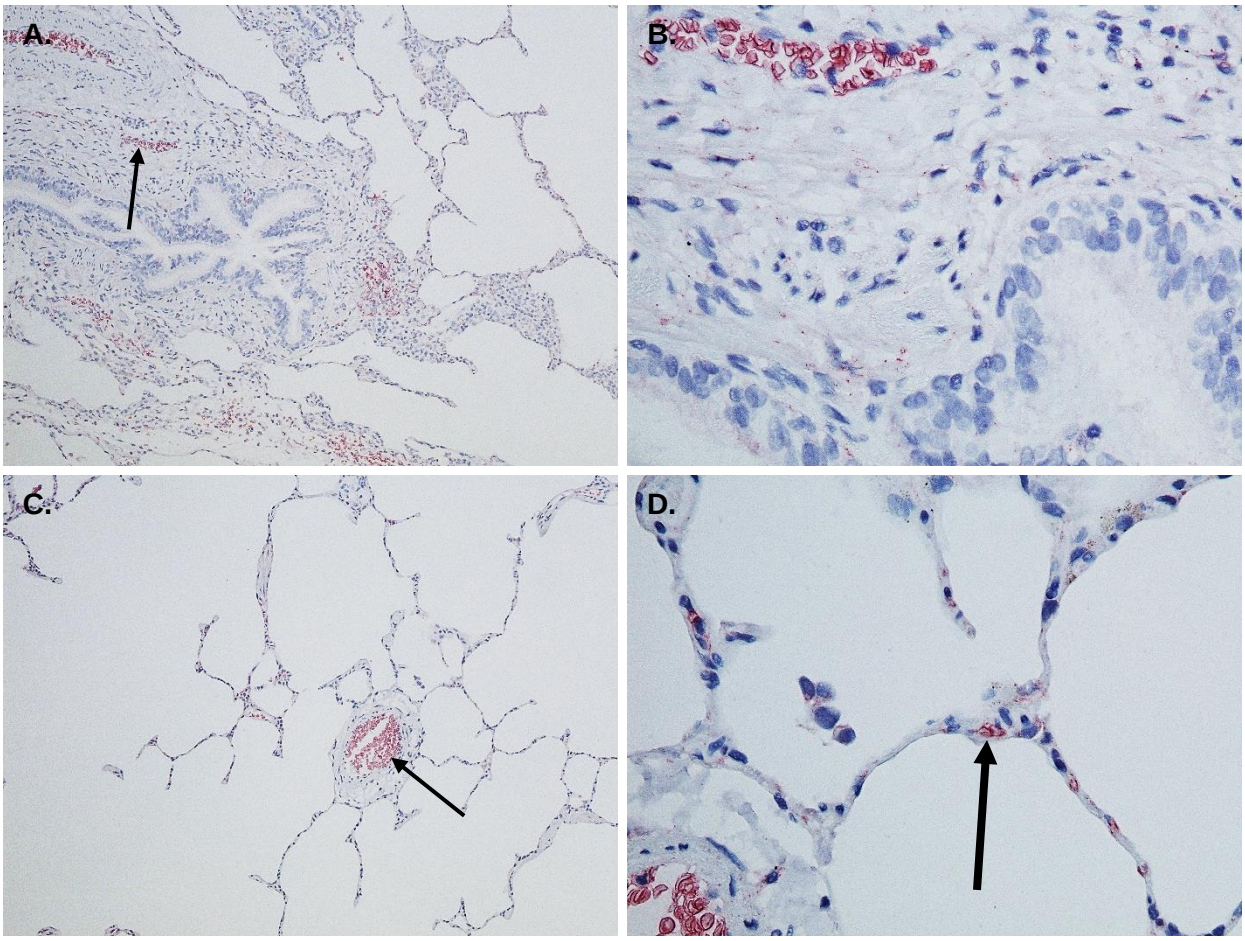

Figure 12. Microscopic Image of Lung Tissue with Ss (GYPB) Polyclonal Antibody
Negative labeling of bronchial epithelium (A.) 10x, (B.) 50x. Negative labeling of alveolar cells and macrophages (C., D.) 50x. Positive labeling of RBCs (arrow).

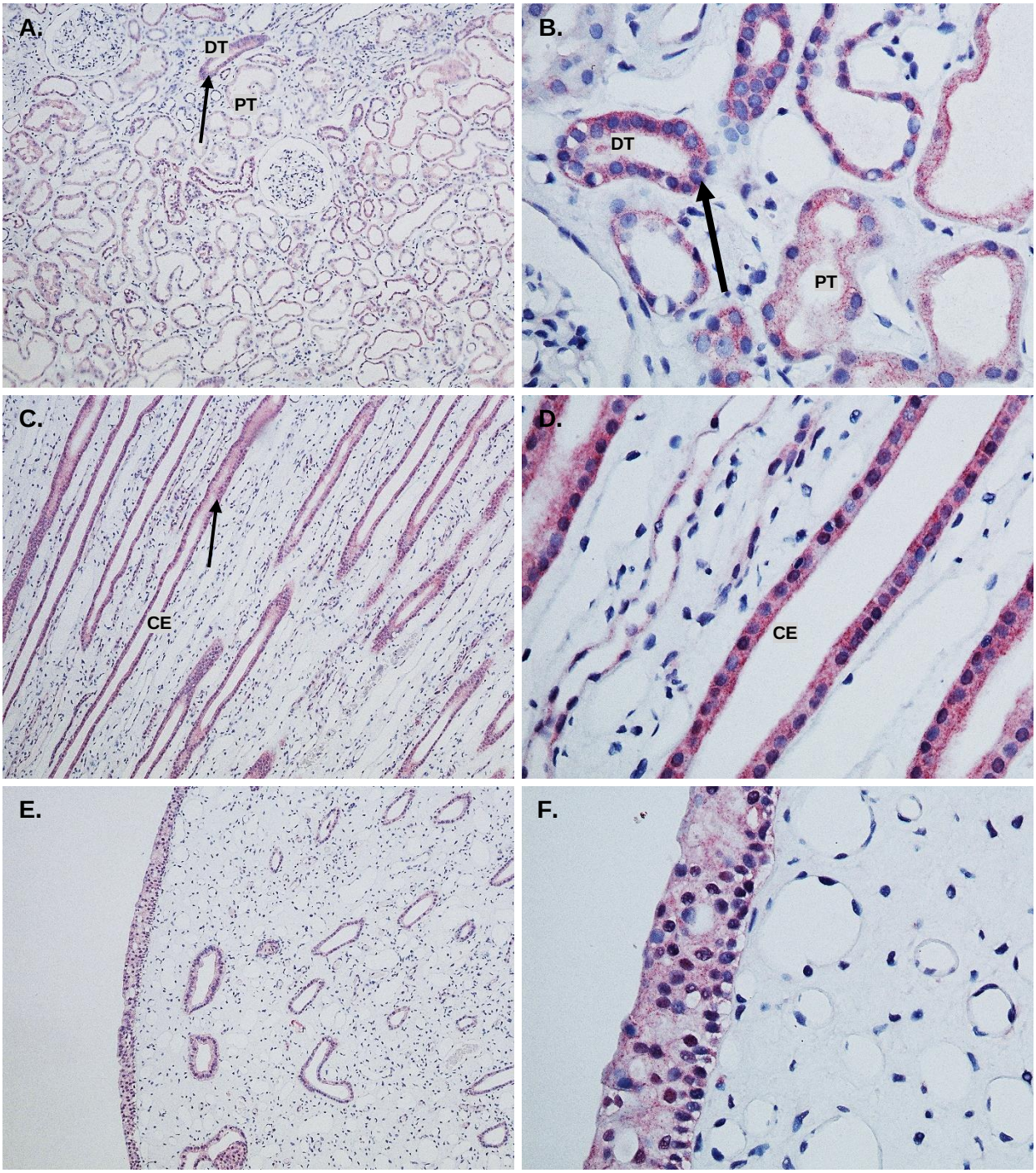

Figure 13. Microscopic Image of Kidney Tissue with Ss (GYPB) Polyclonal Antibody
Positive labeling of the proximal (PT) and distal (DT) (arrow) tubules of the cortex (A.) 10x, (B.) 50x. Positive labeling of the tubules (mostly cuboidal epithelium (CE)) (arrow) of the medulla (C.) 10x, (D.) 50x. Positive labeling of urothelium (E.) 10x, (F.) 50x.

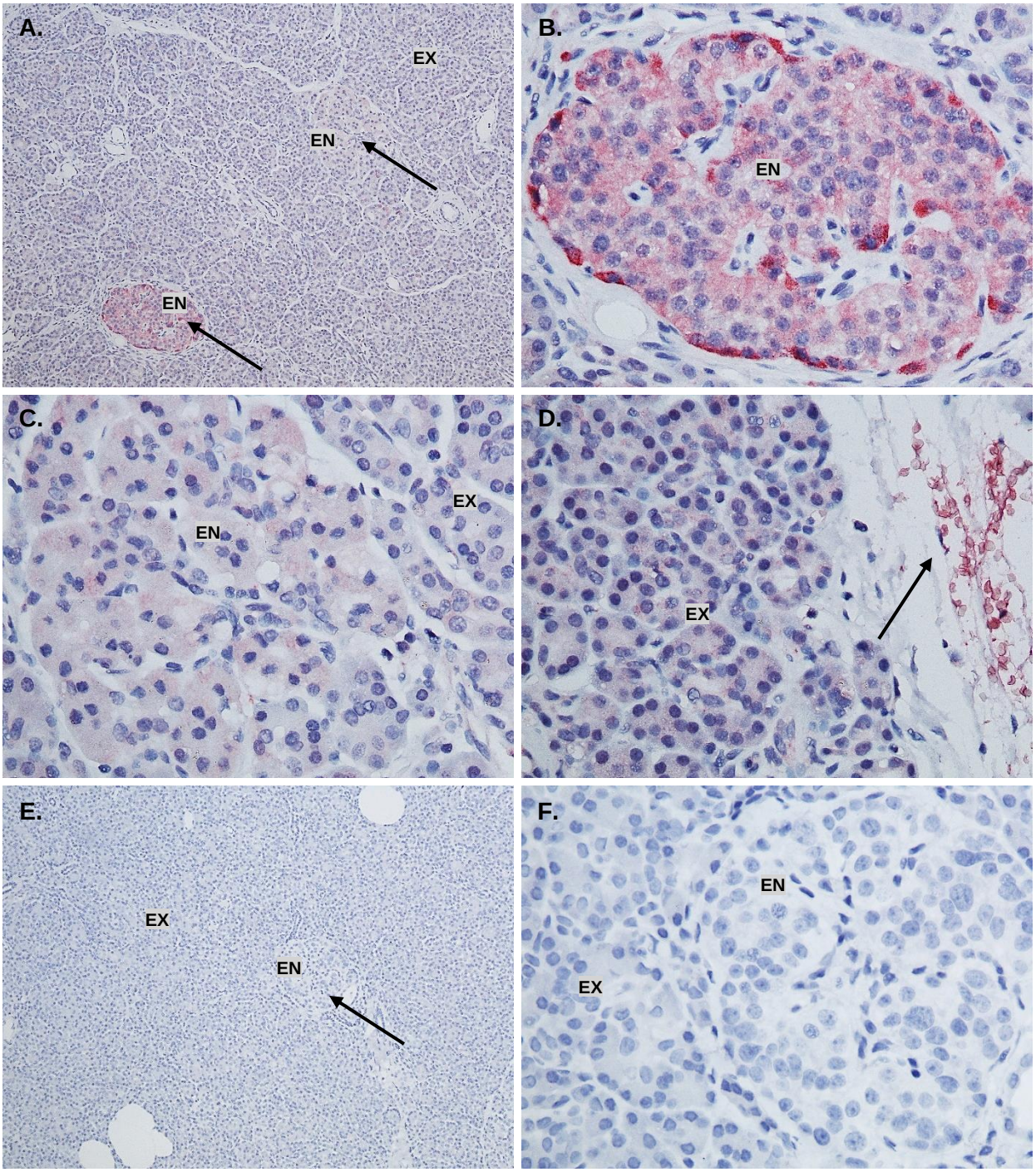

Figure 14. Microscopic Image of Pancreas Tissue with Ss (GYPB) Polyclonal Antibody Positive labeling of endocrine cells (EN) (arrow), negative exocrine cells (EX) (A.) 10x, (B., C.) 50x. Positive labeling of RBCs (D.) 50x (arrow). Negative control (E.) 10x, (F.) 50x.

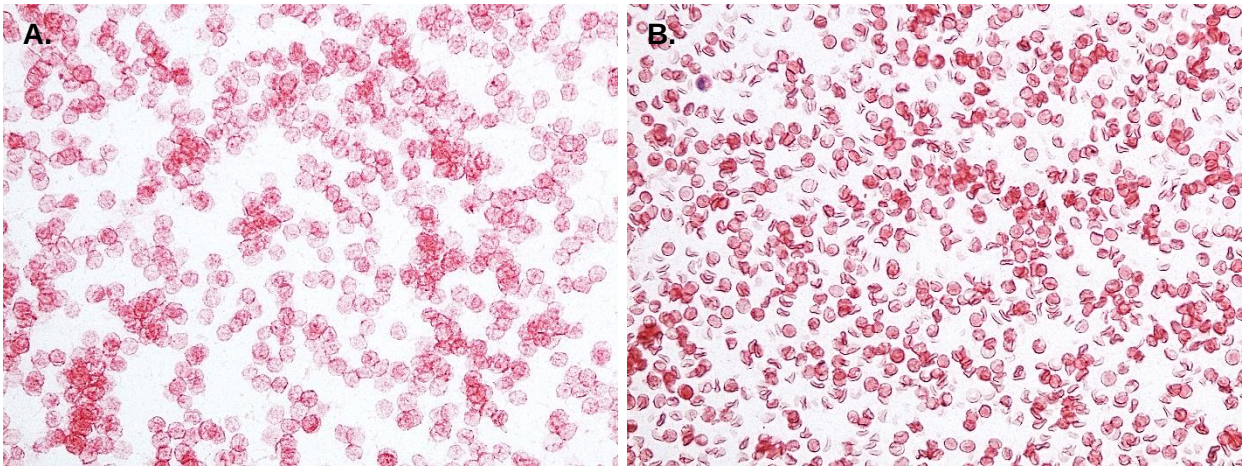

Figure 15. Microscopic Image of RBC Control with Ss (GYPB) Polyclonal Antibody
Positive labeling of Ss antigen-positive RBCs via blood smear technique (A.), and positive labeling of Ss antigen-positive RBCs with FFPE RBC cell pellet (B.) 50x.

2.4.2. Rh System

2.4.2.1. RHD

There was no detection of the RhD protein in heart (Figure 16), lung (Figure 17), kidney (Figure 18), or pancreas tissue (Figure 19). These results were consistent across all biopsy samples of all tissue types (Table 21). RBCs within biopsy samples were also negative.

The anti-RhD antibody produced negative labeling for the FFPE cell pellet, and positive labeling for the RBC blood smear technique (Figure 20).

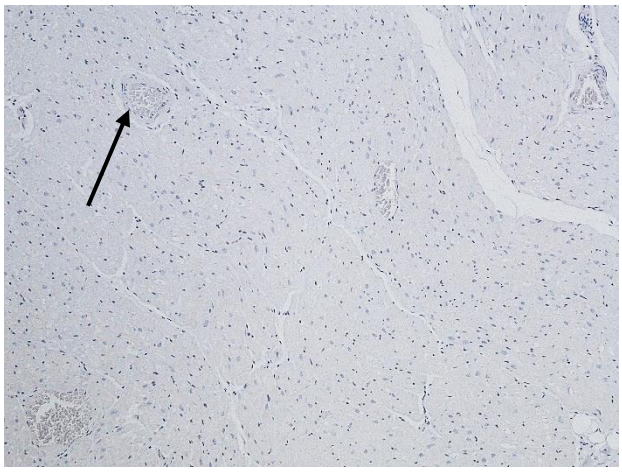

Figure 16. Microscopic Image of Heart Tissue with RhD Polyclonal Antibody
Negative labeling of cardiomyocytes (10x). Negative labeling of RBCs (arrow).

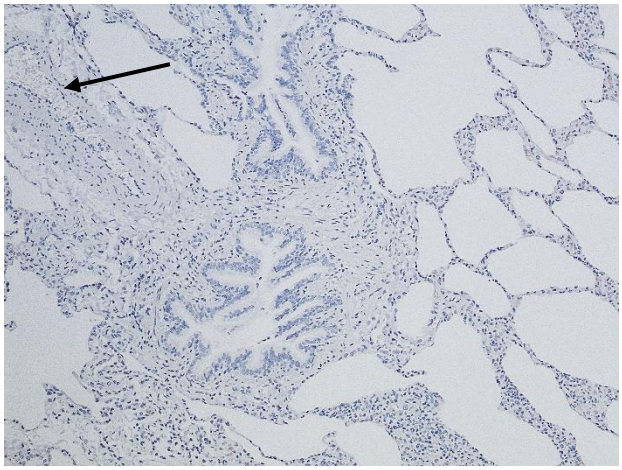

Figure 17. Microscopic Image of Lung Tissue with RhD Polyclonal Antibody
Negative labeling of alveolar cells, and macrophages (10x). Negative labeling of RBCs is shown (arrow).

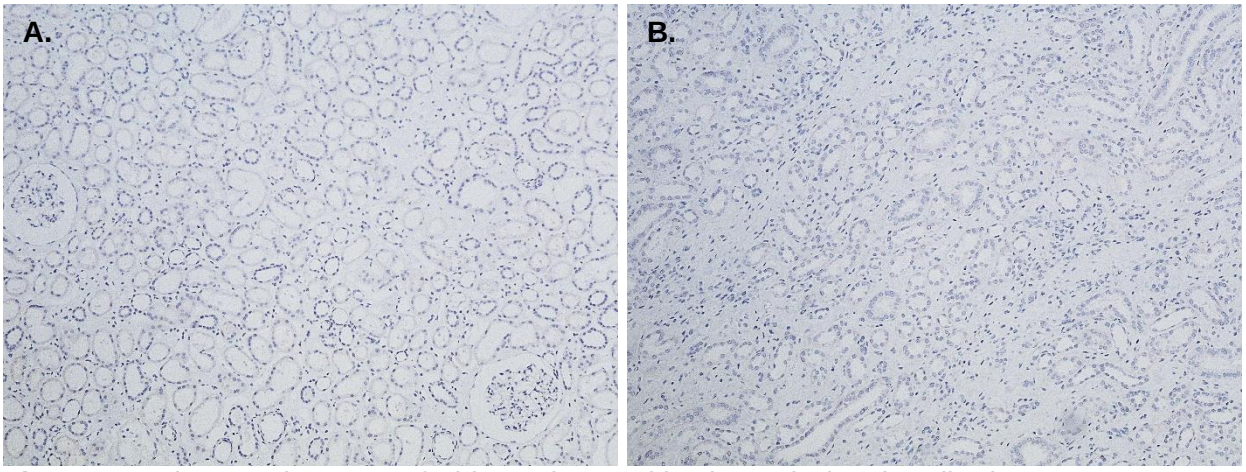

Figure 18. Microscopic Image of Kidney Tissue with RhD Polyclonal Antibody
Negative labeling of (A.) cortex, (B.) medulla (10x).

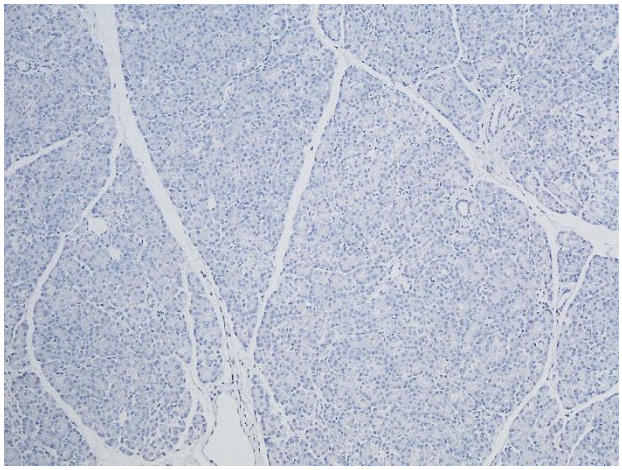

Figure 19. Microscopic Image of Pancreas Tissue with RhD Polyclonal Antibody
Negative labeling of exocrine and endocrine cells (10x).

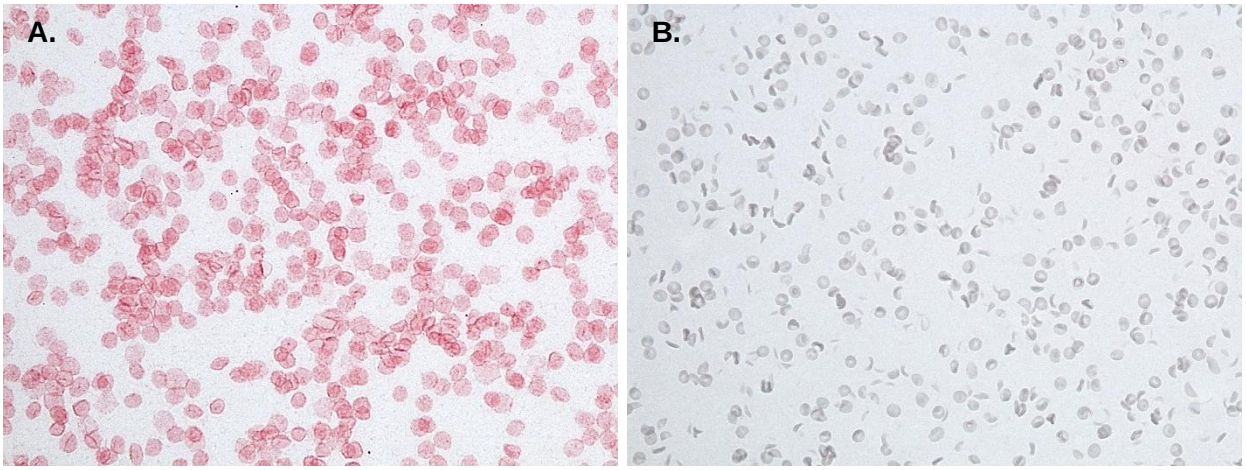

Figure 20. Microscopic Image of RBC Control with RhD Polyclonal Antibody
Positive labeling of RhD antigen-positive RBCs via blood smear technique (A.) 50x. Negative labeling of RhD antigen-positive RBCs with FFPE RBC cell pellet (B.) 50x.

2.4.2.2. RHCE

There was no detection of the RhCE protein in heart (Figure 21), lung (Figure 22), kidney (Figure 23), or pancreas tissue (Figure 24) (Table 21), except for positive labeling of RBCs, and this reactivity was consistent across all biopsy samples of all tissue types.

Diffuse labeling of RBCs within each organ served as the best positive control. Additionally, the anti-RhCE antibody produced negative RBC labeling for the FFPE cell pellet and positive labeling for the RBC blood smear technique (Figure 25).

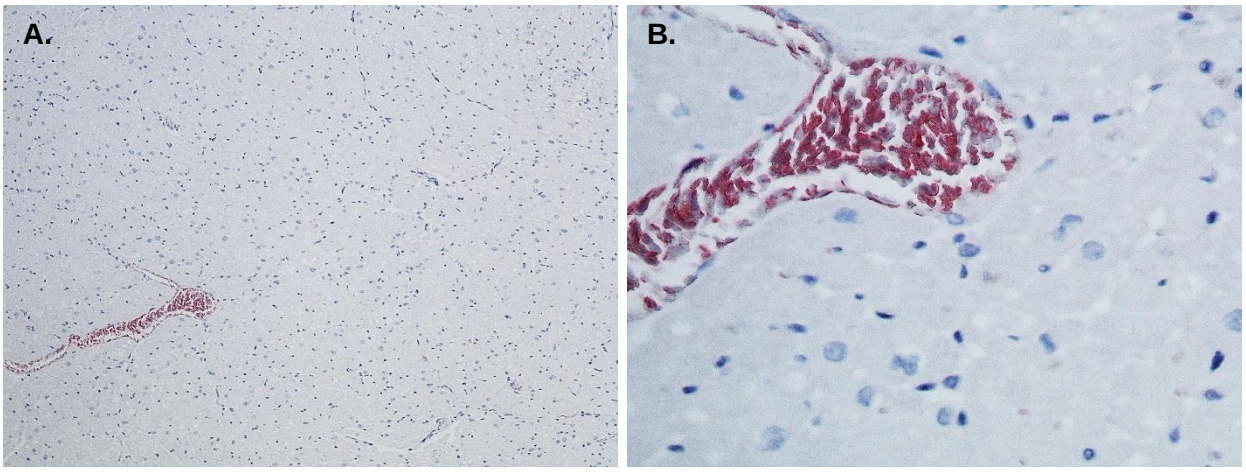

Figure 21. Microscopic Image of Heart Tissue with RhCE Polyclonal Antibody
Negative labeling of cardiomyocytes (A.) 10x, (B.) 50x. Positive labeling is seen on RBCs.

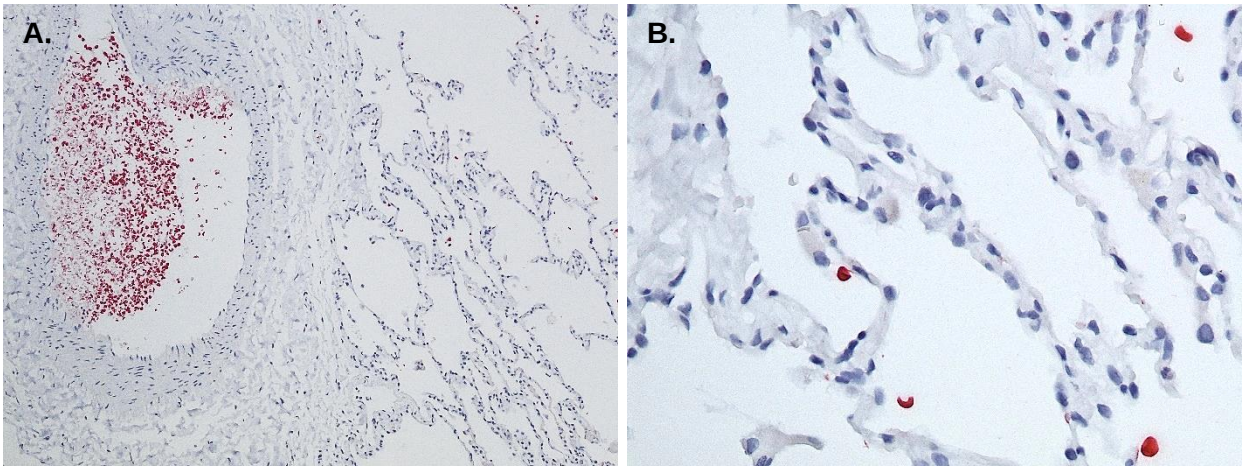

Figure 22. Microscopic Image of Lung Tissue with RhCE Polyclonal Antibody
Negative labeling of alveolar cells, and macrophages (A.) 10x, (B.) 50x. Positive labeling is seen on RBCs.

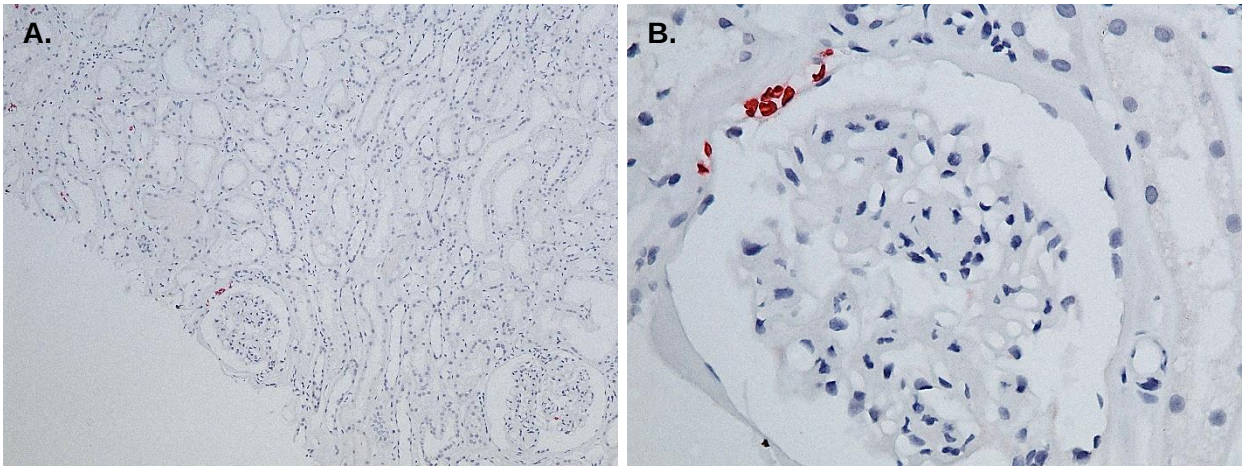

Figure 23. Microscopic Image of Kidney Tissue with RhCE Polyclonal Antibody
Negative labeling of the cortex (A.) 10x, (B.) 50x. Positive labeling is seen on RBCs. Negative labeling of the medulla was also seen (not shown).

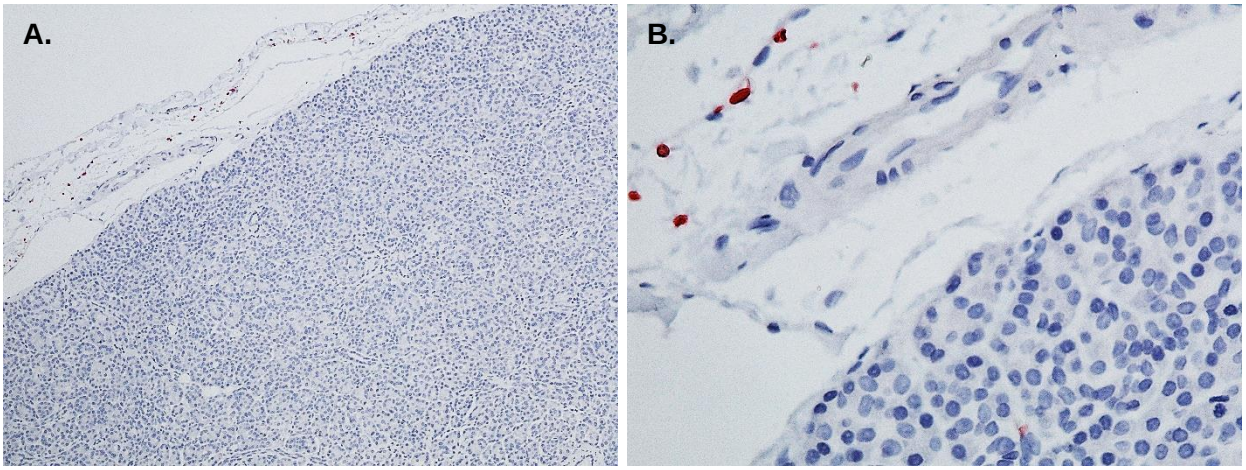

Figure 24. Microscopic Image of Pancreas Tissue with RhCE Polyclonal Antibody
Negative labeling of exocrine and endocrine cells (A.) 10x, (B.) 50x. Positive labeling is seen on RBCs.

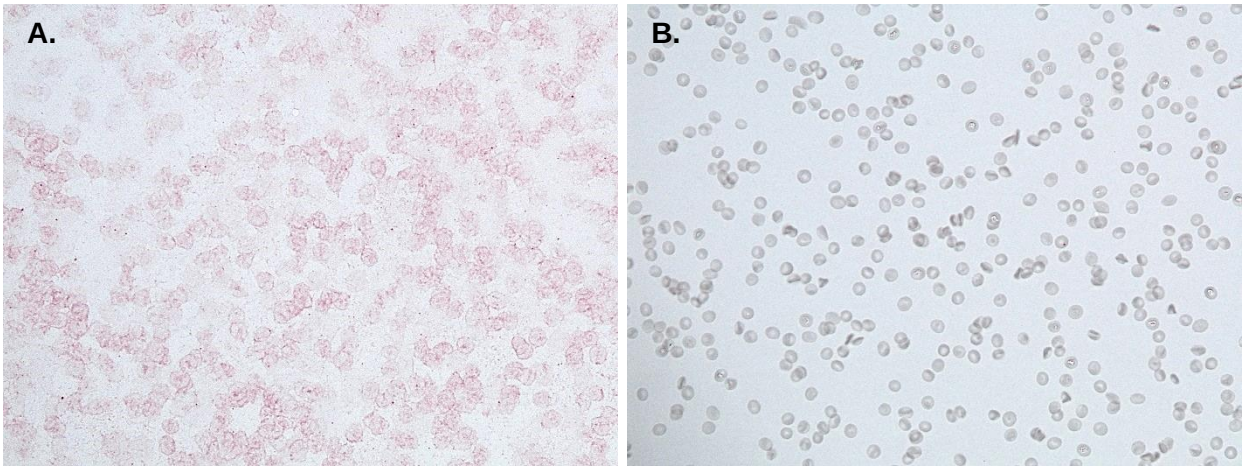

Figure 25. Microscopic Image of RBC Control with RhCE Polyclonal Antibody
Positive labeling of RhCE antigen-positive RBCs via blood smear technique (A.) 50x. Negative labeling of RhCE antigen-positive RBCs with FFPE RBC cell pellet (B.) 50x.

2.4.3. Kell System

The Kell glycoprotein was detected in the heart, lung, kidney, and pancreas (Table 21).

In heart tissue, there was positive cytoplasmic labeling throughout the cardiomyocytes of each biopsy sample, and stronger labeling of the intercalated discs (*n*=6) (Figure 26). Positive cytoplasmic labeling was detected in pulmonary macrophages and bronchial epithelium (*n*=6) (Figure 27). In the kidney, positive cytoplasmic labeling was detected in the proximal and distal tubular epithelial cells of the cortex (the proximal tubules demonstrated stronger labeling) (*n*=6), and in the medullary tubular epithelium (mostly cuboidal epithelium) and urothelium, where

included in the sections (biopsy K1 and K6) (Figure 28). In pancreas tissue, positive labeling was noted in endocrine cells (islets of Langerhans) (*n*=7) (Figure 29). RBCs were not labeled.

For RBC quality control, the Kell antibody produced positive labeling for both the FFPE cell pellet and the RBC blood smear technique (Figure 30).

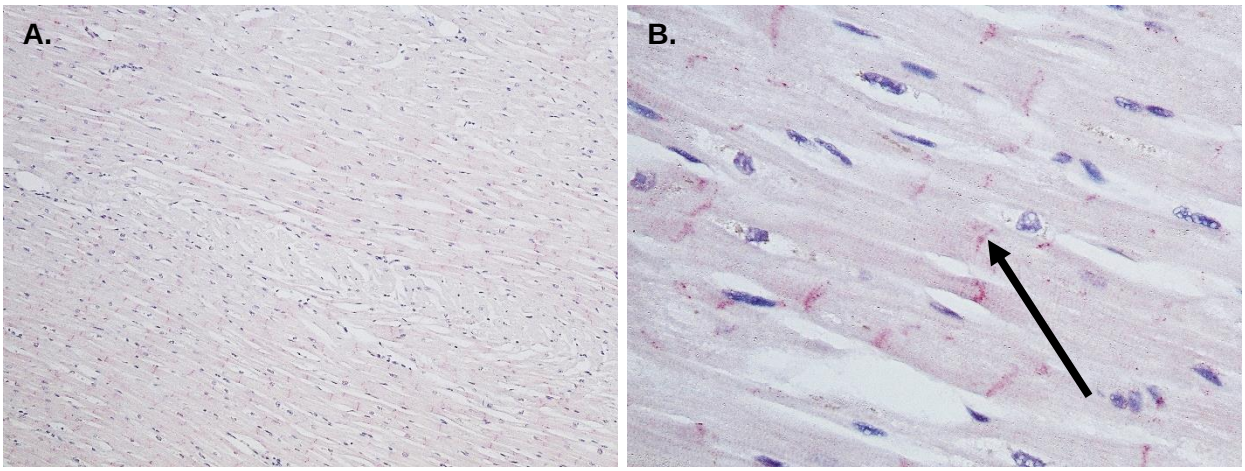

Figure 26. Microscopic Image of Heart Tissue with Kell Polyclonal Antibody
Positive labeling of the intercalated discs of cardiomyocytes (arrow).

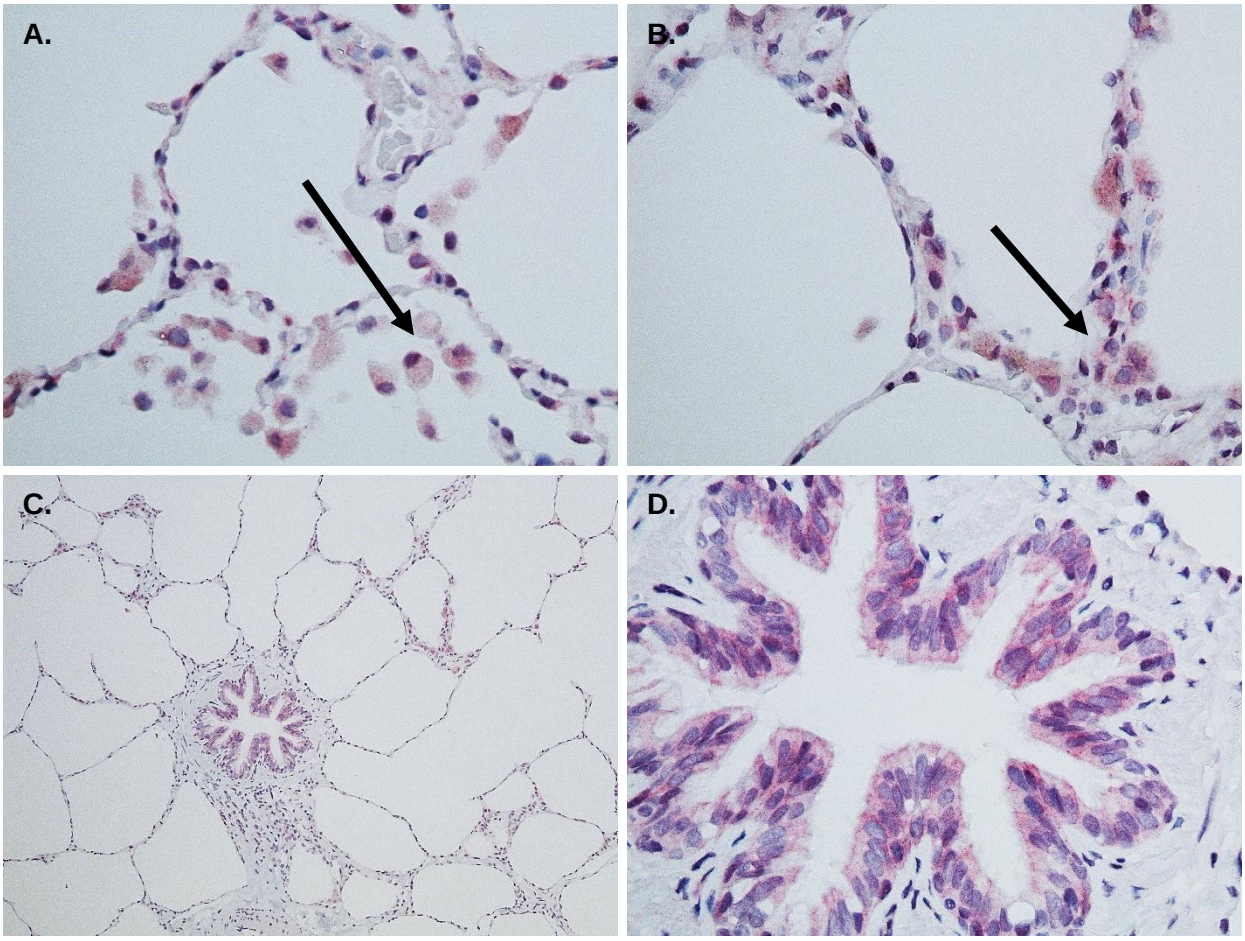

Figure 27. Microscopic Image of Lung Tissue with Kell Polyclonal Antibody
Positive labeling of macrophages (arrow) (A., B.) 50x. Negative labeling of RBCs is also present. Positive labeling of bronchial epithelium (C.) 10x, (D.) 50x.

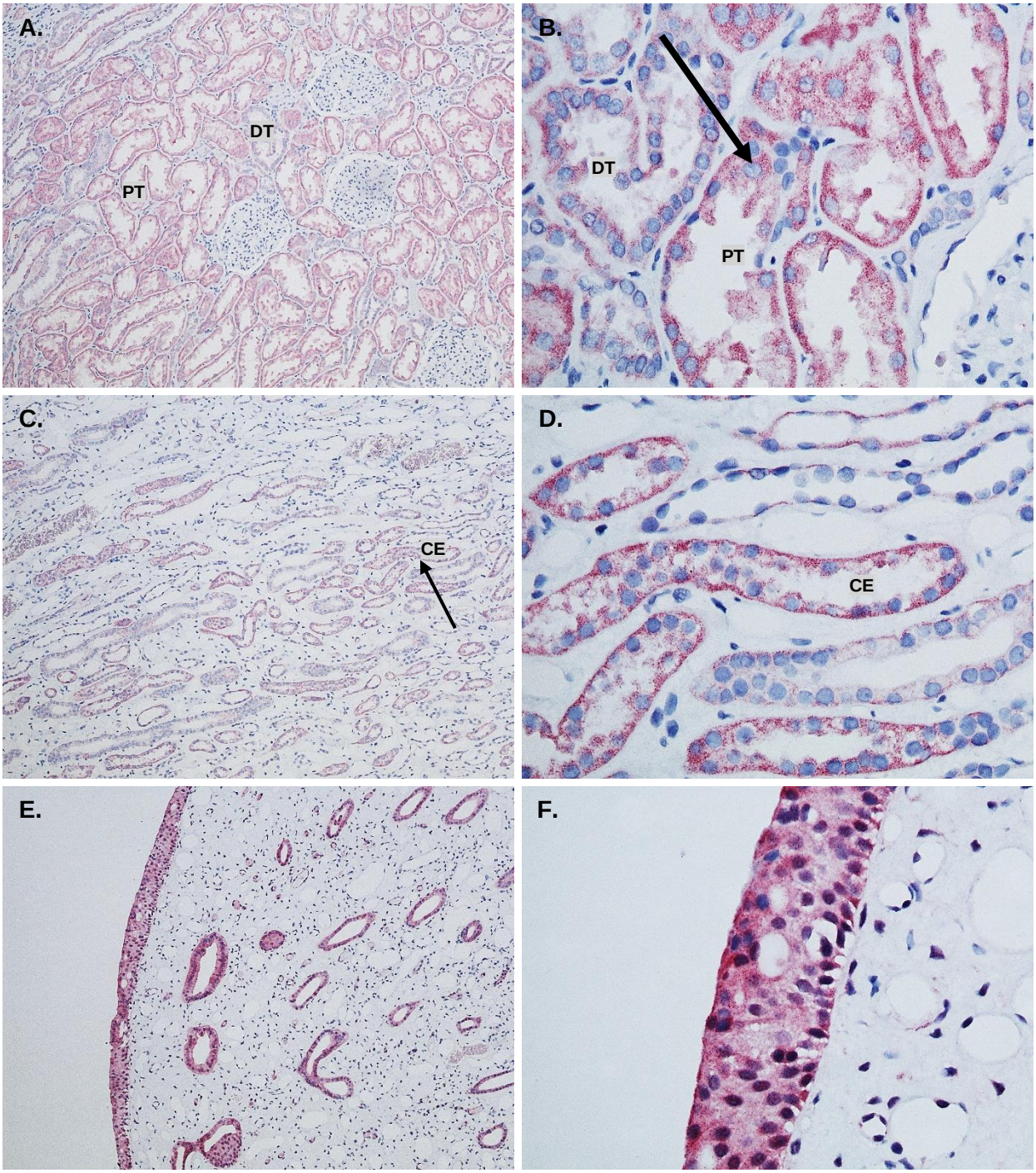

Figure 28. Microscopic Image of Kidney Tissue with Kell Polyclonal Antibody
Positive labeling of the proximal (PT) (arrow) and distal (DT) tubules of the cortex (A.) 10x, (B.) 50x. Positive labeling of the tubules (mostly cuboidal epithelium (CE)) (arrow) of the medulla (C.) 10x, (D.) 50x. Positive labeling of urothelium (E.) 10x, (F.) 50x.

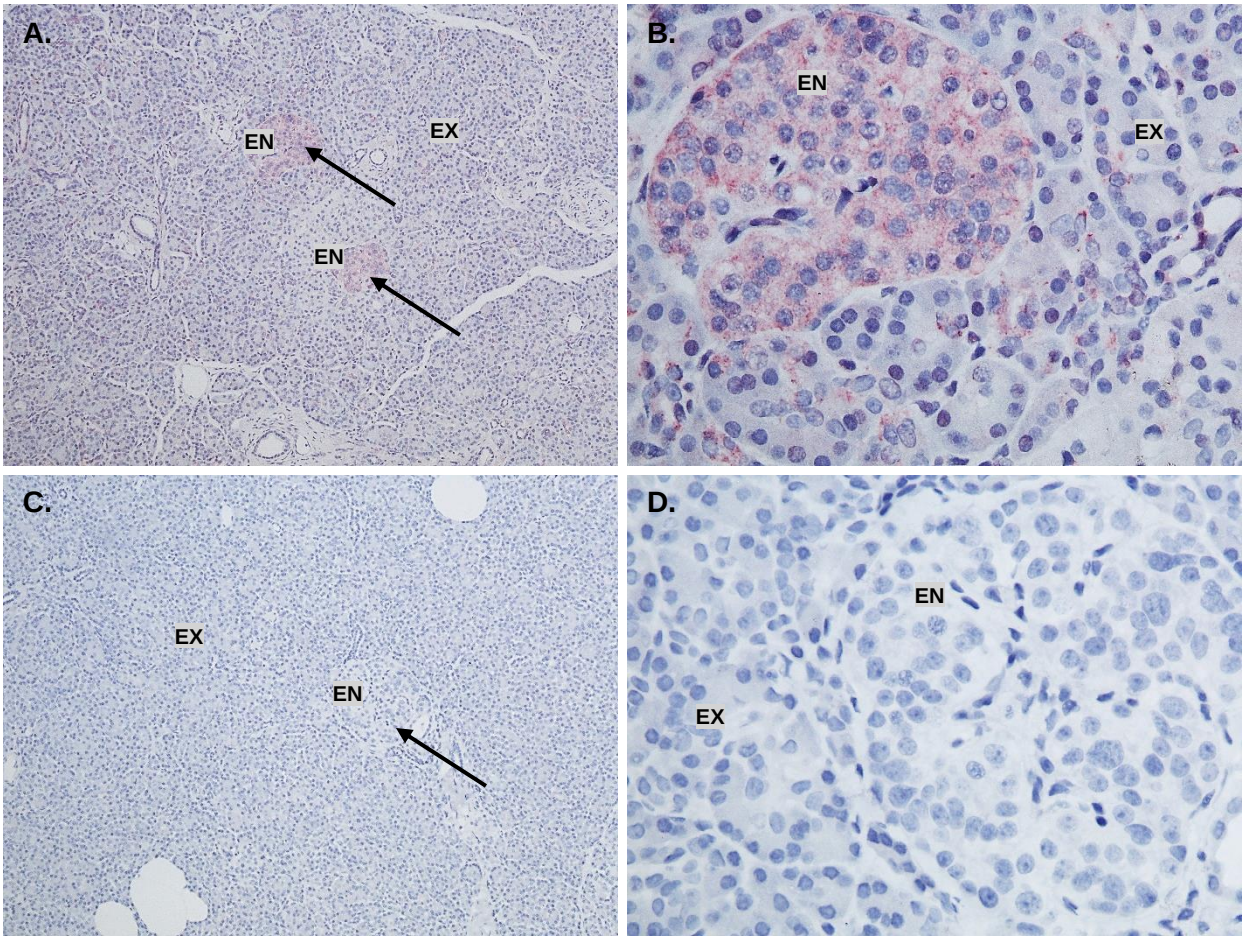

Figure 29. Microscopic Image of Pancreas Tissue with Kell Polyclonal Antibody
Positive labeling of endocrine cells (EN) (arrow), negative exocrine cells (EX) (A.) 10x, (B.) 50x. Negative control (C.) 10x, (D.) 50x.

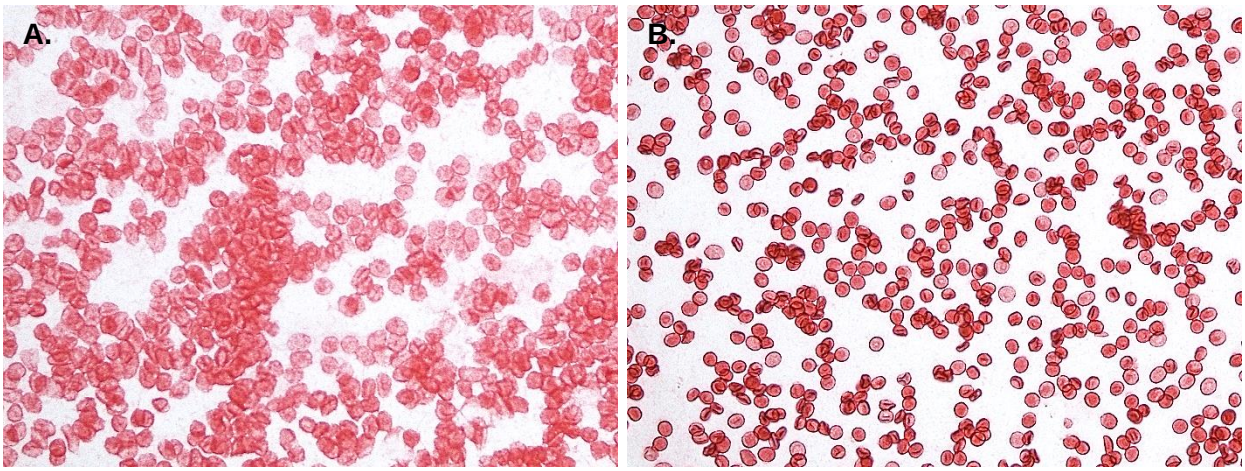

Figure 30. Microscopic Image of RBC Control with Kell Polyclonal Antibody
Positive labeling of Kell antigen-positive RBCs via blood smear technique (A.), and positive labeling of Kell antigen-positive RBCs with FFPE RBC cell pellet (B.) 50x.

2.4.4. Lewis System

The Lewis (FUT3) antigen was detected in heart, kidney, and pancreas tissue (Table 21).

In heart tissue, there was positive labeling throughout the cardiomyocytes (*n*=6) (Figure 31). In lung tissue, positive labeling was seen in the bronchial epithelium (*n*=6) (Figure 32). In the kidney, Lewis (FUT3) was detected in the cells of both the distal and proximal tubules (the proximal tubules had stronger labeling) (*n*=6), and on the urothelium, where included in the sections (biopsy K1 and K6) (Figure 33). In pancreatic tissue, positive labeling was noted in endocrine cells (islets of Langerhans) (*n*=7) (Figure 34). Positive labeling of RBCs was not detected.

The Lewis (FUT3) antibody produced negative labeling for the FFPE cell pellet and positive labeling for the RBC blood smear technique (Figure 35).

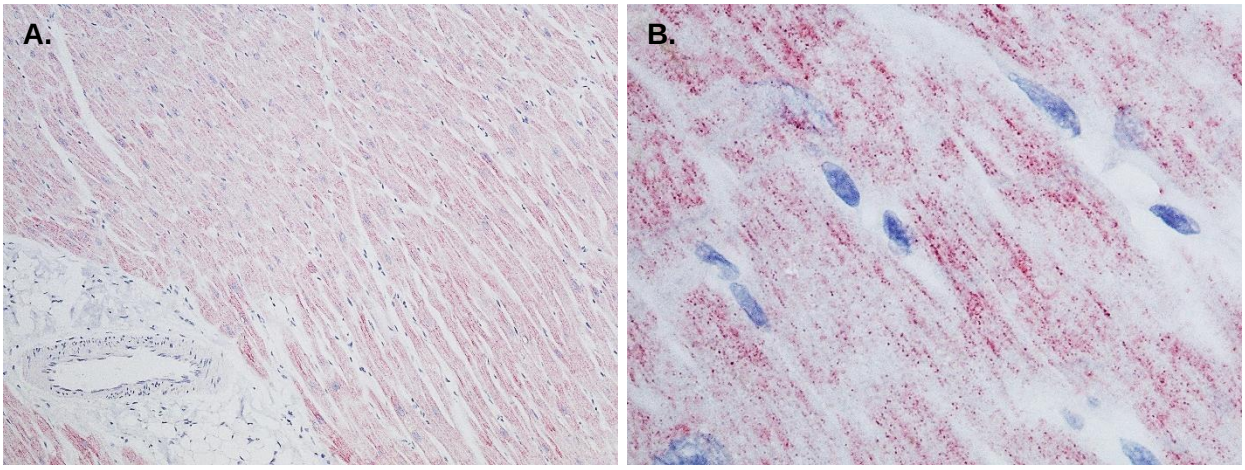

Figure 31. Microscopic Image of Heart Tissue with Lewis (FUT3) Polyclonal Antibody
Positive labeling of cardiomyocytes (A.) 10x, (B.) 100x.

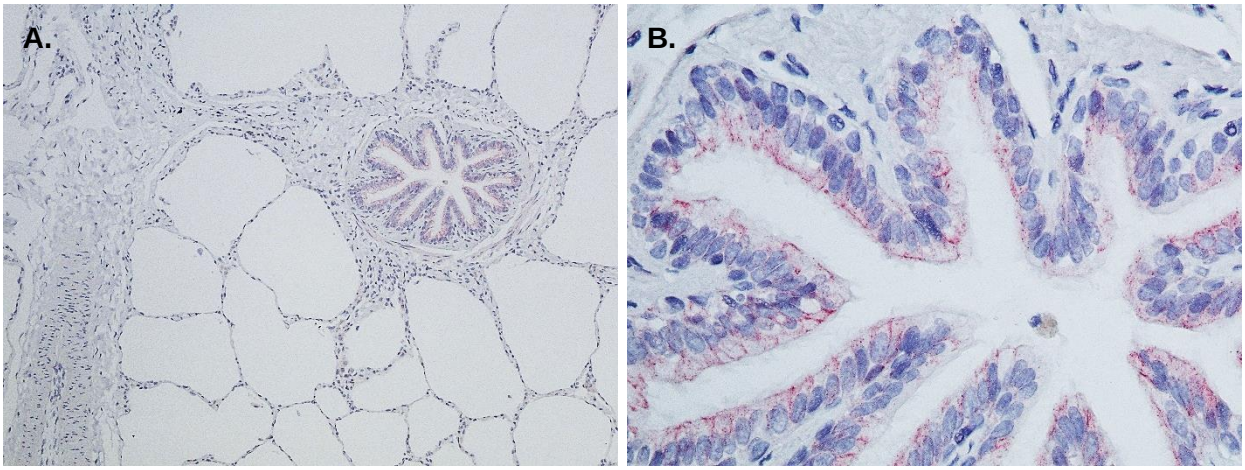

Figure 32. Microscopic Image of Lung Tissue with Lewis (FUT3) Polyclonal Antibody Positive labeling of bronchial epithelium (A.) 10x, (B.) 50x.

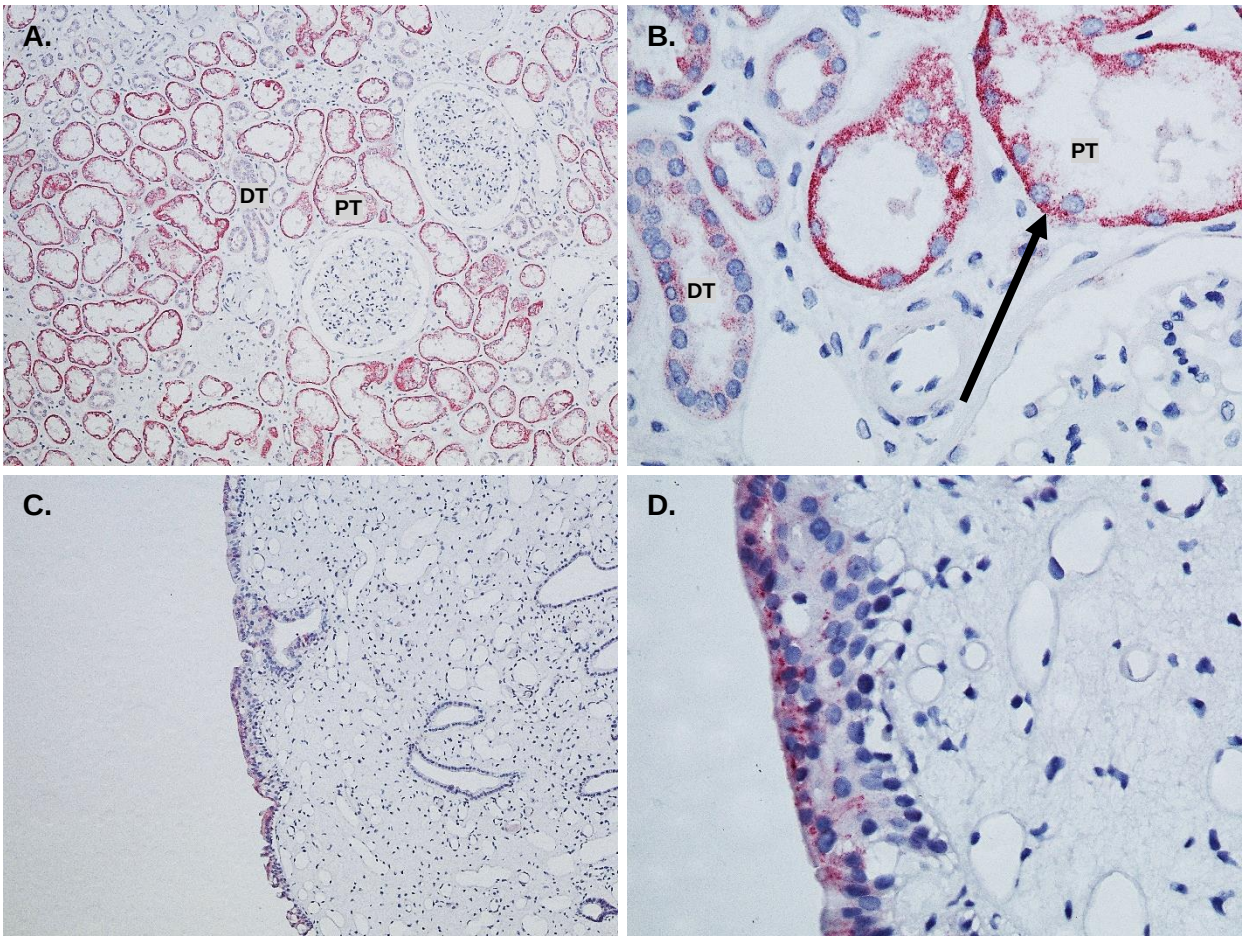

Figure 33. Microscopic Image of Kidney Tissue with Lewis (FUT3) Polyclonal Antibody Positive labeling of proximal (PT) (arrow) and distal (DT) tubules of the cortex (A.) 10x, (B.) 50x. Negative labeling of the medulla was also seen (not shown). Positive labeling of urothelium (C.) 10x, (D.) 50x.

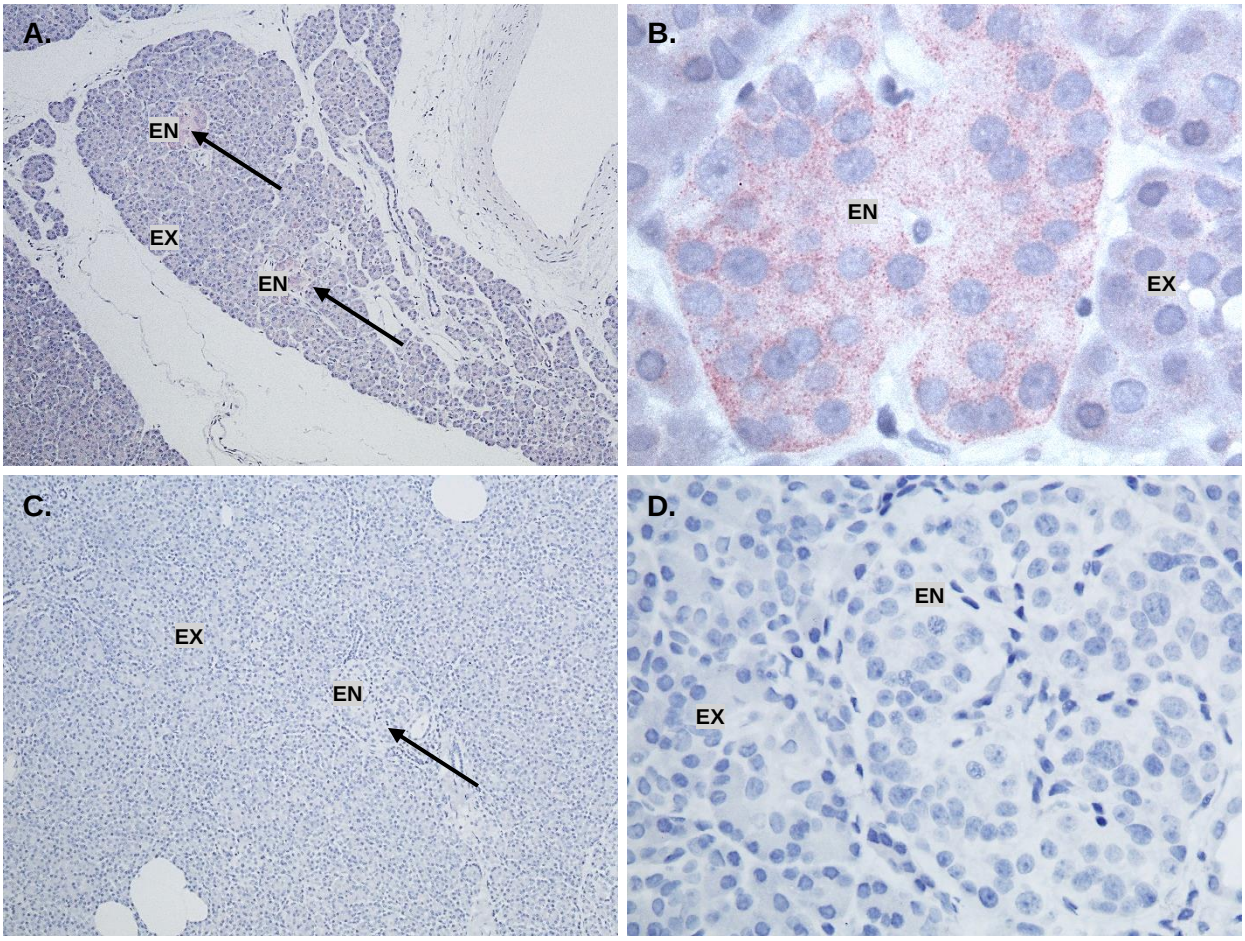

Figure 34. Microscopic Image of Pancreas Tissue with Lewis (FUT3) Polyclonal Antibody Positive labeling of endocrine cells (EN) (arrow), negative exocrine cells (EX) (A.) 10x, (B.) 100x. Negative control (C.) 10x, (D.) 50x.

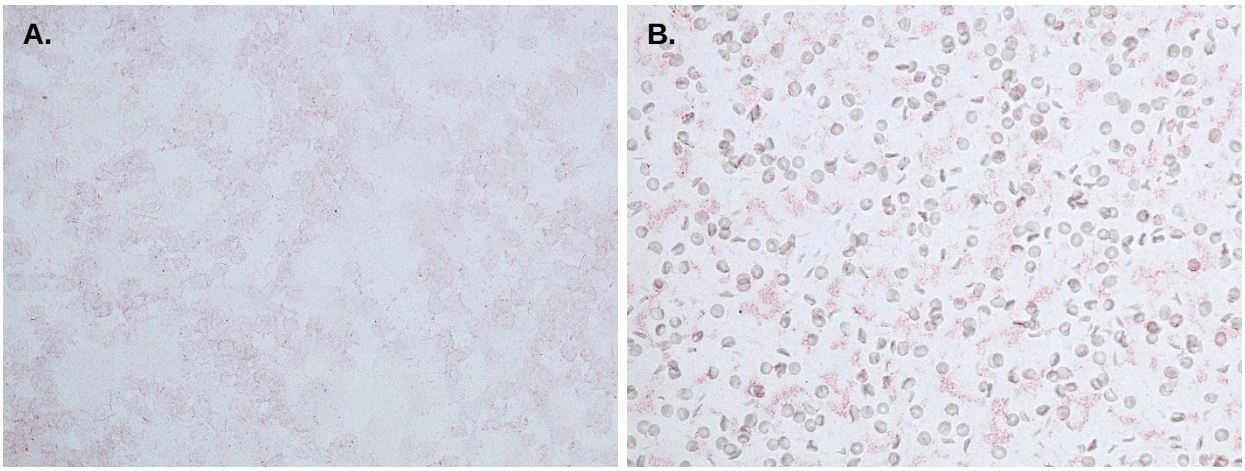

Figure 35. Microscopic Image of RBC Control with Lewis (FUT3) Polyclonal Antibody Positive labeling of Lewis antigen-positive RBCs via blood smear technique (A.) 50x. Negative labeling of Lewis antigen-positive RBCs with FFPE RBC cell pellet (B.) 50x. (note: chromogen substrate seen on background only for FFPE cell pellet).

2.4.5. Duffy System

The Duffy (ACKR1) glycoprotein was detected in heart and kidney tissues, and not detected in lung or pancreas (Table 21) except within endothelial cells of various blood vessels.

In heart tissue, labeling was noted in cardiomyocytes and intercalated discs, but this was only found in heart biopsies H4, H5, and H6. The remaining biopsies lacked positive labeling of cardiomyocytes and intercalated discs. All heart biopsy samples had positive labeling of endothelial cells throughout blood vessels of the myocardium and pericardium (*n*=6) (Figure 36). In lung tissue, negative labeling was seen for alveolar cells and macrophages, and positive labeling was noted along endothelial cells of various blood vessels (*n*=6) (Figure 37). In kidney tissue, labeling was present along the microvilli of the proximal tubules (*n*=6), in the urothelium, where included in the sections (biopsy K1 and K6), and along endothelial cells of various blood vessels (*n*=6) (Figure 38). In pancreas tissue, positive labeling was seen along endothelial cells of various blood vessels (*n*=7) (Figure 39). RBCs did not label.

The anti- Duffy (ACKR1) antibody produced negative labeling for the FFPE cell pellet and positive labeling for the RBC blood smear technique (Figure 40).

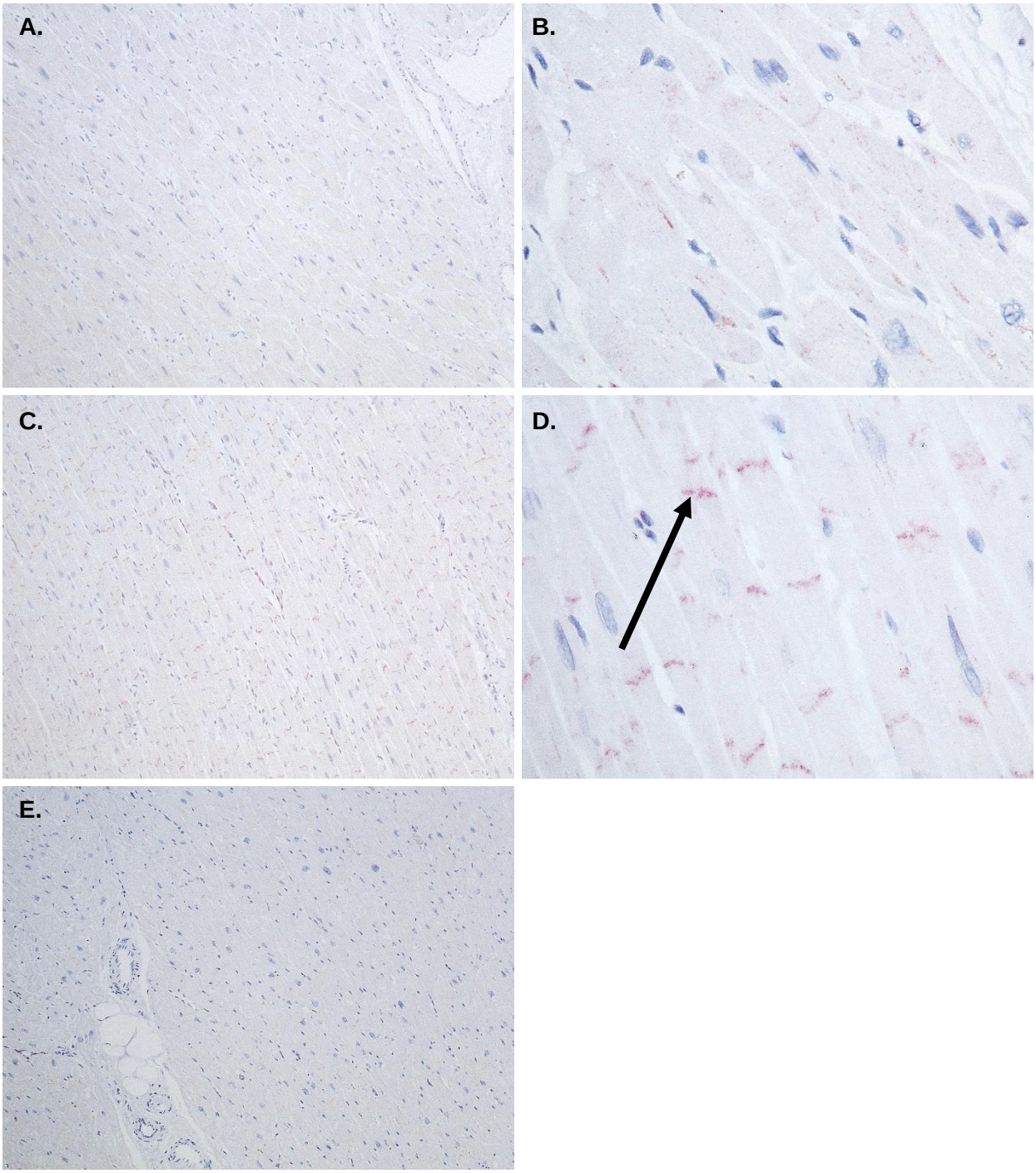

Figure 36. Microscopic Image of Heart Tissue with Duffy (ACKR1) Polyclonal Antibody
Positive labeling of cardiomyocytes (A.) 10x, (B.) 50x. Positive labeling of the intercalated discs of cardiomyocytes (arrow) (C.) 10x, (D.) 50x. Negative labeling (E.) 10x, seen in biopsies H1, H2, H3. Positive labeling of endothelial cells of blood vessels in the pericardium (F.) 10x, (G.) 50x. Negative labeling of RBCs is also present (arrow). Positive labeling of endothelial cells in blood vessels in the myocardium (H.) 50x, (I.) 100x.

Figure 36. (cont'd)

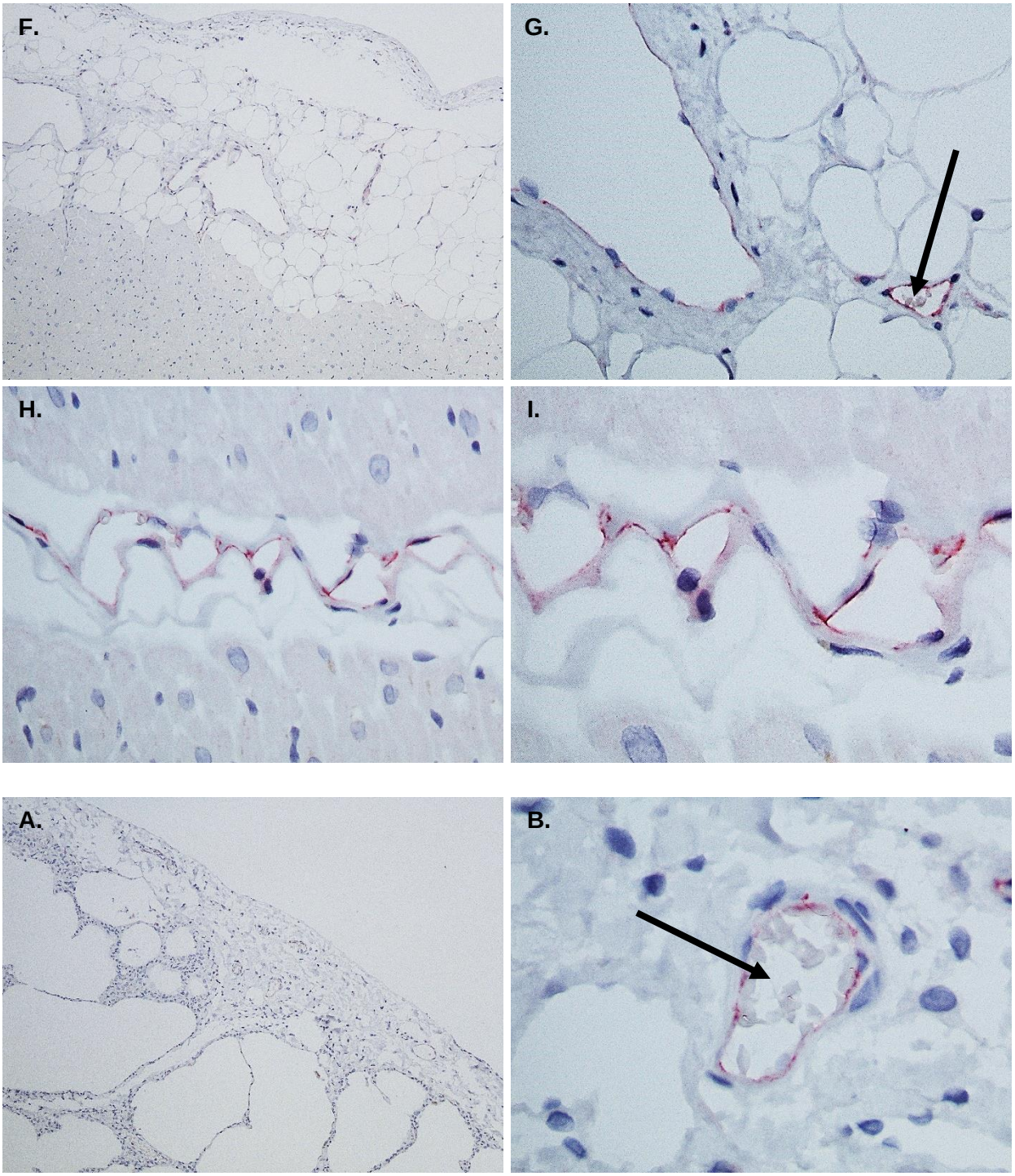

Figure 37. Microscopic Image of Lung Tissue with Duffy (ACKR1) Polyclonal Antibody
Positive labeling of endothelial cells of blood vessels (A.) 10x, (B.) 100x. Negative labeling of RBCs is also present (arrow).

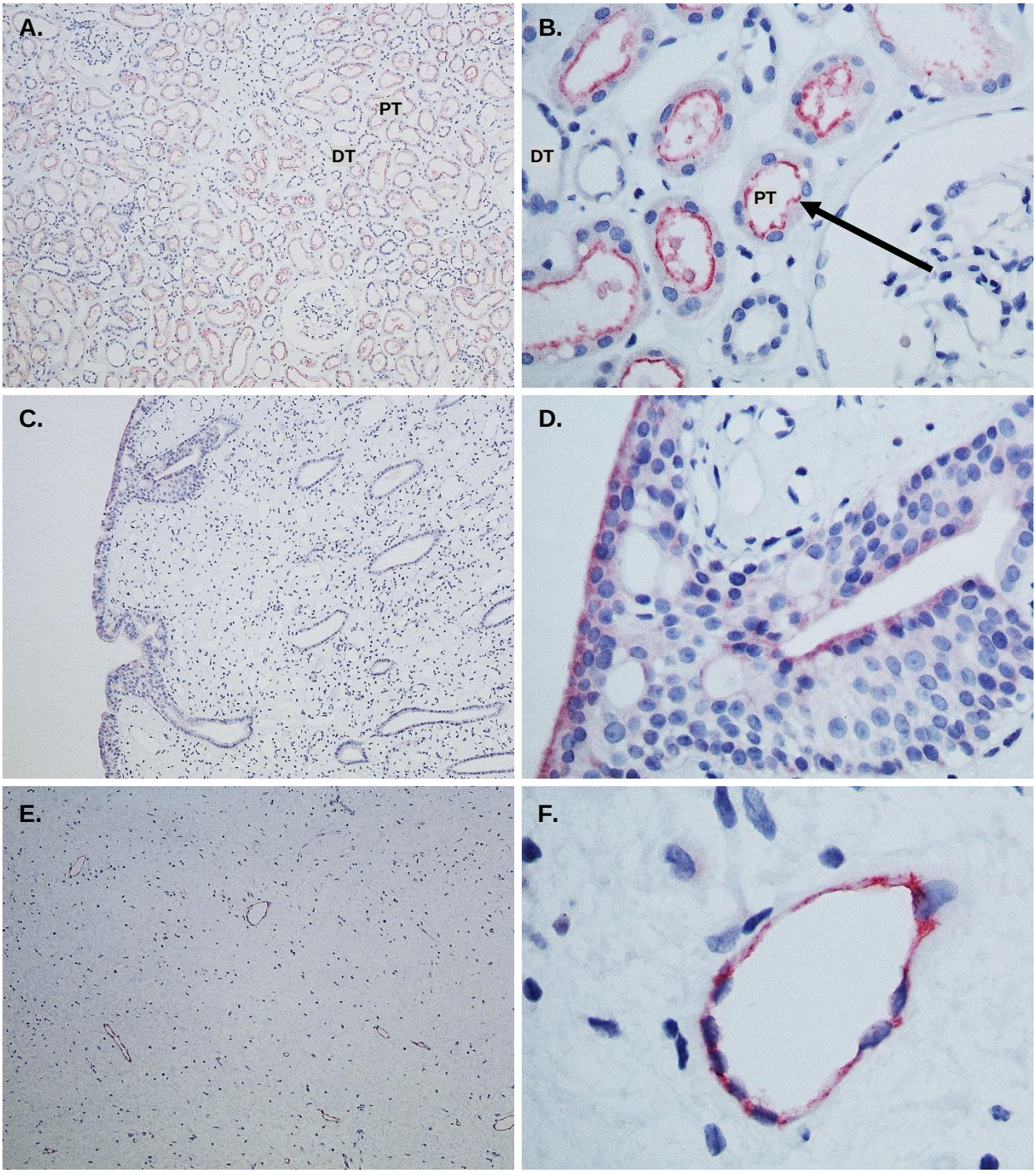

Figure 38. Microscopic Image of Kidney Tissue with Duffy (ACKR1) Polyclonal Antibody Positive labeling of the microvilli of the proximal (PT) tubules of the cortex (arrow), and negative labeling of distal (DT) tubules (A.) 10x, (B.) 50x. Negative labeling of the medulla was also seen (not shown). Positive labeling of urothelium (C.) 10x, (D.) 50x. Positive labeling of endothelial cells of blood vessels (E.) 10x, (F.) 100x.

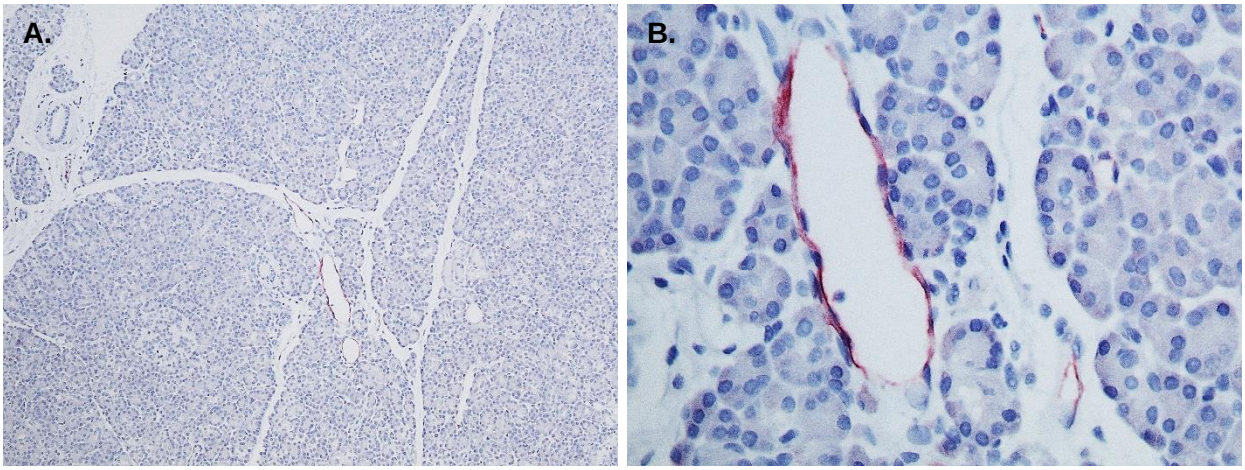

Figure 39. Microscopic Image of Pancreas Tissue with Duffy (ACKR1) Polyclonal Antibody Positive labeling of endothelial cells of blood vessels (A.) 10x, (B.) 50x.

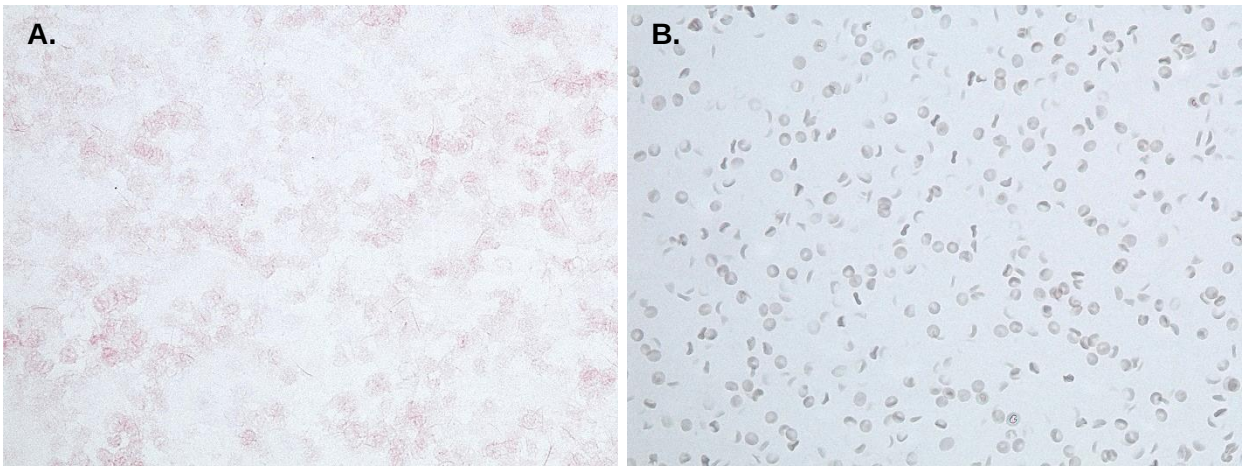

Figure 40. Microscopic Image of RBC Control with Duffy (ACKR1) Polyclonal Antibody Positive labeling of Duffy antigen-positive RBCs via blood smear technique (A.) 50x. Negative labeling of Duffy antigen-positive RBCs with FFPE RBC cell pellet (B.) 50x.

2.4.6. Kidd System

The Kidd (SLC14A1) glycoprotein was detected in heart, kidney, and pancreas tissue (Table 21). Additionally, positive labeling was seen on infiltrating leukocytes (likely granulocytes), in each tissue type.

In heart tissue, positive labeling was seen in cardiomyocytes and particularly in intercalated discs (*n*=6) (Figure 41). In lung tissue, positive labeling was seen in the bronchial epithelium (*n*=6) (Figure 42). In kidney tissue, positive labeling was noted in the squamous epithelium of the medulla (thin loop of Henle) and the urothelium, when present in the sections (biopsy K1 and K6) (Figure 43). In pancreas tissue, positive labeling was noted on endocrine cells. The intensity of endocrine cell (islets of Langerhans) labeling varied (low detection in biopsies P2, P3, P4, P5, and P6; high detection in biopsies P1 and P7) within and between biopsy samples (Figure 44). Positive labeling of Kidd (SLC14A1) was also detected on pancreatic ductal epithelial cells (*n*=7) (Figure 44). RBCs did not label.

The Kidd (SLC14A1) antibody produced negative labeling for the FFPE cell pellet and positive labeling for the RBC blood smear technique (Figure 45).

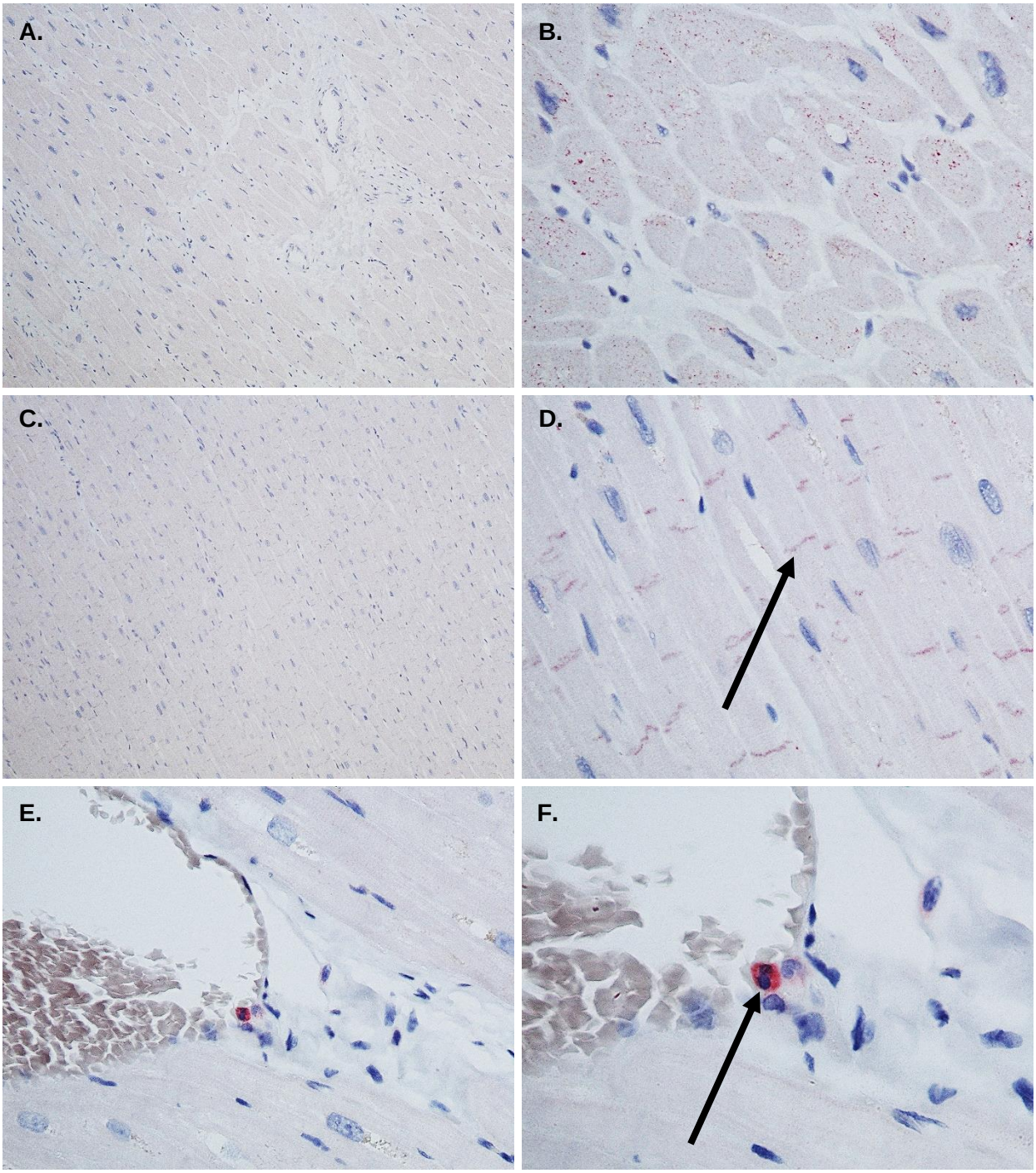

Figure 41. Microscopic mage of Heart Tissue with Kidd (SLC14A1) Polyclonal Antibody Positive labeling of cardiomyocytes (A.) 10x, (B.) 50x. Positive labeling of the intercalated discs of cardiomyocytes (arrow) (C.) 10x, (D.) 50x. Positive labeling of leukocytes (arrow) (E.) 50x, (F.) 100x. Negative labeling of RBCs is also present.

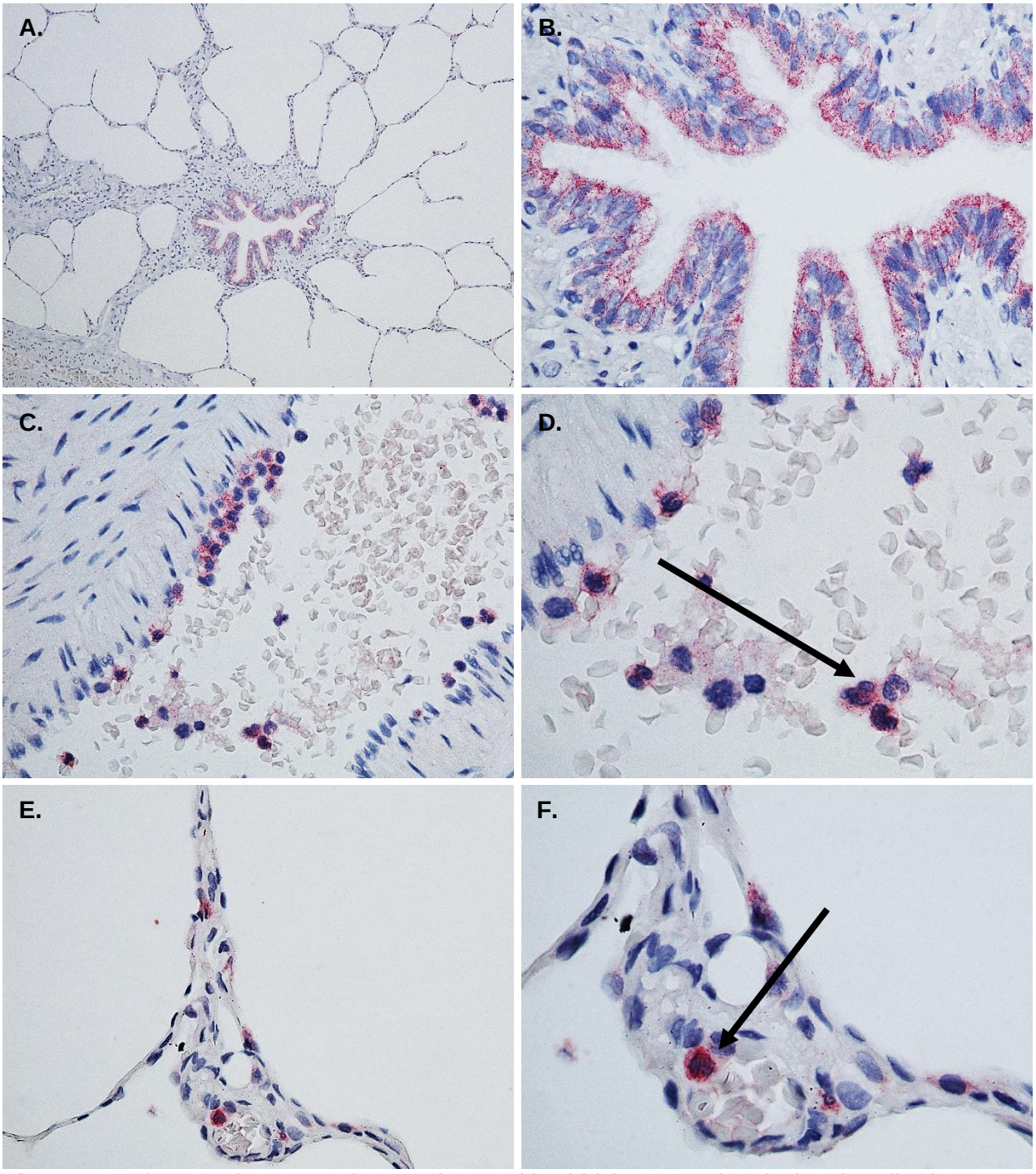

Figure 42. Microscopic Image of Lung Tissue with Kidd (SLC14A1) Polyclonal Antibody Positive labeling of bronchial epithelium (A.) 10x, (B.) 50x. Positive labeling of leukocytes (arrow) (C., E.) 50x, (D., F.) 100x. Negative labeling of RBCs is also present.

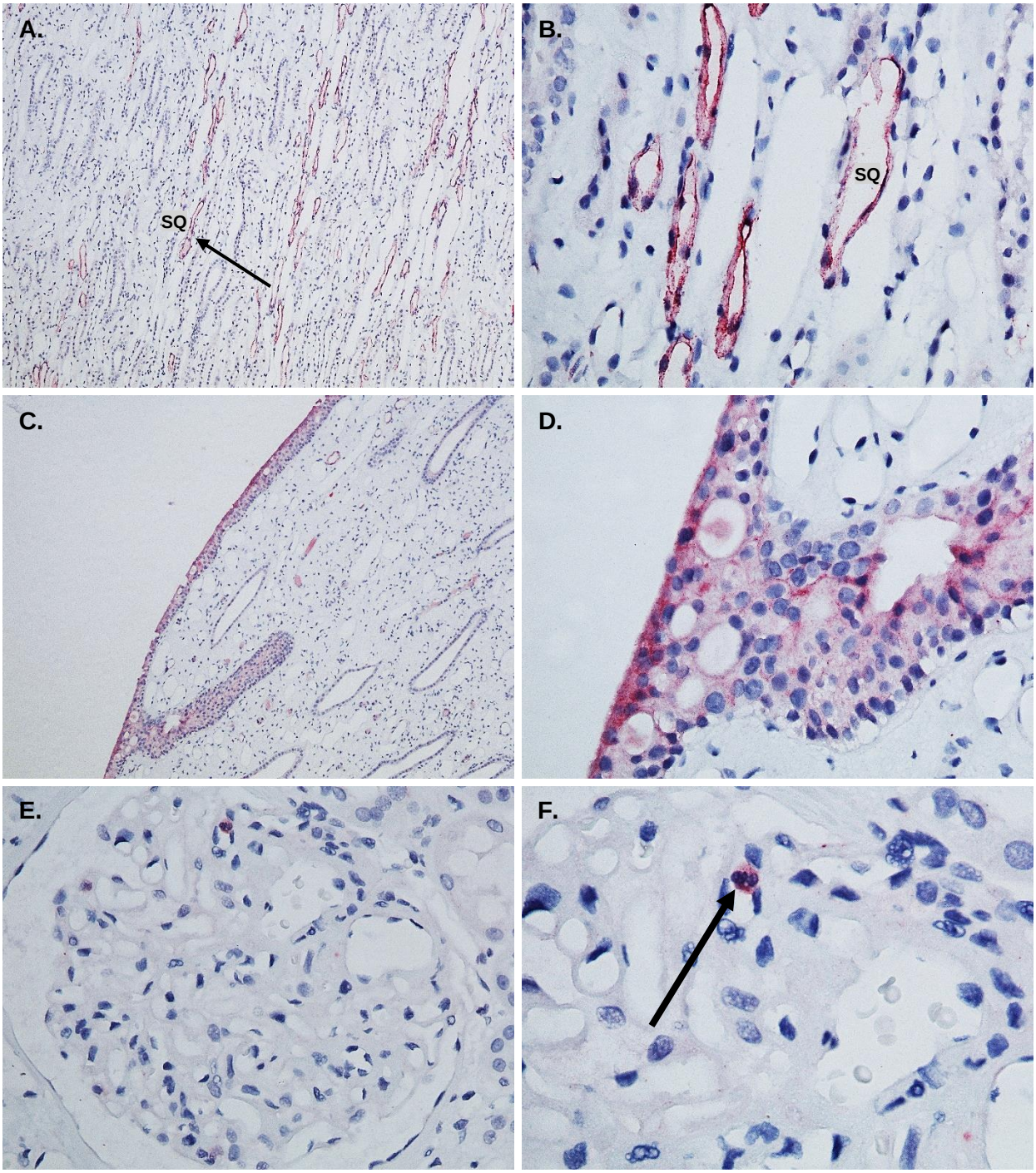

Figure 43. Microscopic Image of Kidney Tissue with Kidd (SLC14A1) Polyclonal Antibody Positive labeling of squamous epithelium (SQ) in the medulla (arrow) (A.) 10x, (B.) 50x. Negative labeling of the cortex was also seen (not shown). Positive labeling of urothelium (C.) 10x, (D.) 50x. Positive labeling of leukocytes (arrow) (E.) 50x, (F.) 100x.

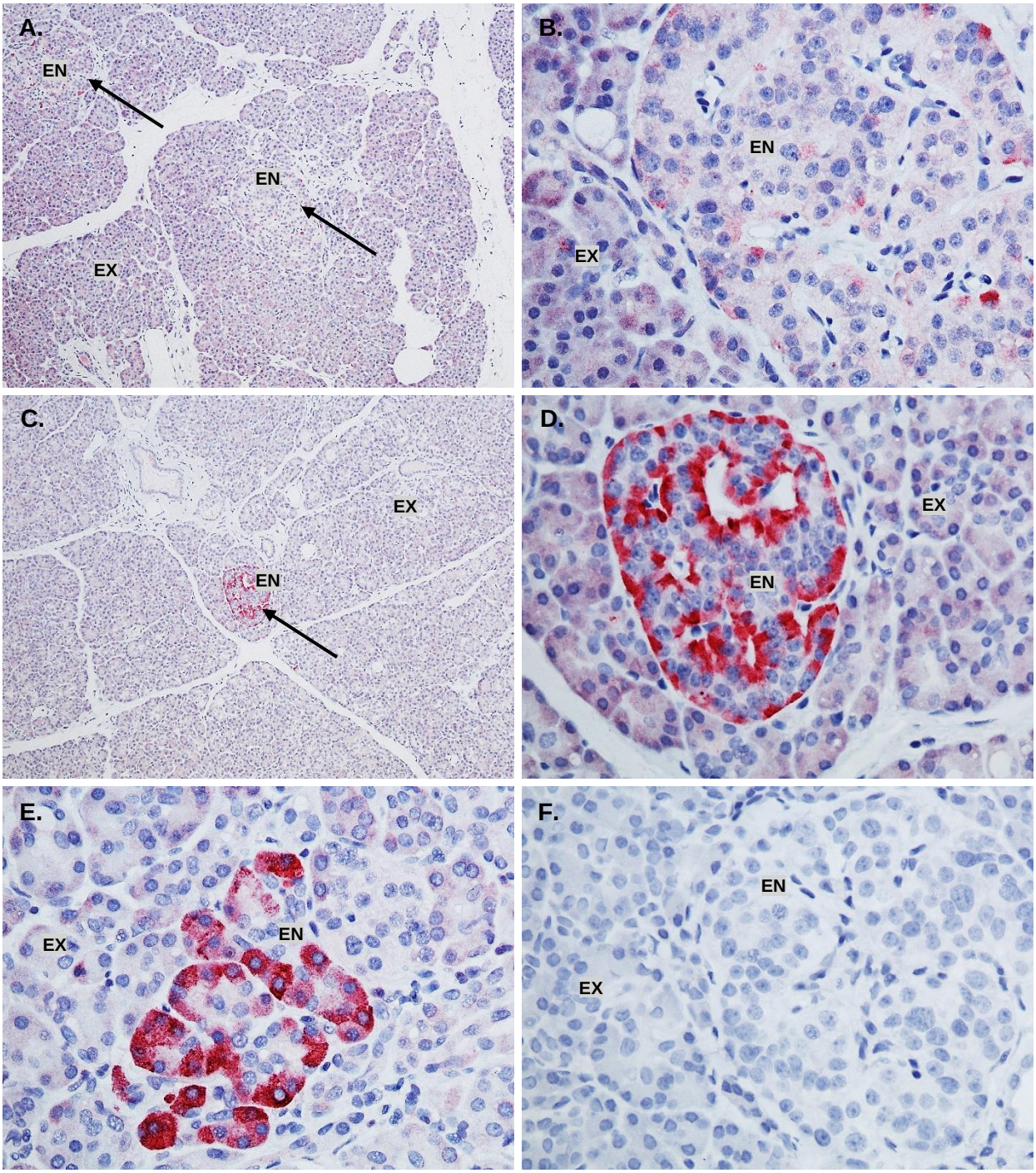

Figure 44. Microscopic Image of Pancreas Tissue with Kidd (SLC14A1) Polyclonal Antibody

Positive labeling of endocrine cells (EN) (arrow), negative exocrine cells (EX) (A., C.) 10x, (B., D., E.) 50x. Positive labeling of ductal epithelial cells (G.) 10x, (H.) 50x. Negative control (F.) 50x. Positive labeling of leukocytes (I.) 50x, (J.) 100x.

Figure 44. (cont'd)

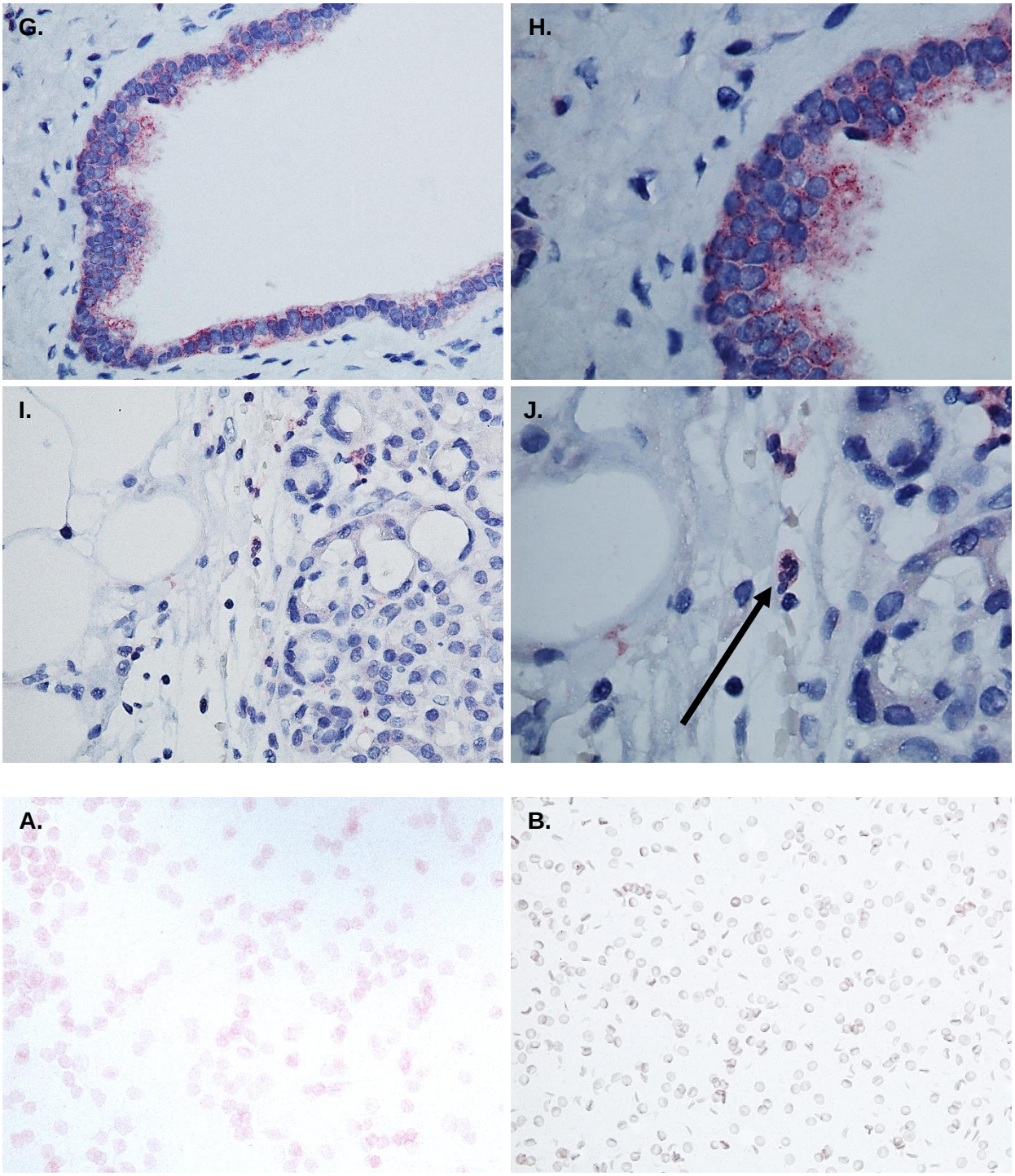

Figure 45. Microscopic Image of RBC Control with Kidd (SLC14A1) Polyclonal Antibody Positive labeling of Kidd antigen-positive RBCs via blood smear technique (A.) 50x. Negative labeling of Kidd antigen-positive RBCs with FFPE RBC cell pellet (B.) 50x.

2.4.7. XG System

XG (CD99) was detected in heart and pancreas but not in lung or kidney tissue (Table 21), except that lymphocytes and fibroblasts were positively labeled wherever they were seen.

Positive labeling was present in cardiomyocytes and intercalated discs, (*n*=6) (Figure 46), and in the pericardium (not shown). Negative labeling was seen in both lung (*n*=6) (Figure 47) and kidney (*n*=6) tissue (Figure 48) except for positive labeling of lymphocytes and fibroblasts. In pancreas tissue, positive labeling was seen in endocrine cells (islets of Langerhans) (*n*=7) (Figure 49). RBCs did not label.

The XG (CD99) antibody produced negative labeling for the FFPE cell pellet and positive labeling for the RBC blood smear technique (Figure 50).

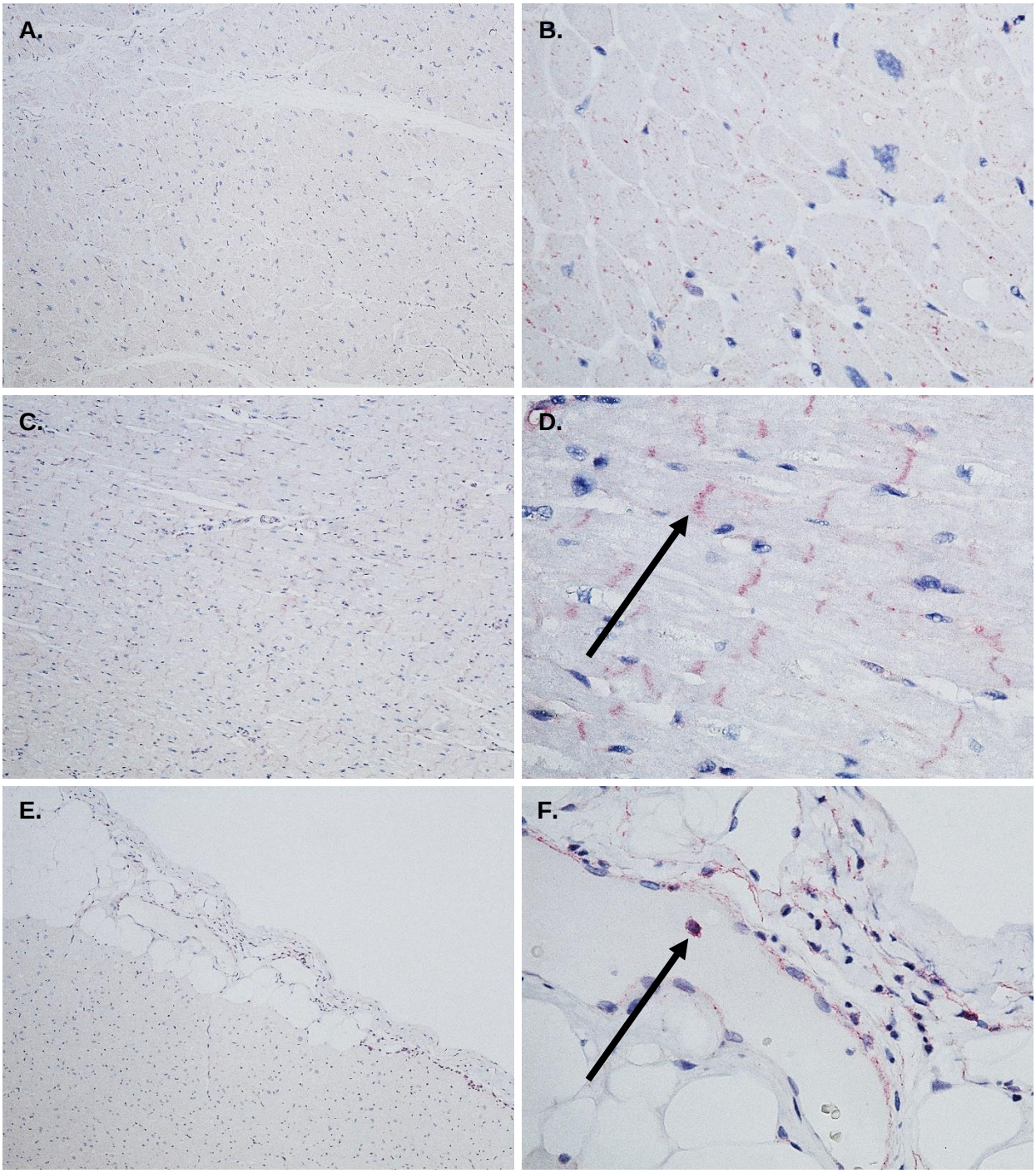

Figure 46. Microscopic Image of Heart Tissue with XG (CD99) Polyclonal Antibody
Positive labeling of cardiomyocytes (A.) 10x, (B.) 50x. Positive labeling of the intercalated discs of cardiomyocytes (C.) 10x, (D.) 50x. Positive labeling of fibroblasts and lymphocytes (arrow) (E.) 10x, (F.) 50x. Negative labeling of RBCs is also present.

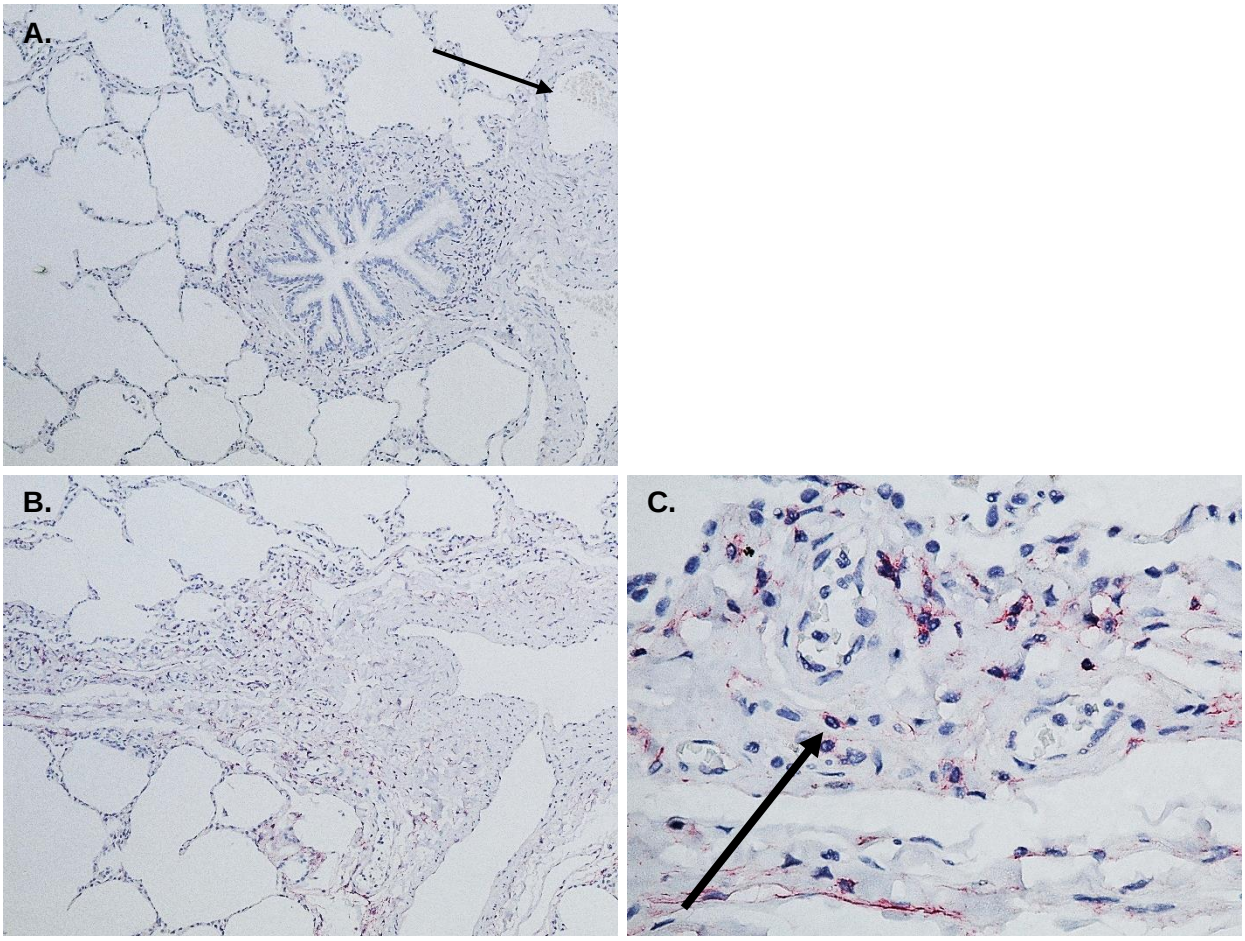

Figure 47. Microscopic Image of Lung Tissue with XG (CD99) Polyclonal Antibody
Negative labeling of alveolar cells (A.) 10x. Negative labeling of RBCs (arrow). Positive labeling of fibroblasts and lymphocytes (arrow) (B.) 10x, (C.) 50x.

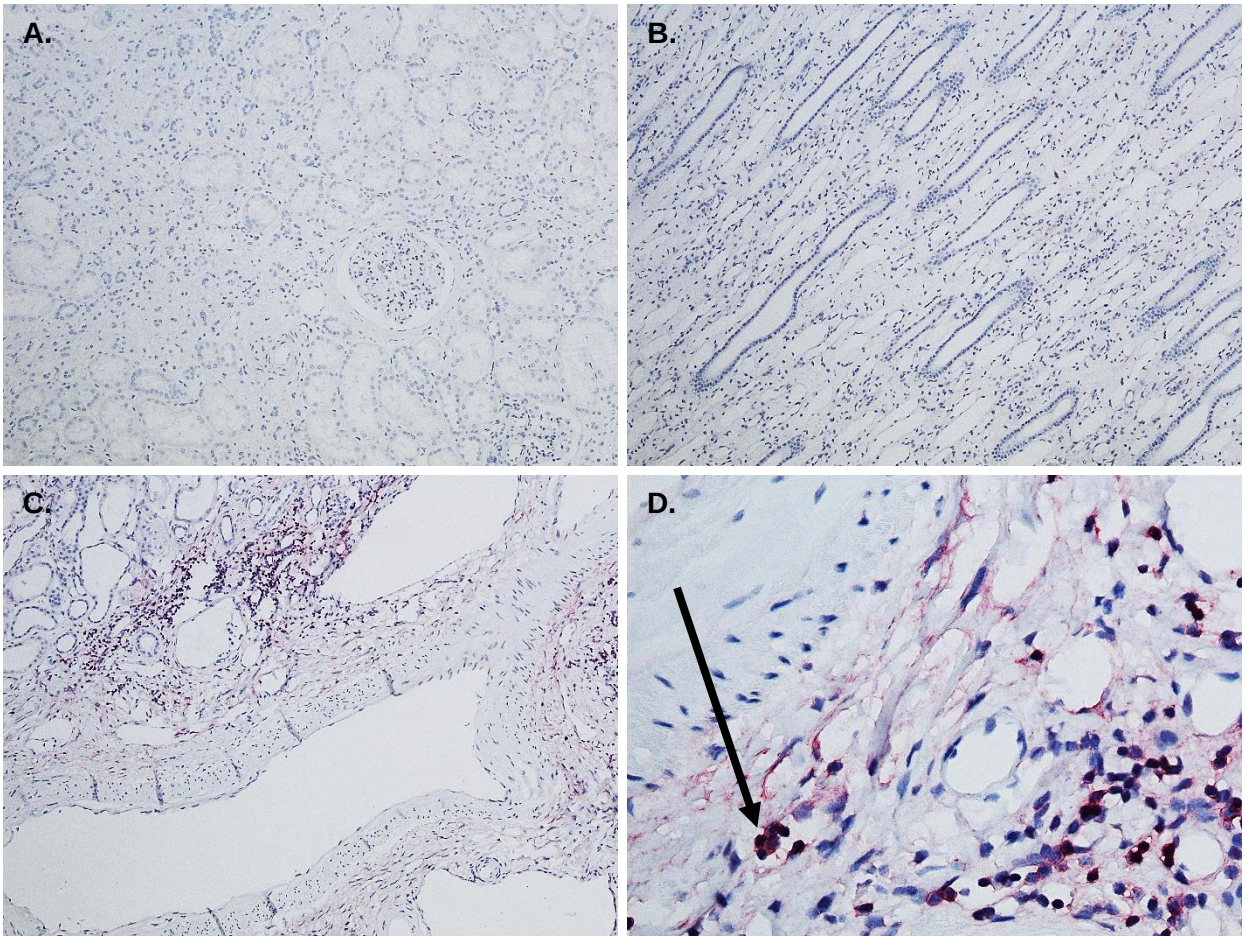

Figure 48. Microscopic Image of Kidney Tissue with XG (CD99) Polyclonal Antibody Negative labeling (A.) cortex, (B.) medulla, 10x. Positive labeling of fibroblasts and lymphocytes (arrow) (C.) 10x, (D.) 50x.

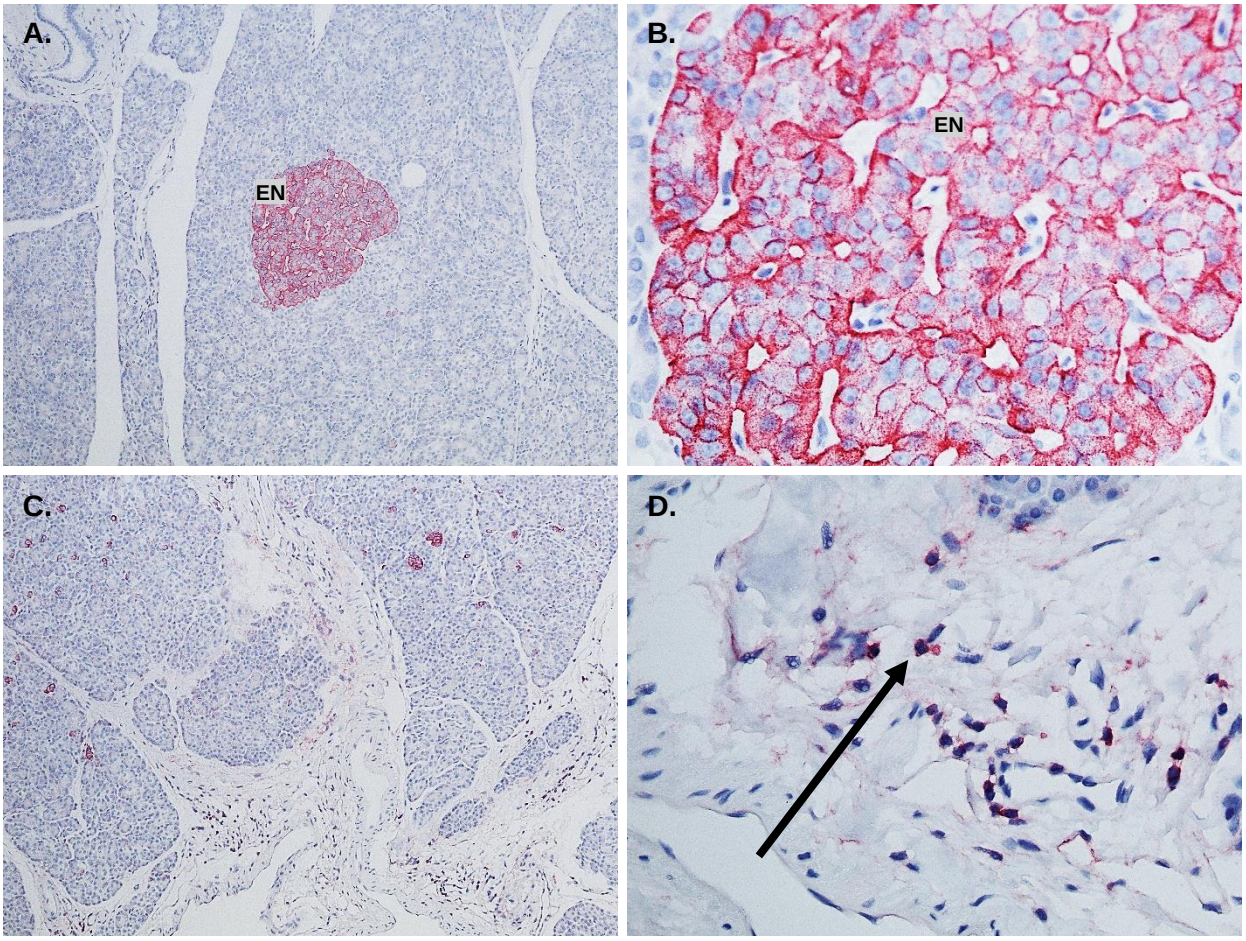

Figure 49. Microscopic Image of Pancreas Tissue with XG (CD99) Polyclonal Antibody
Positive labeling of endocrine cells (EN) (A.) 10x, (B.) 50x. Positive labeling of fibroblasts and lymphocytes (arrow) (C.) 10x, (D.) 50x.

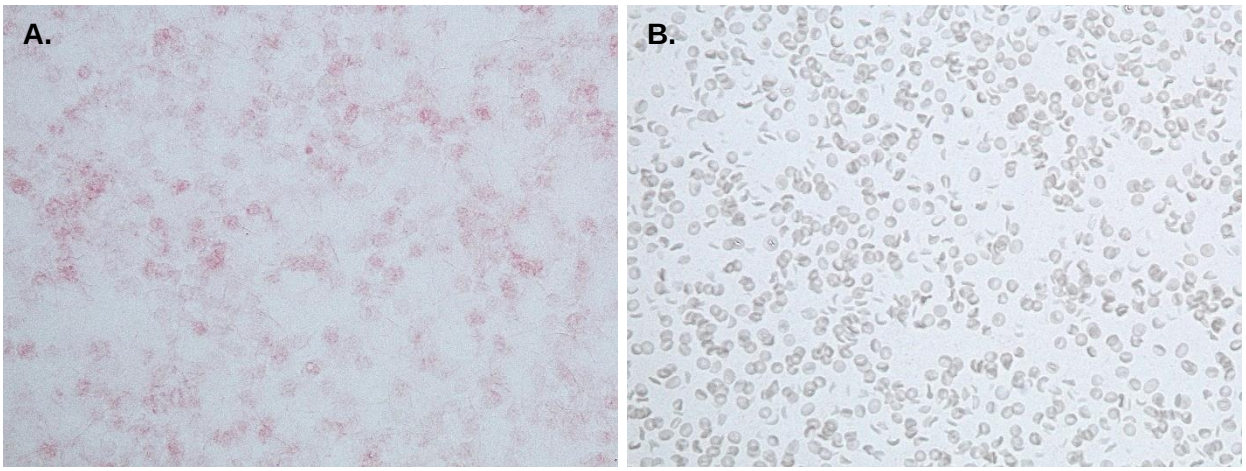

Figure 50. Microscopic Image of RBC Control with XG (CD99) Polyclonal Antibody
Positive labeling of presumed XG antigen-positive RBCs via blood smear technique (A.) 50x. Negative labeling of presumed XG antigen-positive RBCs with FFPE RBC cell pellet (B.) 50x.

2.4.8. Chido/Rodgers System

Chido/Rodgers antigen (C4) was detected in the heart, lung, kidney, and pancreas (Table 21). Positive labeling was also seen in the plasma within vessels.

In heart tissue, positive labeling was seen in the cardiomyocytes and intercalated discs (*n*=6) (Figure 51). In lung tissue, positive labeling was noted in the alveolar macrophages and on the bronchial epithelium (*n*=6) (Figure 52). For kidney tissue, Chido/Rodgers (C4) was seen in both the proximal and distal tubular epithelium of the cortex (distal tubules had stronger labeling) (*n*=6), and in the medullary tubules (cuboidal epithelium), and urothelium, when present in the sections (biopsy K1 and K6) (Figure 53). For pancreas biopsies, expression of Chido/Rodgers (C4) was seen on endocrine cells (islets of Langerhans) for biopsies P1, P2, P3, P4, P5,and P6, but reactivity was not detected in biopsy P7 (Figure 54). RBCs did not label.

The Chido/Rodgers (C4) antibody produced negative labeling for the FFPE cell pellet and positive labeling for the RBC blood smear technique (Figure 55).

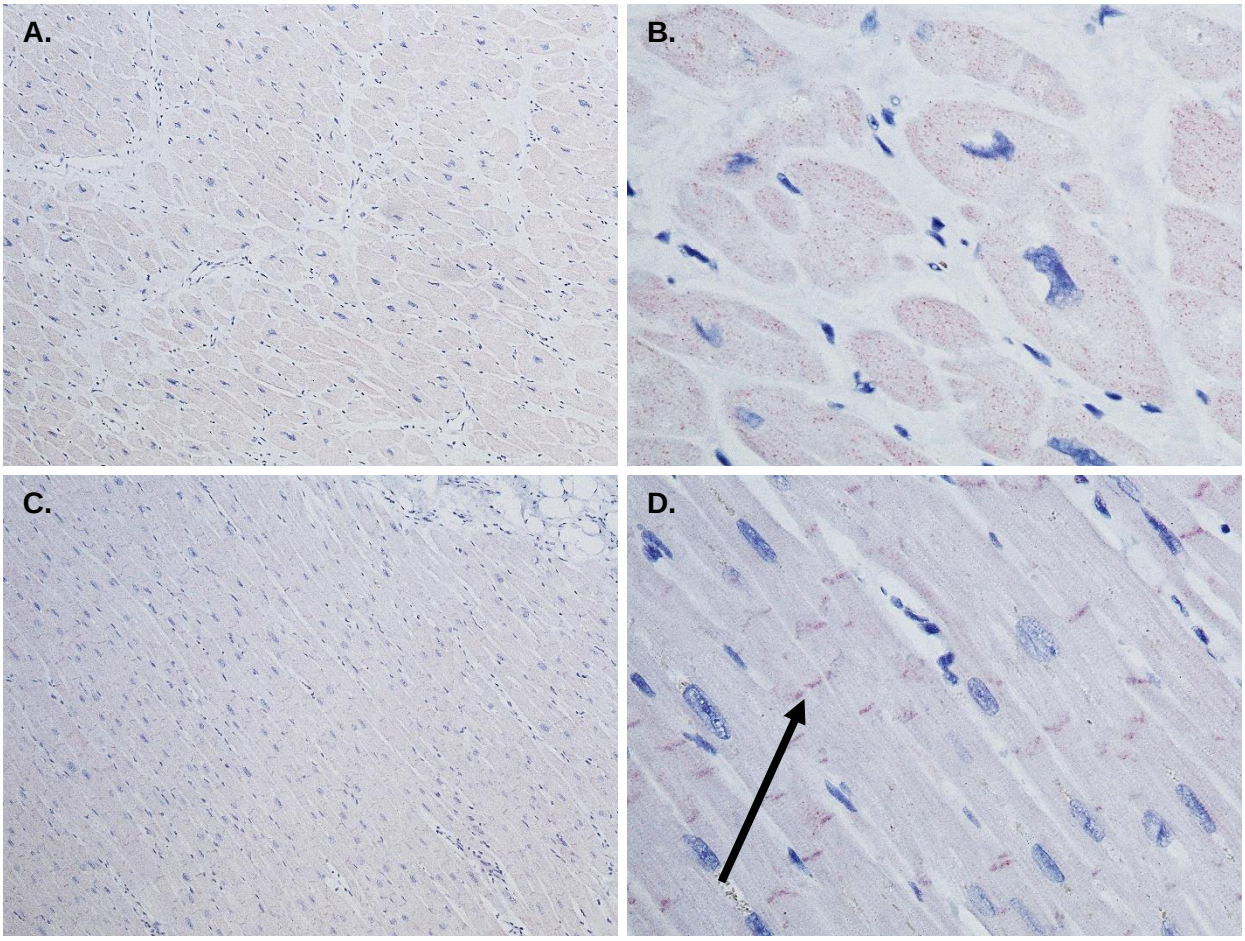

Figure 51. Microscopic Image of Heart Tissue with Chido/Rodgers (C4) Polyclonal Antibody
Positive labeling of cardiomyocytes (A.) 10x, (B.) 50x. Positive labeling of the intercalated discs of cardiomyocytes (arrow) (C.) 10x, (D.) 50x.

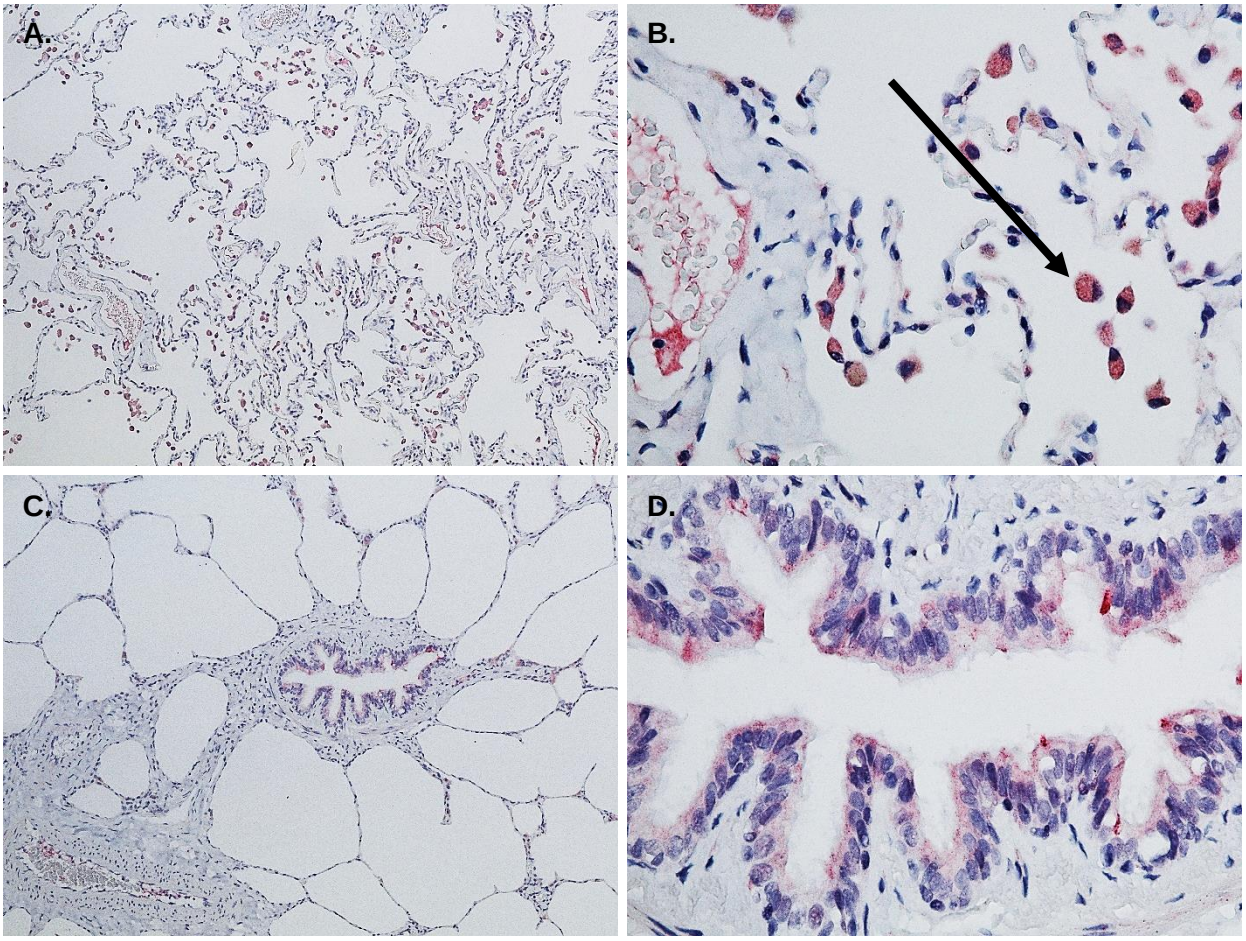

Figure 52. Microscopic Image of Lung Tissue with Chido/Rodgers (C4) Polyclonal Antibody

Positive labeling of macrophages (arrow) (A.) 10x, (B.) 50x. Negative labeling of RBCs, with positive labeling of plasma. Positive labeling of bronchial epithelium (C.) 10x, (D.) 50x.

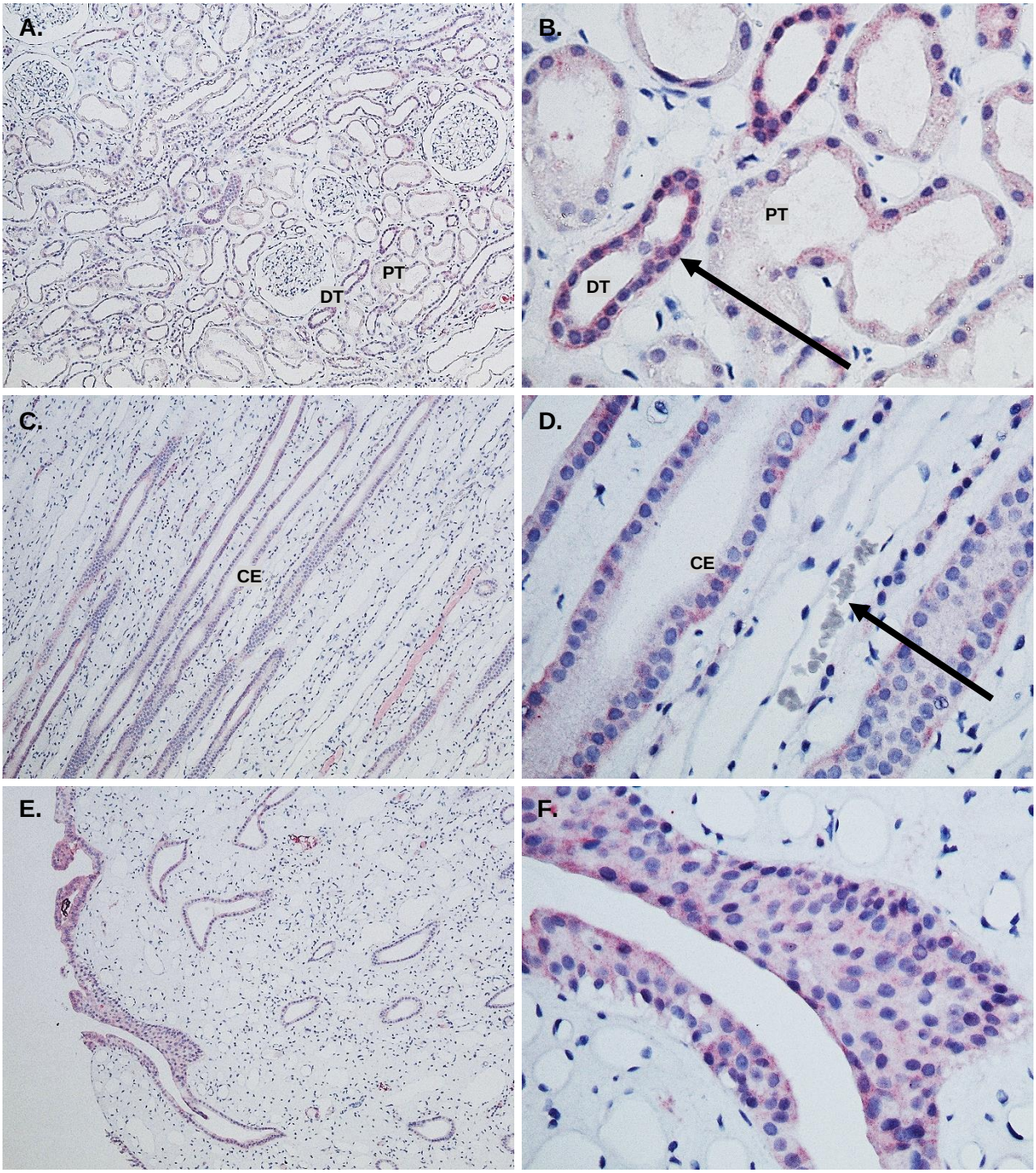

Figure 53. Microscopic Image of Kidney Tissue with Chido/Rodgers (C4) Polyclonal Antibody

Positive labeling of the proximal and distal (arrow) tubules of the cortex (A.) 10x, (B.) 50x. Positive labeling of the tubules (cuboidal epithelium (CE)) of the medulla (C.) 10x, (D.) 50x. Negative labeling of RBCs (arrow). Positive labeling of urothelium (E.) 10x, (F.) 50x.

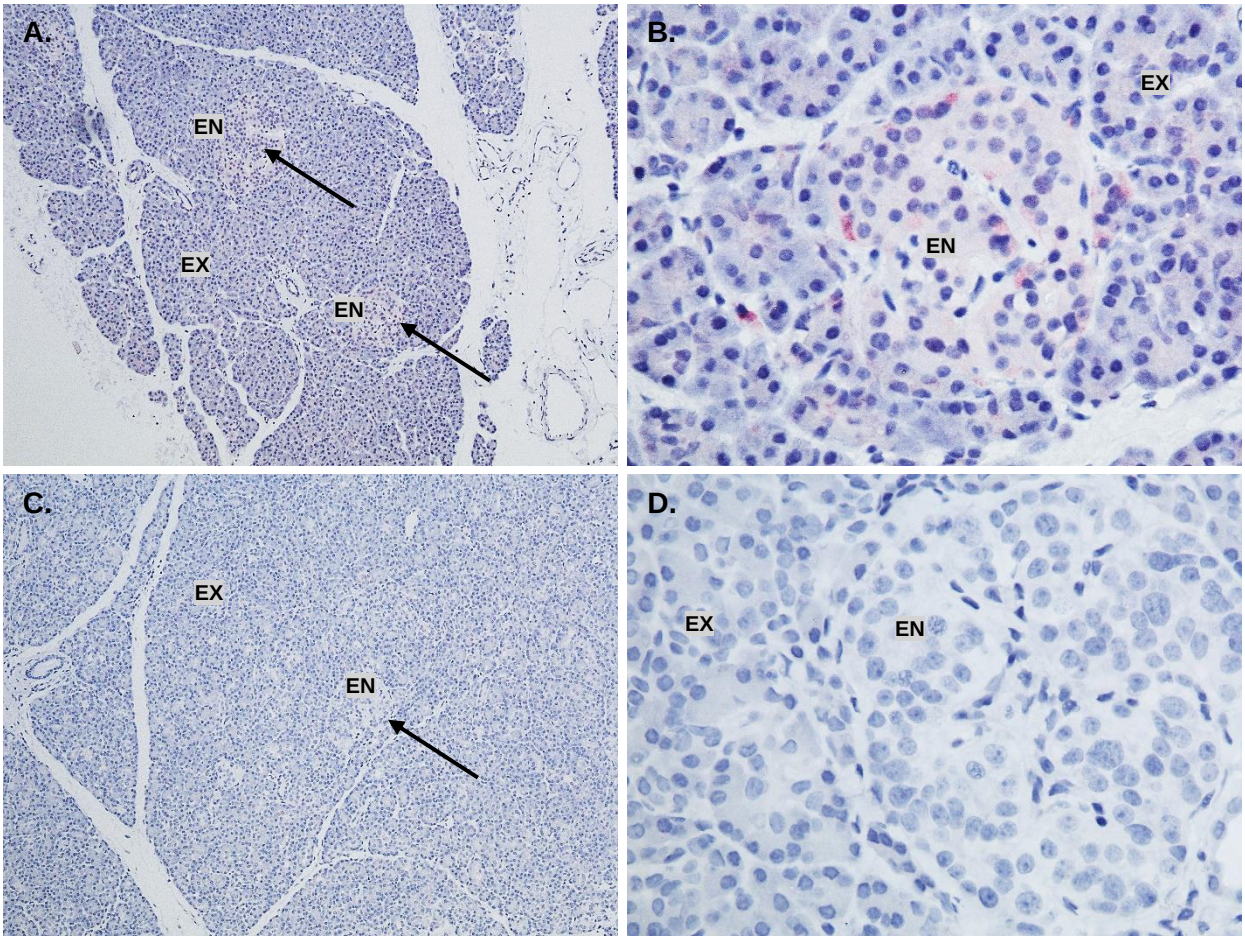

Figure 54. Microscopic Image of Pancreas Tissue with Chido/Rodgers (C4) Polyclonal Antibody
Positive labeling of endocrine cells (EN) (arrow), negative exocrine cells (EX) (A.) 10x, (B.) 50x. Negative labeling of exocrine and endocrine cells in biopsy P7 (C.) 10x (arrow). Negative control (D.) 50x.

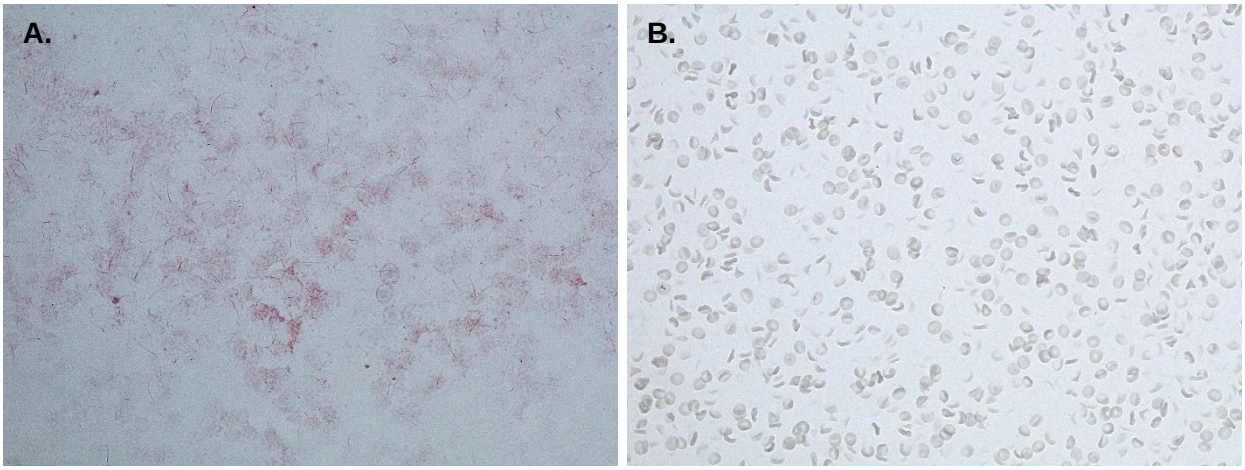

Figure 55. Microscopic Image of RBC Control with Chido/Rodgers (C4) Polyclonal Antibody
Positive labeling of presumed Chido/Rodgers antigen-positive RBCs via blood smear technique (A.) 50x. Negative labeling of presumed Chido/Rodgers antigen-positive RBCs with FFPE RBC cell pellet (B.) 50x.

2.4.9. Knops System

The Knops (CD35) glycoprotein was detected in the heart, lung, kidney, and pancreas (Table 21).

In heart tissue, there was positive labeling throughout the cardiomyocytes (*n*=6) (Figure 56). In lung tissue, positive labeling was noted in the alveolar macrophages and bronchial epithelial cells (*n*=6) (Figure 57). In the kidney, Knops (CD35) was detected in the glomeruli, the proximal and distal tubular epithelial cells of the cortex (distal tubules had stronger labeling) (*n*=6), and in the medullary tubules (squamous and cuboidal epithelium) and urothelium, when present in the sections (biopsies K1 and K6) (Figure 58). In pancreas tissue, positive labeling was noted on the exocrine and endocrine cells (islets of Langerhans) (*n*=7). Positive labeling of leukocytes was also seen (Figure 59). RBCs did not label.

The Knops (CD35) antibody produced negative labeling for the FFPE cell pellet and positive labeling for the RBC blood smear technique (Figure 60).

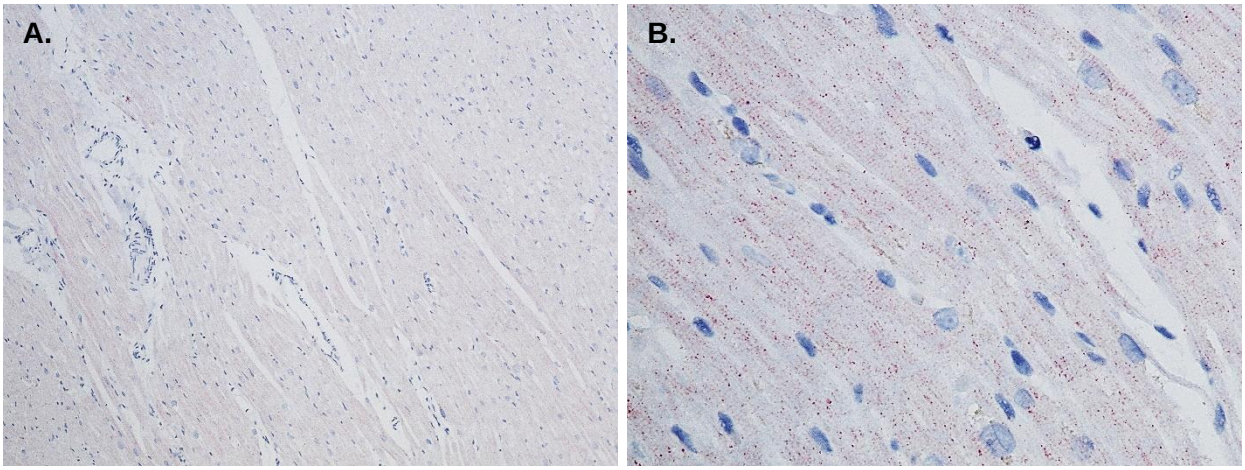

Figure 56. Microscopic Image of Heart Tissue with Knops (CD35) Polyclonal Antibody Positive labeling of cardiomyocytes (A.) 10x, (B.) 100x.

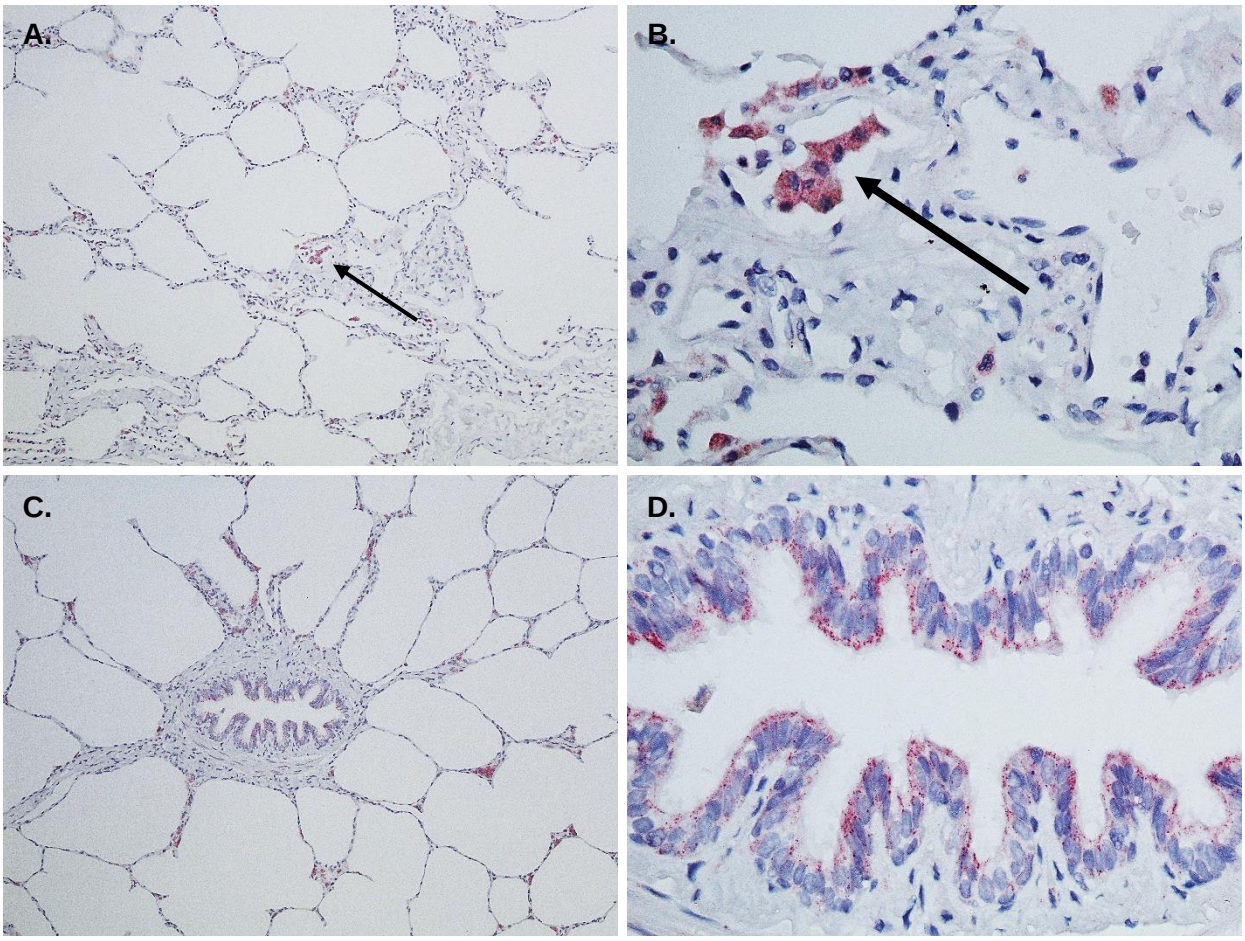

Figure 57. Microscopic Image of Lung Tissue with Knops (CD35) Polyclonal Antibody Positive labeling of macrophages (arrow) (A.) 10x, (B.) 50x. Negative labeling of RBCs is also present. Positive labeling of bronchial epithelium (C.) 10x, (D.) 50x.

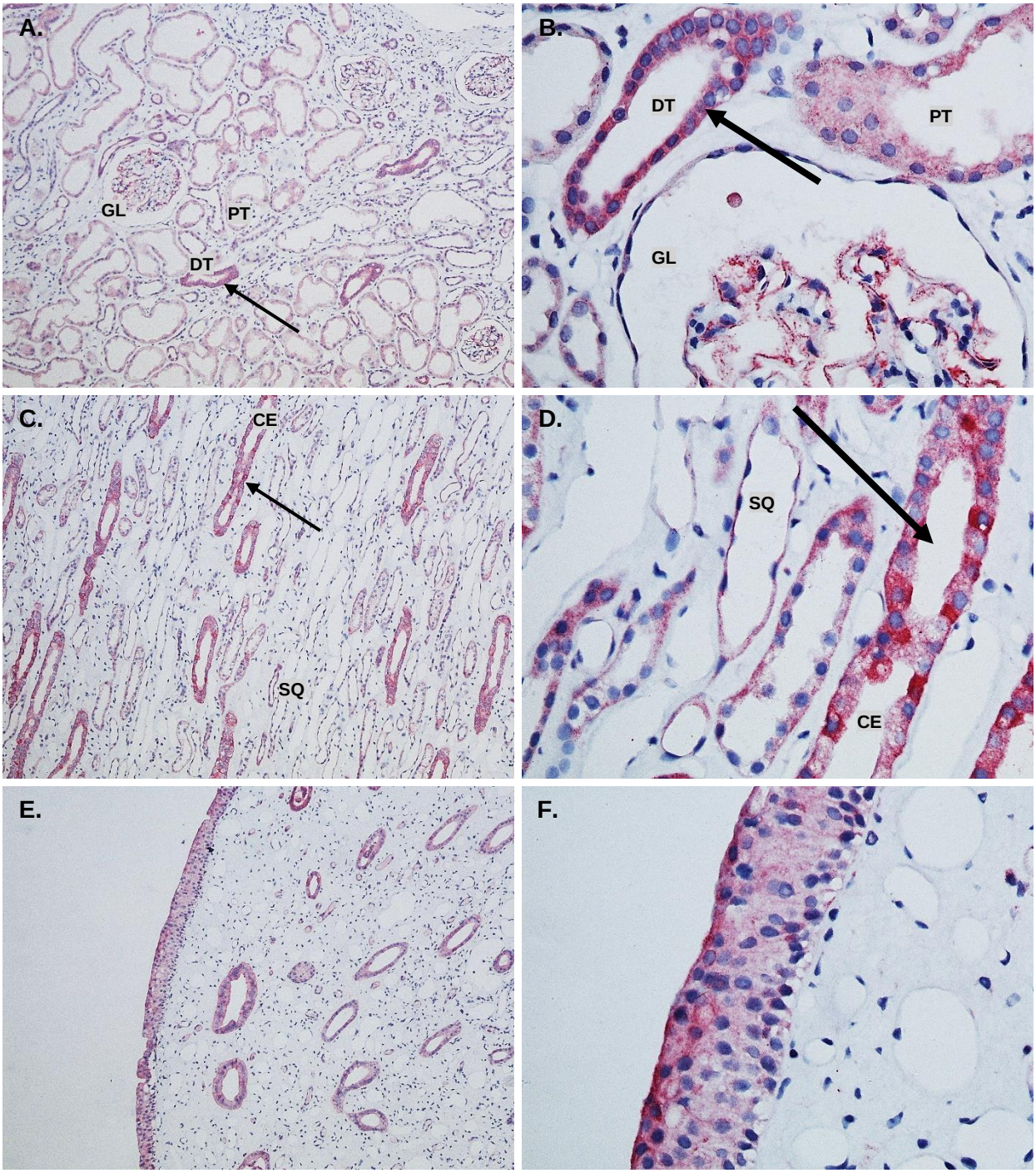

Figure 58. Microscopic Image of Kidney Tissue with Knops (CD35) Polyclonal Antibody Positive labeling of the glomeruli (GL), proximal (PT) and distal (DT) (arrow) tubules of the cortex (A.) 10x, (B.) 50x. Positive labeling of the tubules (squamous (SQ) and cuboidal epithelium (CE) (arrow)) of the medulla (C.) 10x, (D.) 50x. Positive labeling of urothelium (E.) 10x, (F.) 50x.

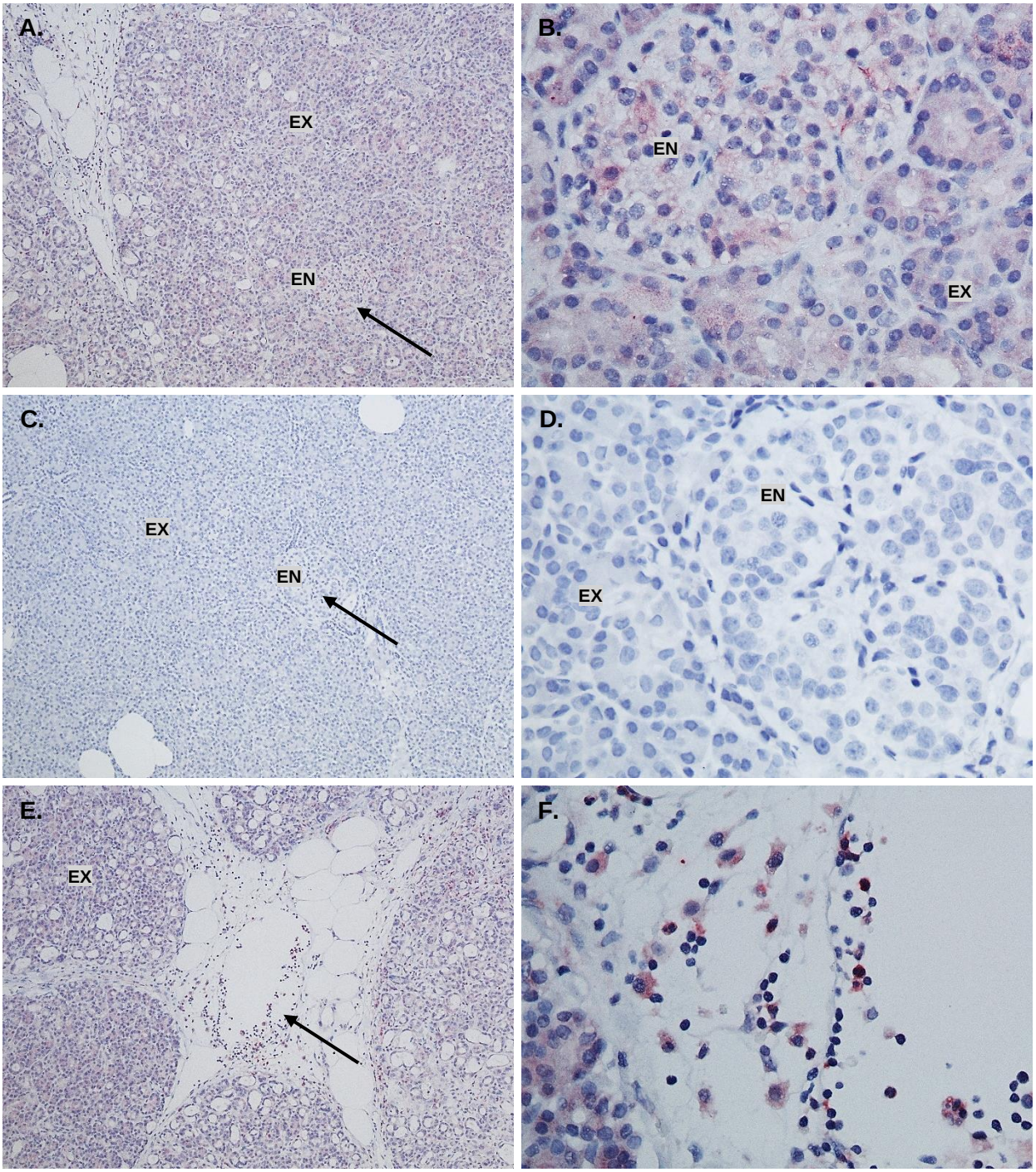

Figure 59. Microscopic Image of Pancreas Tissue with Knops (CD35) Polyclonal Antibody Positive labeling of exocrine (EX) and endocrine cells (EN) (arrow) (A.) 10x, (B.) 50x. Negative control (C.) 10x, (D.) 50x Positive labeling of leukocytes (arrow) (E.) 10x, (F.) 50x.

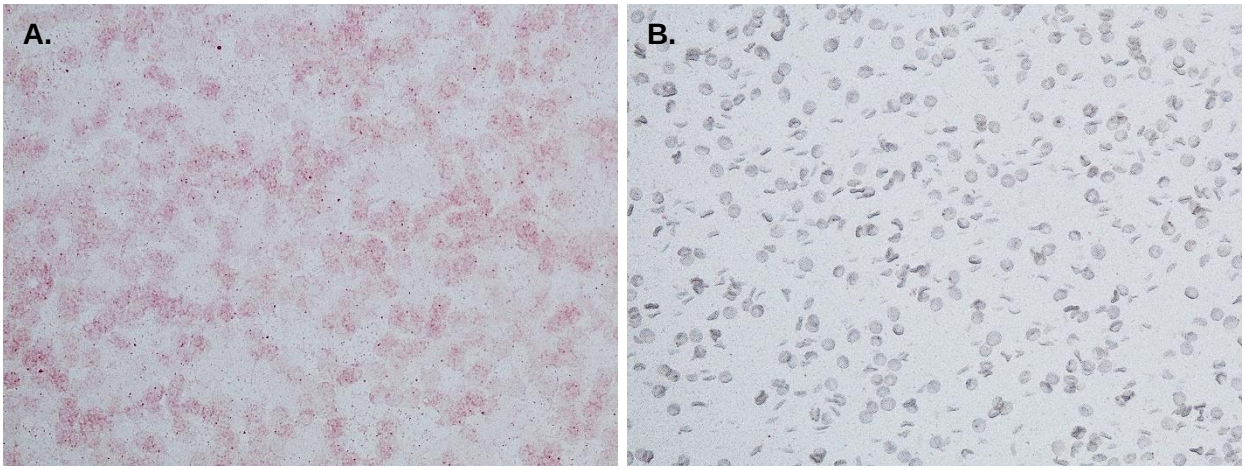

Figure 60. Microscopic Image of RBC Control with Knops (CD35) Polyclonal Antibody
Positive labeling of presumed Knops antigen-positive RBCs via blood smear technique (A.) 50x.
Negative labeling of presumed Knops antigen-positive RBCs with FFPE RBC cell pellet (B.) 50x.

2.4.10. Overview of IHC Detection of Histoblood Group Antigens in Deceased Donor Tissues

Table 21. Histoblood Group Antigen Detection by IHC in the Heart, Lung, Kidney, and Pancreas of Deceased Donors

Tissue Types		Blood Group Systems			
		MNS		RH	
		MN (CD235a)	Ss (GYPB)	RHD	RHCE
Heart	Cardiomyocytes	**Medium**	**Medium**	Not Detected	Not Detected
	Intercalated Discs:	Not Detected	Not Detected	Not Detected	Not Detected
Lung	Alveolar Cells:	Not Detected	Not Detected	Not Detected	Not Detected
	Macrophages:	Not Detected	Not Detected	Not Detected	Not Detected
	Bronchial Epithelium:	**Medium**	Not Detected	Not Detected	Not Detected
Kidney	Urothelium:	**Low**	**Medium**	Not Detected	Not Detected
	Glomeruli:	Not Detected	Not Detected	Not Detected	Not Detected
	Medullary Tubules:	**Low**	**High**	Not Detected	Not Detected
	Distal Tubules:	**Medium**	**Medium**	Not Detected	Not Detected
	Proximal Tubules (Cell Body):	**Low**	**Low**	Not Detected	Not Detected
	Proximal Tubules (Microvilli):	Not Detected	Not Detected	Not Detected	Not Detected
Pancreas	Exocrine Glandular Cells:	Not Detected	Not Detected	Not Detected	Not Detected
	Endocrine Cells:	**Low**	**High ***	Not Detected	Not Detected

Qualitative annotation of the degree of labeling (high, medium, low) was based on the perspective of the investigator of this study.
*Variable detection between biopsies tested

Table 21. (cont'd)

Tissue Types		Blood Group Systems			
		Kell	**Lewis (FUT3)**	**Duffy (ACKR1)**	**Kidd (SLC14A1)**
Heart	Cardiomyocytes	**Medium**	**High**	**Low**	**Low**
	Intercalated Discs:	**High**	Not Detected	**High**	**Low**
Lung	Alveolar Cells:	Not Detected	Not Detected	Not Detected	Not Detected
	Macrophages:	**Low**	Not Detected	Not Detected	Not Detected
	Bronchial Epithelium:	**Low**	**Low**	Not Detected	**Medium**
Kidney	Urothelium:	**High**	**Low**	**Low**	**Medium**
	Glomeruli:	Not Detected	Not Detected	Not Detected	Not Detected
	Medullary Tubules:	**Medium**	Not Detected	Not Detected	**High**
	Distal Tubules:	**Low**	**Low**	Not Detected	Not Detected
	Proximal Tubules (Cell Body):	**Medium**	**High**	Not Detected	Not Detected
	Proximal Tubules (Microvilli):	Not Detected	Not Detected	**High**	Not Detected
Pancreas	Exocrine Glandular Cells:	Not Detected	Not Detected	Not Detected	Not Detected
	Endocrine Cells:	**Low**	**Low**	Not Detected	**High ***

Table 21. (cont'd)

Tissue Types		Blood Group Systems		
		XG (CD99)	**Chido/Rodgers (C4)**	**Knops (CD35)**
Heart	Cardiomyocytes:	**Low**	**Low**	**Medium**
	Intercalated Discs:	**Medium**	**Low**	Not Detected
Lung	Alveolar Cells:	Not Detected	Not Detected	Not Detected
	Macrophages:	Not Detected	**High**	**High**
	Bronchial Epithelium:	Not Detected	**Low**	**Low**
Kidney	Urothelium:	Not Detected	**Medium**	**High**
	Glomeruli:	Not Detected	Not Detected	**High**
	Medullary Tubules:	Not Detected	**Low**	**High**
	Distal Tubules:	Not Detected	**Medium**	**High**
	Proximal Tubules (Cell Body):	Not Detected	**Low**	**Medium**
	Proximal Tubules (Microvilli):	Not Detected	Not Detected	Not Detected
Pancreas	Exocrine Glandular Cells:	Not Detected	Not Detected	**Low**
	Endocrine Cells:	**High**	**Low ***	**Low**

DISCUSSION

1. Experiment One: Unexpected RBC Antibodies in Transplant Patients

The 5% positivity for RBC alloantibodies found in this study population align with the general population having an estimated 2-5% of transfused individuals developing RBC antibodies.[7-8] However, considering each individual transplant population separately, hemagglutination testing did not identify unexpected RBC antibodies in the lung transplant patient population (0%), heart transplant patients had 2% positivity, and renal transplant patients had 13% positivity. Variation in antibody detection among transplant populations could be due to selection bias because of the limited information available for patient transfusion history. Only patients with documented transfusion histories were selected for RBC antibody screening, so any patients with undocumented transfusion history could not be identified and considered for this cohort.

Variation in antibody detection between transplant populations (heart, lung, and kidney) could also occur from differences in immunosuppressive regimes. Immunosuppression for solid organ transplant varies by organ type and by specific recipient factors.[143-144] Most samples from this investigation were post-transplant (Table 9), and these patients have undergone varying degrees of immunosuppression. Immunosuppressed patients would be expected to produce a lower antibody response compared to someone who is not immunosuppressed; however, if transplant patients undergo less induction and/or post-transplant maintenance immunosuppression,[143-144] there may be an increased likelihood of making RBC alloantibodies depending on the transfusion support received.

Transfusion rates among solid organ transplant recipients are highly variable and not well documented. As mentioned previously, there was inconsistency regarding documentation of blood products transfused to the patients investigated in this study, as well as variability in the number of blood products received. Kidney patients had the longest duration between documented transfusion and sample collection (>120 months) and three of those patients had

positive antibody screens (Table 10). Considering that IgG antibody titers typically begin to decline roughly four weeks after primary exposure,[33] it is unknown whether the antibody identification for these patients reflects those longstanding historical transfusion events, or if more recent sensitizing events occurred (e.g. pre-, during, or post-transplant transfusion support), but were not documented.

The clinically significant antibodies identified in this investigation were anti-K (four patients) and anti-Fy^a (two patients) (Table 8). As mentioned in the literature review section, the K antigen is very immunogenic (second only to RhD within the minor blood group systems) and is the third most common RBC antibody identified, behind ABO and RhD.[9,20] The prevalence of the phenotype lacking the K antigen (K-k+) is high, about 91% among Whites and 97% among Blacks.[20] Individuals with this phenotype can make an anti-K if exposed to the K antigen, typically through transfusion or pregnancy. Of the phenotypes that have the K antigen, heterozygous expression (K+k+) is found in 8.8% of Whites and in 3.5% of Blacks, and homozygous expression (K+k-) is found in 0.2% of Whites and in <0.1% of Blacks.[20] Calculating the probability of alloexposure using antigen frequencies by race (antigen-positive × antigen-negative), first developed by Giblett, shows that there could be up to a 9% chance that someone lacking the K antigen would be exposed to the K antigen.[145] During this investigation, anti-K was identified in four (57%) samples with positive antibody screens (Table 8), including one Hispanic male, one White male, and two White females. Given the high immunogenicity of the K antigen, the frequency with which this antibody is identified in the blood bank, and the fact that >90% of the population can make anti-K antibodies, the results of this investigation fall within expectations for the identification of anti-K antibodies.[9,20]

Anti-Fy^a antibodies are commonly encountered RBC alloantibodies and are said to occur around one-third as frequently as anti-K antibodies.[20,47] One study corroborated these claims, finding that anti-K antibodies accounted for 22% and anti-Fy^a antibodies accounted for 6% of their study population.[146] The prevalence of phenotypes lacking the Fy^a antigen are (i) Fy(a-b+), which

is present in 32% of Whites and in 20% of Blacks, and (ii) Fy(a-b-), which is very rare in Whites and present in 67% of Blacks.[9] The likelihood of alloexposure is increased for the Fy^a antigen compared to the K antigen; there is about a 67% chance that any random blood unit will possess the Fy^a antigen.[20] During this investigation, anti-Fy^a was identified in two (29%) samples with positive antibody screens (Table 8), including one White male and one Hispanic female. Given the frequency of Fy(a+) blood units, and that anti-Fy^a is a commonly encountered RBC antibody, the results obtained during this investigation fall within the expectations for identifying the Fy^a antibody.[9,20]

Limitations regarding hemagglutination testing include alloantibody detection limits of the assay that could miss the presence of an antibody when: (i) there is an antibody titer that is lower than what the assay can detect, (ii) screening cells used in the assay are missing the corresponding antigen, (iii) screening cells are heterozygous for the corresponding antigen and cannot detect a low titer of a particular antibody.

At the completion of testing, a possible limitation of the antibody screen was identified: only heterozygous K (K+k+) cells were provided. This is a common occurrence for reagent RBCs selected as antibody screening cells because homozygous K (K+k-) RBCs are rare and found in <1% of the population.[9] RBCs with heterozygous K expression may not detect lower-titer anti-K antibodies, thus incorrectly classifying these samples as negative. Though this limitation exists, this study otherwise provided optimal testing conditions to detect K antibodies, and K antibodies were identified in multiple patients at lower reaction strengths (1+, 2+) using both screening cells and antibody identification panel cells. Other RBC antigen systems that produce antibodies which demonstrate dosage (antibodies that have a stronger reaction with cells that have homozygous antigen expression compared to cells with heterozygous expression) are Rh (except RhD), Kidd, Duffy, and MNS systems.[20] Both the screening cells and panel cells used in this study included donors with homozygous (double dose) expression for each of these antigen systems.

2. Experiment Two: Histologic Distribution of RBC Group Antigens in Deceased Donor Tissues

During IHC testing, areas resembling pathologic changes were noted within some biopsies, but could not be confirmed due to only limited demographic information being provided for each sample used in this study (Table 14). Despite this finding, IHC results were consistent among biopsies of each tissue type whether pathology was present or not. Additionally, fixation times for each biopsy sample were variable (Table 15), but this does not appear to have impacted immunodetection as labeling was consistent throughout biopsy samples. The polymer-based IHC assay used in this investigation was a non-biotin system, removing the interference of endogenous biotin in tissues. Additionally, all biopsies were tested for the presence of endogenous AP (enzyme substrate) and found to be negative. The sample size requested for each organ this study (n=6) was selected without the guidance of literature support, and consistent labeling of biopsies did not warrant additional samples to be collected.

For RBC quality control, the FFPE cell pellet was inconsistent for immunodetection of RBC antigens. The FFPE cell pellet samples were fixed for the same duration as the biopsies to mimic testing conditions of both the control and testing samples. However, the FFPE cell pellet did not produce positive labeling for eight of the primary antibodies used in this study. Though there were three antibodies that did produce positive labeling with the FFPE cell pellet (MN, Ss, and Kell), there were no conformational similarities shared between these antigens (Table 16, Figure 5). The RBC blood smear technique was successful in producing positive labeling for all primary antibodies used in this investigation, supporting that each antibody could bind to antigen when present. One explanation for the lack of FFPE cell pellet labeling could relate to the fixation time of the cells. In the RBC blood smear technique, cells were fixed for ten minutes, whereas in the FFPE RBC cell pellet, cells were fixed for a minimum of four days. Varying results between FFPE cell pellets and RBC blood smears could also occur from sample preparation and processing. Though fixation time was addressed when comparing the FFPE cell pellet and RBC blood smear results, it is acknowledged that a FFPE cell pellet with RBCs fixed in 10% NBF for a shorter

duration (e.g., ten minutes) was not created for this study, and could provide insight into additional processes that could impact RBC antigen recognition prior to IHC detection.

Since histoblood group antigen expression was unknown for the tissues used in this study, RBCs with known antigen expression were utilized as a positive control. However, internal controls (both positive and negative) are the gold standard for IHC detection, but the presence of RBCs in sample tissues was unknown prior to testing. As mentioned, the primary antibodies selected for this investigation eliminate the need for phenotyping/genotyping of samples prior to IHC testing, because they do not discriminate between the polymorphisms of alleles within each blood group system (e.g., Fy^a versus Fy^b) (see section 2.3.4.3). Given the immunogen targets of the selected primary antibodies (see appendix A), positive labeling was expected for RBCs within biopsies, as well as RBC quality control samples. Unexpectedly, sample biopsies did not produce positive labeling of RBCs with the RhD, Kell, Lewis (FUT3), Duffy (ACKR1), Kidd (SLC14A1), XG (CD99), Chido/Rodgers (C4), and Knops (CD35) antibodies. Positive labeling of RBCs within sample biopsies were detected with the MN (CD235a), Ss (GYPB), and RhCE antibodies; there are no structural similarities specific for these antigens (Table 16, Figure 5). Considering that positive labeling was detected in tissue substructures of sample biopsies and not RBCs (apart from RhD), these results suggest that histoblood group antigens may be expressed differently on RBCs compared to other tissues, or that sample processing affects the expression of histoblood group antigens on RBCs, making epitopes inaccessible and/or unrecognizable to antibodies.

The effects of tissue processing on IHC, outside of fixation, are not completely known and reports are limited. One study detected the effects of each step in formalin-fixed tissue processing prior to IHC, using multiple cancer cell lines and anti-nuclear antibodies.[141] Overall, this study reported the greatest decrease in immunorecognition following the use of xylene (clearing step), even after antigen retrieval.[141] Reports such as these highlight the variability in immunodetection that may occur following tissue processing and the complexity surrounding the expression of the same antigen in or on different cells.[141] The conformation of histoblood group antigens by cells

other than RBCs is unknown. There is a need to identify steps in sample processing that can influence the expression of these antigens on RBCs and non-RBCs; this will help create optimal testing conditions for specific detection of histoblood group antigens, when present.

2.1 Data by Organ Type

Differences in IHC detection between the HPA (Table 2) and this investigation (Table 21) varied by organ type and are detailed below.

Differences in results reported by the HPA and this investigation could be due to differences such as (i) testing platform (e.g., automated versus manual IHC), (ii) endogenous interference (e.g., biotin), (iii) enzyme-conjugated secondary antibody and enzyme substrate (e.g., horseradish peroxidase with 3,3'-diaminobenzide (DAB) versus AP with ImmPACT Vector Red®), or (iv) experience of the researcher performing testing.

Qualitative annotation (high, medium, low) was based on the perspective of the investigator of this study following review of the collective results of each tissue type. Differences between results reported for IHC detection between the HPA and this investigation could be due to subjective qualitative interpretation, as there is no set standard for qualitative annotation of IHC labeling.

2.1.1. Heart Tissue

Labeling of cardiomyocytes was detected with both the XG (CD99) and Knops (CD35) polyclonal antibodies, but the HPA did not report expression of these antigens by cardiomoycytes.[13] Additionally, positive cardiomyocyte labeling (medium detection) was note for both the Ss (GYPB) and Kell polyclonal antibodies (Kell also showed high labeling of intercalated discs), but the HPA does not have data for either of these antigens.[13] Despite similar results for cardiomyocytes, it is unknown if the HPA has data for the detection of MN (CD235a), Lewis (FUT3), Duffy (ACKR1), Kidd (ALC14A1), XG (CD99), or Chido/Rodgers (C4) antigens in intercalated discs.[13]

Low detection of XG (CD99) antigen detected on cardiomyocytes could be due to the phenotype of biopsies. The Xg(a) phenotype of donors cannot be inferred through the detection of CD99, but females that are Xg(a+) have CD99H on their RBCs and Xg(a-) females have CD99L on their RBCs.[20] Additionally, 68% of Xg(a-) males have CD99H and 32% have CD99L.[20,106] The biopsies used in the HPA had both males (two) and females (three).[13] It is also unknown if XG (CD99) antigens are represented differently by cells other than RBCs. Additional differences in results reported by the HPA and this investigation are detailed at the beginning of this section (2.1).

Medium expression of the Knops (CD35) antigen in this study could be related to the low number of antigen sites and variable expression of CR1 that has been documented on RBCs,[20,44] however, it is not known if CR1 is represented differently by cells other than RBCs. Though the tissues used in the HPA were classified as normal, an explanation for a lack of detection could arise from autoimmune disease that may cause a decrease in Knops antigen on cell membranes,[44] but it is unknown if this influence goes beyond RBC CR1. Additional differences in results reported by the HPA and this investigation are detailed at the beginning of this section (2.1).

Variable detection on cardiomyocytes was seen in this study with the Duffy (ACKR1) antibody (three low detection, three not detected), and these results are comparable with the HPA (three low detection, three not detected).[13] Slight variation in documented positive detection between this study and the HPA for MN (CD235a), Lewis (FUT3), and Kidd (SLC14A1) antigens are unremarkable and could be due to subjective qualitative interpretation of data, and/or the experience of the researcher performing testing.

Overall, the documented differences in IHC detection between the HPA and this investigation are unremarkable. Table 22 provides a comparison of IHC detection in the heart between this study and the HPA.

Table 22. Comparison of Histoblood Group Antigen IHC Detection in Heart Deceased Donor Tissues

Primary Antibody	Cardiomyocytes	
	HPA	Our Study
CD235a	**Low ***	**Medium**
GYPB	-	**Medium**
RhD	Not Detected	Not Detected
RhCE	Not Detected	Not Detected
KEL	-	**Medium**
FUT3	**Medium**	**High**
ACKR1	**Low ***	**Low ***
SLC14A1	**Medium**	**Low**
CD99	Not Detected	**Low**
C4	**Low ***	**Low**
CD35	Not Detected	**Medium**

* Highest IHC detection documented for all biopsies tested.
- No IHC data available

2.1.2. Lung Tissue

This study did not detect antigen in alveolar cells with any of the primary antibodies tested, but the HPA documented expression in alveolar cells with MN (CD235a) (three samples had low detection, three samples had no detection)[13], Kidd (SLC14A1) (all samples had low detection (*n*=3))[13], and Knops (CD35) (three samples had low detection, six samples had no detection) antibodies.[13] The HPA also documented expression in macrophages with MN (CD235a) (three samples had low detection, three samples had no detection)[13], Lewis (FUT3) (all samples had low detection (*n*=3))[13], Kidd (SLC14A1) (all samples had low detection (*n*=3))[13], and XG (CD99) (all samples had low detection (*n*=6)) antibodies. This expression was not detected in this study.[13]

This investigation documented low labeling of Kell glycoprotein in lung macrophages, but the HPA does not have data for this antigen.

Additionally, this investigation documented medium labeling in the bronchial epithelium for MN (CD235a), and Kidd (SLC14A1), and low detection with Kell, Lewis (FUT3), Chido/Rodgers (C4), and Knops (CD35). It is unknown if the HPA has data for the detection of these antigens in bronchial epithelium.[13]

Considering that the HPA did not report positivity of all biopsies tested with the MN (CD235a) and Knops (CD35) antibodies, the negative results obtained from this investigation with the MN (CD235a) and Knops (CD35) antibodies, are unremarkable (Table 21).

Detection reported by the HPA for low expression of Kidd (SLC14A1) antigen in alveolar cells and macrophages does not align with documented literature for the location of the antigens of this blood group system (Table 4). EST data does support the presence of Kidd antigens in the lung (Table 1), but this detection is not specific. More testing needs to be performed for Kidd (SLC14A1) antigens in lung tissue to assess the potential impact of this antigen for histocompatibility. Additional differences in results reported by the HPA and this investigation are detailed at the beginning of this section (2.1).

Detection reported by the HPA for low expression of Lewis (FUT3) antigen by macrophages is consistent with documented literature stating that Lewis antigens are present on monocytes (Table 4).[44] This study was unable to detect Lewis (FUT3) antigen in macrophages, and though it is unknown if the Lewis antigen behaves similarly by cells other than RBCs, this detection could be due to Lewis antigens not being intrinsic to RBC membranes, where the antigen is passively adsorbed from soluble Lewis glycolipid present in the plasma.[9,20,44] Also, donor management such as transfusion and dialysis could cause Lewis antigen to be shed from cell membranes.[20,44,80,81] Additional differences in results reported by the HPA and this investigation are detailed at the beginning of this section (2.1).

Detection reported by the HPA for low expression of XG (CD99) antigen by macrophages, is consistent with documented literature that states the CD99 antigen is ubiquitously expressed at low levels on almost all human cell types (Table 4).[108] This study was unable to detect XG (CD99) antigen in macrophages, and this could be due to the phenotype of biopsies. As mentioned earlier, the Xg(a) phenotype of donors cannot be inferred through the detection of CD99, and females that are Xg(a+) have CD99H on their RBCs and Xg(a-) females have CD99L on their RBCs,[20] while 68% of Xg(a-) males have CD99H and 32% have CD99L.[20,106] The lung biopsies used in this study were from males (four) and females (two) (Table 14). It is also unknown if XG (CD99) antigens are represented differently by cells other than RBCs. Additional differences in results reported by the HPA and this investigation are detailed at the beginning of this section (2.1).

The highest detection reported by the HPA for the expression of Chido/Rodgers (C4) antigen in lung macrophages was low (one sample had low detection, two samples had no detection),[13] and this investigation documented high detection (Table 21). The Chido/Rodgers antigens are on the fourth component of complement (C4), and though it is unknown if the Chido/Rodgers antigens behave similarly by cells other than RBCs, the increased variability seen during comparison could be due to Chido/Rodgers antigens not being intrinsic to RBC membranes, where the antigen is passively adsorbed from the plasma.[9,20,44] The Chido/Rodgers antigens are synthesized by monocytes/macrophages, so the detection seen in the lung by both the HPA and this investigation correlate with what is documented in the literature (Table 4).[9,20,44] If the deposition of C4d was also tested for, and found to be present in deceased donor biopsy samples, the high detection documented in this investigation could be influenced by increased involvement of the classical or lectin complement pathways, for which this activation may not be as prevalent in normal tissues.[44,110] Additional differences in results reported by the HPA and this investigation are detailed at the beginning of this section (2.1).

Slight variation in reported positive detection between this study and the HPA by lung macrophages with the Knops (CD35) antibody is unremarkable and could be due to subjective qualitative interpretation of data, and/or the experience of the researcher performing testing.

Overall, the reported differences in IHC detection between the HPA and this investigation are unremarkable. Table 23 provides a comparison of IHC detection in the lung between this study and the HPA.

Table 23. Comparison of Histoblood Group Antigen IHC Detection in Lung Deceased Donor Tissues

Primary Antibody	Alveolar Cells		Macrophages	
	HPA	**Our Study**	**HPA**	**Our Study**
CD235a	**Low ***	Not Detected	**Low ***	Not Detected
GYPB	-	Not Detected	-	Not Detected
RhD	Not Detected	Not Detected	Not Detected	Not Detected
RhCE	Not Detected	Not Detected	Not Detected	Not Detected
KEL	-	Not Detected	-	**Low**
FUT3	Not Detected	Not Detected	**Low**	Not Detected
ACKR1	Not Detected	Not Detected	Not Detected	Not Detected
SLC14A1	**Low**	Not Detected	**Low**	Not Detected
CD99	Not Detected	Not Detected	**Low**	Not Detected
C4	Not Detected	Not Detected	**Low ***	**High ***
CD35	**Low ***	Not Detected	**Medium ***	**High ***

* Highest IHC detection documented for all biopsies tested.
- No IHC data available

2.1.3. Kidney Tissue

This study identified positive labeling of the proximal, and medullary tubules for MN (CD235a), whereas the HPA did not report expression by these substructures. Though the HPA did not report MN antigens in proximal and medullary tubules,[13] only low detection was documented in this investigation. MN antigens are largely thought to be restricted to RBCs, but MNS system antigen expression has been documented on renal endothelium and epithelium (Table 4),[1,9,44] consistent with the findings of this study. Additional differences in results reported by the HPA and this investigation are detailed at the beginning of this section (2.1). Positive labeling was also noted on the cortical and medullary tubules with both the Ss (GYPB) and Kell polyclonal antibodies, but the HPA does not report data for either of these antigens.[13]

This investigation documented low urothelial expression of MN (CD235a), Lewis (FUT3), and Duffy (ACKR1), medium expression of Ss (GYPB), Kidd (SLC14A1), and Chido/Rodgers (C4), and high expression of Kell, and Knops (CD35). It is unknown if the HPA has data regarding the expression of these antigens on urothelium.[13]

The remaining differences involve antigen expression in glomeruli. Documented detection from the HPA shows expression of Duffy (ACKR1) (three samples had low detection, three samples had no detection)[13], Kidd (SLC14A1) (all samples had low detection (*n*=3))[13], Chido/Rodgers (C4) (three samples had low detection, six samples had no detection),[13] and XG (CD99) (three samples had medium detection, three samples had low detection).[13] Considering the HPA did not report positivity for all biopsies tested with Duffy (ACKR1), and Chido/Rodgers (C4) antibodies,[13] the negative results obtained from this investigation with the Duffy (ACKR1), and Chido/Rodgers (C4) antibodies, are unremarkable (Table 21).

Detection documented by the HPA for low expression of Kidd (SLC14A1) antigen in the glomeruli (*n*=3) does not align with documented literature for the location of these antigens beyond the medulla (Table 4).[20,26,44] However, given the role of the Jk antigen as a urea transporter, it is not unexpected to detect Kidd (SLC14A1) antigen in the glomeruli. Additional differences in

results reported by the HPA and this investigation are detailed at the beginning of this section (2.1).

Detection reported by the HPA for expression of XG (CD99) in the glomeruli (three samples had medium detection, three samples had low detection) is consistent with documented literature that states the CD99 antigen is ubiquitously expressed at low levels on almost all human cell types (Table 4).[108] This study was unable to detect XG (CD99) in the glomeruli, and this could be due to the phenotype of biopsies. As mentioned earlier, the Xg(a) phenotype of donors cannot be inferred through the detection of CD99, and females that are Xg(a+) have CD99H on their RBCs and Xg(a-) females have CD99L on their RBCs,[20] while 68% of Xg(a-) males have CD99H and 32% have CD99L.[20,106] The kidney biopsies used in this study were from males (three) and females (three) (Table 14). It is also unknown if XG (CD99) antigens are expressed differently by cells other than RBCs. Additional differences in results reported by the HPA and this investigation are detailed at the beginning of this section (2.1).

Positive detection was documented by the HPA and this investigation for the tubules of the kidney, however, documented detection from the HPA for the Lewis (FUT3), Kidd (SLC14A1), Chido/Rodgers (C4), and Knops (CD35) antigens are limited with their classification of “cells in tubules”. This documented detection does not specify whether detection was found in cortical tubules (distal and/or proximal) and/or medullary tubules (squamous and/or cuboidal) (Table 24).

Detection documented by the HPA for low expression of Lewis (FUT3) antigen in the tubules (n=3)[13] aligns with this investigation for low detection on distal tubules, and no detection on the medulla, but does not align with the high detection documented on proximal tubules (Table 21). Detection of Lewis (FUT3) antigen on tubules of the cortex, and not on tubules of the medulla is consistent with documented literature (Table 4).[44] Though it is unknown if the Lewis antigen behaves similarly by cells other than RBCs, the variability in documented detection between this investigation and the HPA could be due to Lewis antigens not being intrinsic to RBC membranes, where the antigen is passively adsorbed from soluble Lewis glycolipid present in the plasma.[9,20,44]

Also, donor management such as transfusion and dialysis could cause Lewis antigen to be shed from cell membranes.[20,44,80,81] Additional differences in results reported by the HPA and this investigation are detailed at the beginning of this section (2.1).

Though it is not specific, detection documented by the HPA for high expression of Kidd (SLC14A1) antigen on the tubules of the kidney (*n*=3)[13] aligns with this investigation documenting high detection on medullary tubules (Table 21). This detection also aligns with documented literature which states that these antigens are located in the medulla (Table 4).[20,26,44]

Detection documented by the HPA for the Chido/Rodgers (C4) antigen is variable on the tubules (three samples had high detection, three samples had medium detection, three samples had low detection).[13] This detection appears to align with cortical and medullary detection documented in this study (Table 21), though specific labeling is needed to confirm these claims. The variable labeling seen with the Chido/Rodgers (C4) antibody could be due to the presence of Chido/Rodgers antigens on the fourth component of complement (C4); these antigens are known for not being intrinsic to RBC membranes.[9,20,44] The Chido/Rodgers antigens are not documented to have solid tissue localization (Table 4), [9,20,44] but if the deposition of C4d was tested for, and also found to be present in deceased donor biopsy samples, the high detection documented by this investigation could be influenced through the involvement of the classical or lectin complement pathways.[44,110] Additional differences in results reported by the HPA and this investigation are detailed at the beginning of this section (2.1).

Detection documented by the HPA for low expression of the Knops (CD35) antigen on the tubules of the kidney (one sample had low detection, two samples had no detection) does not align with the medium and high expression documented by this investigation (Table 21). Detection of Knops antigens beyond of the glomeruli is also not consistent with documented literature (Table 4).[9,20,44] Variable expression of the Knops (CD35) antigen that was documented between the HPA and this study could be due to subjective qualitative interpretation of data, or could relate to the variable expression of CR1 that has been documented on RBCs.[20,44] However, it is not known if

CR1 is represented differently by cells other than RBCs. Though the tissues used in the HPA are classified as normal, an explanation for a lack of detection could arise from autoimmune disease that may cause a decrease in Knops antigen on cell membranes,[44] but it is unknown if this influence goes beyond RBC CR1. Additional differences in results reported by the HPA and this investigation are detailed at the beginning of this section (2.1).

Slight variation in documented positive detection between this study and the HPA for the MN (CD235a), Kidd (SLC14A1), and Chido/Rodgers (C4) antigens are unremarkable and could be due to subjective qualitative interpretation of data and/or the experience of the researcher performing testing.

Overall, the documented differences in IHC detection between the HPA and this investigation are unremarkable. Table 24 provides a comparison of IHC detection in the kidney between this study and the HPA.

Table 24. Comparison of Histoblood Group Antigen IHC Detection in Kidney Deceased Donor Tissues

Primary Antibody	Distal Tubules		Proximal Tubules	
	HPA	Our Study	HPA	Our Study
CD235a	**Medium ***	**Medium**	Not Detected	**Low**
GYPB	-	**Medium**	-	**Low**
RhD	Not Detected	Not Detected	Not Detected	Not Detected
RhCE	Not Detected	Not Detected	Not Detected	Not Detected
KEL	-	**Low**	-	**Medium**
FUT3	**^**	**Low**	**^**	**High**
ACKR1	Not Detected	Not Detected	**High ***	**High**
SLC14A1	**^**	Not Detected	**^**	Not Detected
CD99	Not Detected	Not Detected	Not Detected	Not Detected
C4	**^**	**Medium**	**^**	**Low**
CD35	**^**	**High**	**^**	**Medium**

* Highest IHC detection documented for all biopsies tested.
- No IHC data available.
^ HPA detection documented as "Cells in Tubules". Detection does not specify cortical or medullary tubules.

Table 24. (cont'd)

Primary Antibody	Glomeruli		Medullary Tubules	
	HPA	Our Study	HPA	Our Study
CD235a	Not Detected	Not Detected	Not Detected	**Low**
GYPB	-	Not Detected	-	**High**
RhD	Not Detected	Not Detected	Not Detected	Not Detected
RhCE	Not Detected	Not Detected	Not Detected	Not Detected
KEL	-	Not Detected	-	**Medium**
FUT3	Not Detected	Not Detected	**^**	Not Detected
ACKR1	**Low ***	Not Detected	Not Detected	Not Detected
SLC14A1	**Low**	Not Detected	**^**	**High**
CD99	**Medium ***	Not Detected	Not Detected	Not Detected
C4	**Low ***	Not Detected	**^**	**Low**
CD35	**High ***	**High**	**^**	**High**

2.1.4. Pancreas Tissue

This study reported positive labeling of endocrine cells with the MN (CD235a), Kidd (SLC14A1), and Chido/Rodgers (C4) antibodies; the HPA did not report detection of these substructures.[13] Additionally, positive labeling was noted for exocrine and endocrine cells with both the Ss (GYPB) and Kell polyclonal antibodies, but the HPA does not have data for either of these antigens.[13] The remaining differences involve detection of exocrine cells. Expression was reported by the HPA for Lewis (FUT3) antigen (all samples had low detection (*n*=3)),[13] XG (CD99) (three samples had medium detection, three samples had no detection),[13] and Duffy (ACKR1) (three samples had high detection, three samples had no detection).[13]

Considering the HPA did not report positivity with all biopsies tested for Duffy (ACKR1), and XG (CD99) antibodies,[13] the negative results obtained from this investigation with the Duffy (ACKR1), and XG (CD99) antibodies are unremarkable (Table 21).

Detection reported by the HPA for low expression of Lewis (FUT3) antigen on exocrine glandular cells is consistent with documented literature stating that Lewis antigen is present in the pancreas (Table 4).[44] This study was unable to detect Lewis (FUT3) antigen in exocrine cells, and though it is unknown if the Lewis antigen behaves similarly by cells other than RBCs, this detection could be due to Lewis antigens not being intrinsic to RBC membranes, where the antigen is passively adsorbed from soluble Lewis glycolipid present in the plasma.[9,20,44] Donor management such as transfusion and dialysis could cause Lewis antigen to be shed from cell membranes.[20,44,80,81] Additional differences in results reported by the HPA and this investigation are detailed at the beginning of this section (2.1).

The highest detection reported by the HPA for the Knops (CD35) antigen on exocrine glandular cells was high (one sample had high detection, one sample had medium detection, one sample had no detection);[13] this investigation documented low detection (Table 21). The variation in reported expression for the Knops (CD35) antigen between this study and the HPA could be related to the low number of antigen sites and variable expression of CR1 that has been

documented on RBCc.[20,44] However, it is not known if CR1 is represented differently by cells other than RBCs. An explanation for the lower detection documented by this investigation and the HPA could arise from autoimmune disease that can cause a decrease in Knops antigen on cell membranes,[44] but it is unknown if this influence goes beyond RBC CR1, and no medical history was provided for the biopsies used in this study, nor is such history available for the samples used in the HPA.[13] Additional differences in results reported by the HPA and this investigation are detailed at the beginning of this section (2.1).

The reported low labeling of endocrine cells in this study with the MN (CD235a), and Chido/Rodgers (C4) antibodies could be due to subjective qualitative interpretation of data. Additionally, biopsy P7 was unable to detect Chido/Rodgers (C4) antigen in this investigation, and the remaining biopsies only showed low detection. Overall, detection of MN (CD235a) and Chido/Rodgers (C4) antigen on endocrine cells does not align with documented literature for the location of these antigens (Table 4). Another explanation for low positive labeling documented on endocrine cells could be due to the highly vascularized nature of endocrine cells (islets of Langerhans).[147] In this study, the MN (CD235a) antibody positively labels RBCs, and the Chido/Rodgers (C4) antibody positively labels plasma, which could influence the perspective labeling of endocrine cells. Additional differences in results reported by the HPA and this investigation are detailed at the beginning of this section (2.1)..

Variation in positive labeling of endocrine cells with the Kidd (SLC14A1), and Ss (GYPB) antibodies were seen between two pancreas donors, P1 and P7. The remaining biopsies had low detection on endocrine cells. Though no pathologic changes were noted, the increased labeling with these donors could be influenced by undetermined morbidity, or through deceased donor management. High positive labeling was noted along what appear to be alpha cells of the islet of Langerhans.[148] Alpha cells are responsible for secreting glucagon, which increases blood sugar.[149] In addition to hypoglycemia, catecholamines can increase glucagon secretion from alpha cells.[150] As mentioned in the literature review section, brain death and organ procurement

can result in an increase in in these hormones,[15,17,120] which may influence the variation in immunoreactivity seen with these antibodies. Additional testing is needed to confirm these claims. Following a BLAST comparison (https://blast.ncbi.nlm.nih.gov/Blast.cgi) both the Ss (GYPB) and Kidd (SLC14A1) antigens do not have AA sequence similarities (see Appendix A) with glucagon (https://www.uniprot.org/uniprotkb/P01275). Sequence similarities could cause the Ss (GYPB) and Kidd (SLC14A1) antibodies to cross-react with glucagon and produce positive labeling. Additional differences in results reported by the HPA and this investigation are detailed at the beginning of this section (2.1).

Since variability in reactivity was not seen throughout all biopsies, and no health history was provided with donor biopsies used in this investigation, the overall documented differences in IHC detection between the HPA and this investigation are unremarkable. Table 25 provides a comparison of IHC detection in the pancreas between this study and the HPA.

Table 25. Comparison of Histoblood Group Antigen IHC Detection in Pancreas Deceased Donor Tissues

Primary Antibody	Exocrine Glandular Cells		Endocrine Cells	
	HPA	Our Study	HPA	Our Study
CD235a	Not Detected	Not Detected	Not Detected	**Low**
GYPB	-	Not Detected	-	**High ***
RhD	Not Detected	Not Detected	Not Detected	Not Detected
RhCE	Not Detected	Not Detected	Not Detected	Not Detected
KEL	-	Not Detected	-	**Low**
FUT3	**Low**	Not Detected	**Low**	**Low**
ACKR1	**High ***	Not Detected	Not Detected	Not Detected
SLC14A1	Not Detected	Not Detected	Not Detected	**High ***
CD99	**Medium ***	Not Detected	**High ***	**High**
C4	Not Detected	Not Detected	Not Detected	**Low**
CD35	**High ***	**Low**	**Low ***	**Low**

* Highest IHC detection documented for all biopsies tested.
- No IHC data available

2.1.5. Conclusions by Organ-Type

Overall, the IHC data collected in this investigation reveals that the expression of histoblood group antigens on deceased donor tissues (heart, lung, kidney, and pancreas) are comparable to that of healthy individuals. Documentation from the HPA showed the most consistency with the detection of Lewis (FUT3) and Kidd (SLC14A1) antigens, and most variability with the detection of Chido/Rodgers (C4), and Knops (CD35) antigens. This investigation had consistent detection of MN (CD235a), Kell, Lewis (FUT3), XG (CD99), and Knops (CD35) antigens. Variability was noted for the detection of Duffy (ACKR1) antigen in the heart, and for the detection of Ss (GYPB), Kidd (SLC14A1), and Chido/Rodgers (C4) antigen in the pancreas.

This investigation did further document substructures for immunodetection of histoblood group antigens where comparable data was unavailable: intercalated discs (heart), bronchial epithelium (lung), and urothelium (kidney). There was also no comparable IHC data for Ss (GYPB) and Kell antigens; this investigation provides classification of these antigens by cells other than RBCs, which has yet to be documented in literature.

2.2. Data by Primary Antibody

Comparisons between data collected from ESTs (Table 1), the HPA (Table 2), and this study (Table 21) are described below for each primary antibody.

2.2.1. MNS System

2.2.1.1. MN

The MN protein was detected with the CD235a polyclonal antibody PA5-80679 in the heart, lung (bronchial epithelium), kidney, and pancreas. (Table 21). This antibody also produced positive labeling of RBCs.

These results are consistent with EST data (Table 1) and with the HPA (Table 2) for detection in heart and kidney tissues, and consistent with the literature for detection in the kidney (Table 4).

These data are inconsistent with EST data in that positive labeling was seen on the bronchial epithelium and in the pancreas with IHC (Table 1), and inconsistent with the HPA due to a lack of detection in lung alveolar cells and macrophages (the HPA reported expression), and detection in pancreas (the HPA did not report expression) (Table 2). Additionally, in this investigation, positive MN labeling was present in bronchial epithelium and the urothelium of the kidney; the HPA has no documentation of this.

2.2.1.2. Ss

Labeling for the Ss protein with GYPB polyclonal antibody PA5-71844 was noted in heart, kidney, and pancreas tissue (Table 21). This antibody also produced positive labeling of RBCs.

These results are consistent with EST data showing detection in heart and kidney tissues (Table 1), and consistent with reports of renal expression (Table 4). There is no comparable IHC data in the HPA for GYPB (Table 2).

These data are inconsistent with EST data since positive labeling was seen in the pancreas with IHC (Table 1).

2.2.2. Rh System

2.2.2.1. RHD

There was no detection of the RhD protein in the heart, lung, kidney, or pancreas using RHD polyclonal antibody PA5-112694 (Table 21). RBCs within biopsy samples also showed negative labeling.

These results are consistent with the HPA (Table 2) and with documented literature stating that RH system antigens are erythroid specific (Table 4).

These results are inconsistent with EST data that shows RH detection in the heart, lung, and kidney (Table 1).

The inability of RhD polyclonal antibody PA5-112694 to positively label RBCs or tissue substructures within biopsies suggests the possibility of false negative labeling. Tissue processing may have altered the expression of the RhD epitopes required for this antibody to bind, but the RBC blood smear technique produced a positive result, indicating the antibody can bind to the RhD antigen, when present. Considering the *RhD* and *RhCE* genes are 97% identical[9], a BLAST sequence analysis of both the RhD and RhCE proteins were performed, and the immunogen sequence of this antibody was found to be identical to the RhCE antigen (See Appendix A). Additionally, the immunogen sequence of the RhCE polyclonal antibody PA5-140007 was also found to be identical to the RhD antigen; therefore, positive labeling of RBCs reported with the RhCE antibody PA5-140007 account for the lack of positive labeling with RhD antibody PA5-112694, validating that the RhD and RhCE antigens are both erythroid-specific.

2.2.2.2. RHCE

There was no detection of the RhCE protein in the heart, lung, kidney, or pancreas using RHCE polyclonal antibody (PA5-140007) (Table 21). This antibody did produce positive labeling of RBCs, and this reactivity was consistent across all biopsy samples, of all tissue types.

These results are consistent with the HPA (Table 2) and with documented literature stating that RH system antigens are erythroid specific (Table 4).

These results are inconsistent with EST data that shows RH detection in the heart, lung, and kidney (Table 1).

Positive labeling of FFPE RBCs was detected for all biopsies tested in this investigation, but the RhCE antibody did not product positive labeling of the FFPE RBC cell pellet. This result was unexpected, and suggests that sample processing may have altered the expression of RhCE epitopes required for antibody detection. Considering both the RBC blood smear technique and internal RBCs within biopsies produced a positive result, sample processes specific for the creation of FFPE cell pellets (see section 2.3.2) need to be investigated further as a cause of this phenomenon.

2.2.3. Kell System

Detection of the Kell glycoprotein with KEL polyclonal antibody (PA5-97891) was noted in the heart, lung, kidney, and pancreas (Table 21).

There is no comparable data with both EST (Table 1) and the HPA (Table 2) for this antibody. Though there is EST data showing XK on heart tissue, the Kell polyclonal antibody used in this investigation does not target this protein (see Appendix A).

These data do correlate with reports that Kell is expressed in the heart (Table 4), but documentation of the Kell glycoprotein in kidney, lung, or pancreas was not found.

The Kell polyclonal antibody PA5-97891 was able to positively label RBCs in the FFPE RBC control pellet and blood smear but was surprisingly unable to label RBCs within sample biopsies. In addition to the FFPE RBC cell pellet, this antibody produced labeling within various

tissue substructures; therefore, the differences in RBC labeling to not appear to be caused from formalin fixation. The lack of RBC labeling in sample tissues could be false negative reactivity or could result from sample processing that may have altered the expression of detectable epitopes required for antibody detection.

2.2.4. Lewis System

Detection of the Lewis antigen using FUT3 polyclonal antibody (PA5-101596) was noted in the heart, lung (bronchial epithelium), kidney, and pancreas (Table 21).

These results are consistent with the HPA for detection in the heart, kidney, and pancreas (Table 2), and consistent with documented literature for detection in the kidney and pancreas (Table 4).

These data are inconsistent with the HPA because this investigation did not detect Le expression by macrophages in the lung (the HPA did report Le expression by lung macrophages) (Table 2). Additionally, this investigation detected antigen in bronchial epithelium and the urothelium of the renal pelvis, but the HPA has no documentation of this. Also, Le expression in the heart or lung has not been reported (Table 4). There are no comparable data for Le using EST (Table 1).

2.2.5. Duffy System

the Duffy glycoprotein was detected in heart and kidney using an ACKR1 polyclonal antibody (PA5-82549) (Table 21). Expression was also detected in endothelial cells of various blood vessels.

These results correlate with EST data and the HPA showing FY expression in heart and kidney (Table 1, Table 2). The detection of Fy antigen on endothelial cells of each tissue type also correlates with the literature (Table 4).

These results are inconsistent with EST data regarding detection of Fy antigen in lung and pancreas (Table 1), and inconsistent with the HPA, as Fy antigen was not detected in the glomeruli or exocrine glandular cells of the pancreas in this study (Table 2). Additionally, this

investigation detected Fy antigen the urothelium of the kidney, whereas the HPA has no documentation of this. Expression was not seen in the collecting ducts of the kidney, or the alveolar epithelium as reported in the literature (Table 4).

2.2.6. Kidd System

Detection of the Kidd glycoprotein with SLC14A1 polyclonal antibody (PA5-110377) was noted in the heart, lung (bronchial epithelium), kidney, and pancreas (Table 21). Positive labeling was seen on infiltrating leukocytes, in each tissue type.

These results correlate with EST data showing Jk system expression in the heart, lung, kidney, and pancreas (Table 1), and with the HPA by detecting Jk antigen on cardiomyocytes, and cells of kidney tubules (specifically the loop of Henle) (Table 2). Documented literature states that Jk antigen is in the kidney, which correlates with results of this investigation (Table 4).

These data are inconsistent with the HPA whereas this study did not detect Jk antigen on the glomeruli of the kidney, on the alveolar cells and macrophages of the lung, or in the pancreas (Table 2). Additionally, this investigation detected Fy antigen the urothelium of the kidney, and the HPA has no documentation of this. The detection of JK antigen in the heart, lung, or pancreas is not documented in literature (Table 4).

2.2.7. XG System

Detection of XG using CD99 polyclonal antibody (PA5-32337) was noted in heart and pancreas tissue (Table 21). Where lymphocytes and fibroblasts were present, positive detection was seen.

These results are consistent with EST data showing detection in only heart and pancreas tissues (Table 1), and there is correlation with the HPA for the detection of CD99 in pancreatic endocrine cells (Table 2). The results of this investigation correlate with documented literature for the detection of CD99 in lymphocytes, fibroblasts, heart, and islet cells of the pancreas (Table 4).

These data are inconsistent with the HPA. Detection of pancreatic exocrine glandular cells, glomeruli of the kidney, or lung macrophages was not duplicated in this study (Table 2), but

this investigation conflicts with the HPA in that detection was seen in the intercalated discs and pericardium of heart tissue.

2.2.8. Chido/Rodgers System

Detection of CH/RG using C4 polyclonal antibody (PA5-29133) was noted in the heart, lung, kidney, and pancreas (Table 21). Positive labeling was also seen in the plasma within vessels.

These results correlate with the HPA for detection on cardiomyocytes, lung macrophages, and tubule cells of the kidney (Table 2).

These data are inconsistent with the HPA for detection in the glomeruli (this investigation did not have detection), and no detection in pancreas tissue (investigation saw positive labeling in both exocrine and endocrine cells) (Table 2). Additionally, this investigation detected CH/RG antigen on bronchial epithelium, and the urothelium of the kidney; the HPA has no documentation of this.

The results of this study do not correlate with documented literature which states that CH/RG does not have solid tissue localization (Table 4), and there is no EST data to correlate CH/RG expression (Table 1).

2.2.9. Knops System

Detection of the KN glycoprotein with CD35 polyclonal antibody (PA5-98627) was noted in the heart, lung, kidney, and pancreas (Table 21).

These results are consistent with EST data for detection in the heart, lung, kidney, and pancreas (Table 1), and consistent with the HPA for detection in lung macrophages, the glomeruli and tubules of the kidney, and the exocrine and endocrine cells of the pancreas (Table 2). The results of this study also correlate with documented literature for the detection of KN in the glomerulus and on macrophages (Table 4).

These data are inconsistent with the HPA for not detecting KN antigen in heart tissue (this investigation saw positive labeling), and for the detection of KN antigen on alveolar cells (this investigation did not have detection) (Table 2).

2.2.10. Conclusions by Primary Antibody

For the histoblood group antigen systems tested in this study, IHC detection was mostly consistent with the literature; most notably, the Rh system antigens (RhD and RhCE) were confined to RBCs.

The Duffy (ACKR1) antigen was found to be expressed by endothelial cells of various blood vessels within each biopsy tested in this investigation. This detection correlates with the reports that Duffy system antigens are located on endothelial cells of capillary and post-capillary venules.[9,20,44] As mentioned in the literature review above, endothelium is an important interface for transplantation because this is where the donor and recipient circulation first make contact; endothelium is a prime target for non-HLA antibodies.[3,6,29] The best-studied non-HLA antibodies include anti-endothelial cell antibodies,[3,9] because antibodies can induce graft injury through endothelial cell activation.[6] Detection of the Duffy (ACKR1) antigen on endothelial cells warrants further studies into this blood group system for non-HLA histocompatibility, and for AMR.

Notable detection that contrasted with the literature was the finding of Kidd (SLC14A1) expression by leukocytes. This detection was seen in all tissue types, and the HPA documented Kidd (SLC14A1) detection on lung macrophages (Table 2). Further investigation is needed to determine if Kidd system antigens are also present on these cells in normal tissues.

There are no comparable IHC data for both the Ss (GYPB) and Kell histoblood group systems, therefore this investigation provides new data for the detection of these antigens by cells other than RBCs.

CONCLUSION

Transplant patients may have circulating non-ABO RBC antibodies, the frequency of which in the total study population of this investigation aligns with the frequency of these antibodies in the general transfused population (2-5%).[7-8] The antibodies detected in this study align with characteristics of the clinically significant antibodies that have been reported, being commonly encountered and targeting antigens that have higher immunogenicity.[9,20]

IHC data collected in this investigation reveals that the expression of histoblood group antigens in deceased donor tissues (heart, lung, kidney, and pancreas) is comparable to that of healthy individuals, with few differences noted. Additionally, this investigation provides further details for the detection of histoblood group antigens on tissue substructures where comparable IHC data were unavailable: intercalated discs (heart), bronchial epithelium (lung), and urothelium (kidney), and new data for the expression of Ss (GYPB) and Kell antigens by cells other than RBCs.

Immune targets of the primary antibodies used in this investigation are associated with epitopes that are common for the protein of interest, and do not discriminate specific polymorphisms of alleles within each blood group system (e.g., Fy^a versus Fy^b). Therefore, these data do not offer indications for the rejection of potential organ donors based on blood group genotyping, but instead allow for the consideration of non-ABO RBC antibodies in cases of suspected non-HLA AMR by correlating the location of these antigens with that of immune destruction seen in allografts.

The clinical significance of RBC alloimmunization is widely recognized in transfusion medicine, and this study provides new evidence for the transplant community by documenting the frequency of non-ABO RBC antibodies in transplant patients. It also identifies the location of histoblood group antigens in deceased donor tissues as potential areas to look for evidence of AMR in post-transplant biopsies. These data facilitate consideration of non-ABO RBC antibodies in cases of suspected AMR when HLA DSAs are absent.

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APPENDIX A: IHC PRIMARY ANTIBODY IMMUNOGEN INFORMATION

A-1. Immunogen Target Amino Acid Sequence of Primary Antibodies, Compared to the HPA

Table A-1: Immunogen Target Amino Acid Sequence of Primary Antibodies, Compared to the HPA

Antibody	Study Polyclonal Antibodies *		HPA Polyclonal Antibodies ^					
	Catalog #	Immunogen Amino Acid Sequence	Catalog #	Immunogen Amino Acid Sequence	Catalog #	Immunogen Amino Acid Sequence	Catalog #	Immunogen Amino Acid Sequence
CD235a	PA5-80679	AA 1-91	HPA014811	AA 31-90	-	-	-	-
GYPB	PA5-71844	AA 22-51	-	-	-	-	-	-
RhD	PA5-112694	AA 32-76	HPA077385	AA 388-417	-	-	-	-
RhCE	PA5-140007	AA 388-417						
KEL	PA5-97891	AA 201-500	-	-	-	-	-	-
FUT3	PA5-101596	AA 94-144	HPA046966	AA 200-235	-	-	-	-
ACKR1	PA5-82549	AA 308-336	HPA016421	AA 308-336	HPA017672	AA 2-60	-	-
SLC14A1	PA5-110377	AA 376-389	HPA059570	AA 192-232	-	-	-	-
CD99	PA5-32337	AA 1-185	HPA035304	AA 143-183	-	-	-	-
C4	PA5-29133	AA 23-302	HPA046356	AA 668-760	HPA048287	AA 247-332	HPA050103	AA 1651-1738
CD35	PA5-98627	AA 420-550	HPA042455	AA 2001-2034	HPA043579	AA 2001-2034	HPA049348	AA 278-334, AA 728-784

* Invitrogen (www.thermofisher.com)
^ Atlas Antibodies (https://www.atlasantibodies.com)

A-2. MN (CD235a)

Full Amino Acid Sequence: (www.uniprot.org/uniprotkb/P02724)

MYGKIIFVLL LSEIVSISAS STTGVAMHTS TSSSVTKSYI SSQTNDTHKR DTYAATPRAH EVSEISVRTV YPPEEETGER VQLAHHFSEP EITLIIFGVM AGVIGTILLI SYGIRRLIKK SPSDVKPLPS PDTDVPLSSV EIENPETSDQ

Polyclonal Antibody Immunogen Target Sequence: AA 1-91

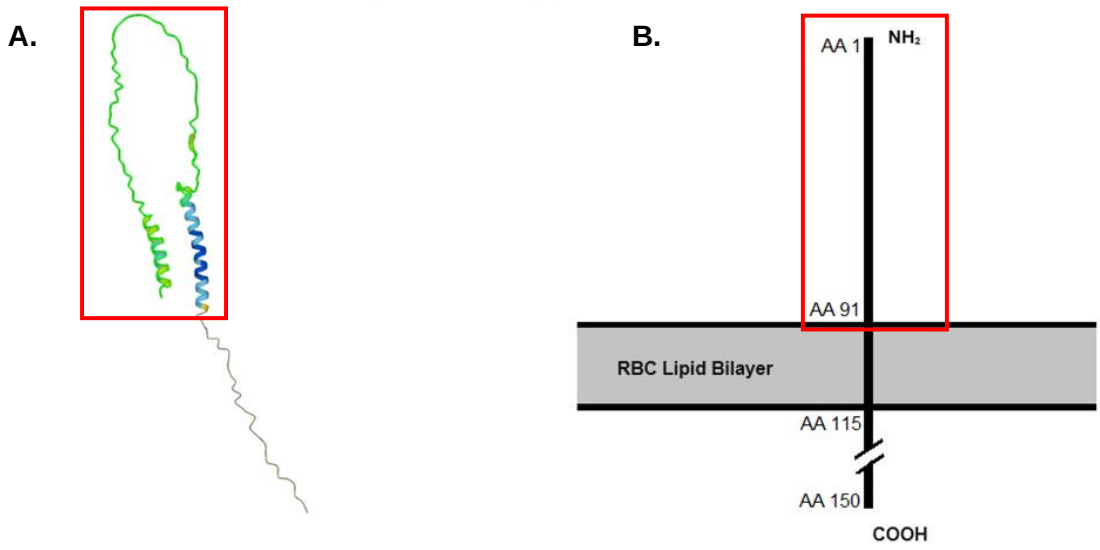


Figure A-2.1. MN (GYPA) Protein Structure
(A.) 3D model of GYPA from www.alphafold.ebi.ac.uk/entry/P02724. Green highlighting and red box represent the target AA sequence of the primary antibody used in this investigation (AA 1-91). **(B.)** Simple diagram of GYPA adapted from: Cohn CS, et al. Technical Manual. AABB (2023)[9]; Reid ME, et al. The Blood Group Antigen Facts Book (2012)[44] ,red box represents the target AA sequence of the primary antibody used in this investigation (AA 1-91).

A-3. Ss (GYPB)

Full Amino Acid Sequence: (www.uniprot.org/uniprotkb/P06028)

MYGKIIFVLL LSEIVSISAL STTEVAMHTS TSSSVTKSYI SSQTNGETGQ LVHRFTVPAP VVIILIILCV MAGIIGTILL ISYSIRRLIK A

Polyclonal Antibody Immunogen Target Sequence: AA 22-51

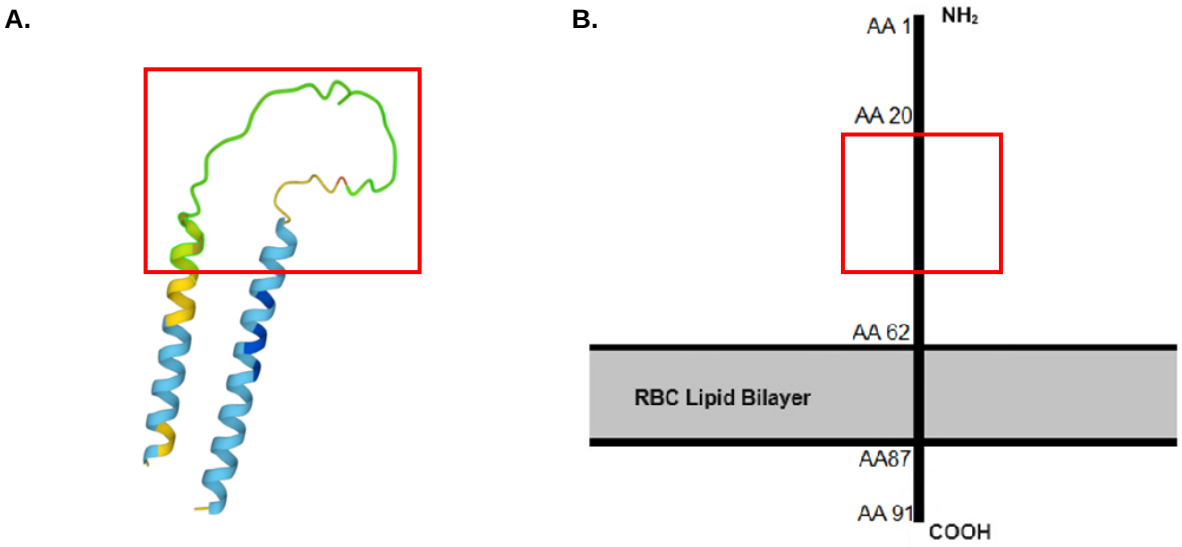


Figure A-3.1. Ss (GYPB) Protein Structure
(A.) 3D model of GYPB from www.alphafold.ebi.ac.uk/entry/P06028. Green highlighting and red box represent the target AA sequence of the primary antibody used in this investigation (AA 22-51). **(B.)** Simple diagram of GYPB adopted from: Cohn CS, et al. Technical Manual. AABB (2023)[9]; Reid ME, et al. The Blood Group Antigen Facts Book (2012)[44], the red box represents the target AA sequence of the primary antibody used in this investigation (AA 22-51).

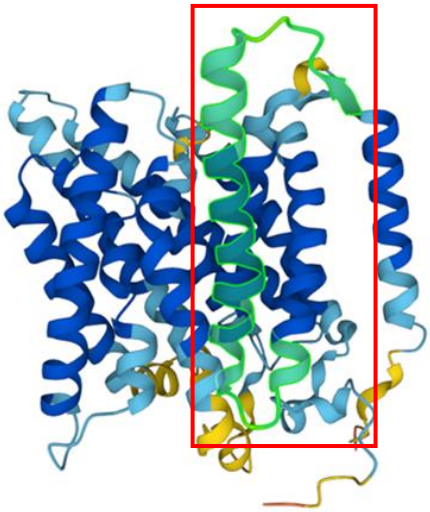
A-4. RhD

Full Amino Acid Sequence: (www.uniprot.org/uniprotkb/Q02161)

MSSKYPRSVR RCLPLWALTL EAALILLFYF FTHYDASLED QKGLVASYQV GQDLTVMAAI GLGFLTSSFR RHSWSSVAFN LFMLALGVQW AILLDGFLSQ FPSGKVVITL FSIRLATMSA LSVLISVDAV LGKVNLAQLV VMVLVEVTAL GNLRMVISNI FNTDYHMNMM HIYVFAAYFG LSVAWCLPKP LPEGTEDKDQ TATIPSLSAM LGALFLWMFW PSFNSALLRS PIERKNAVFN TYYAVAVSVV TAISGSSLAH PQGKISKTYV HSAVLAGGVA VGTSCHLIPS PWLAMVLGLV AGLISVGGAK YLPGCCNRVL GIPHSSIMGY NFSLLGLLGE IIYIVLLVLD TVGAGNGMIG FQVLLSIGEL SLAIVIALMS GLLTGLLLNL KIWKAPHEAK YFDDQVFWKF PHLAVGF

Polyclonal Antibody Immunogen Target Sequence: AA 32-76

A.



B.

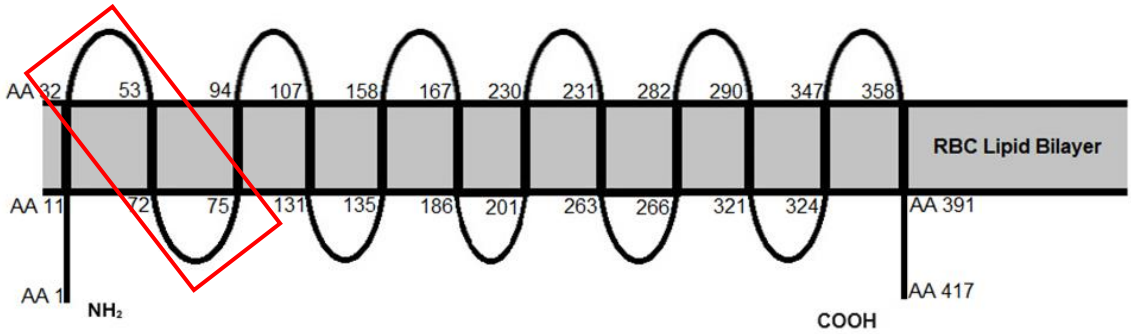


Figure A-4.1. RhD Protein Structure
(A.) 3D model of the RhD protein from www.alphafold.ebi.ac.uk/entry/Q02161. Green highlighting and red box represent the target AA sequence of the primary antibody used in this investigation (AA 32-76). **(B.)** Simple diagram of the RhD protein adopted from: Cohn CS, et al. Technical Manual. AABB (2023)[9]; Reid ME, et al. The Blood Group Antigen Facts Book (2012)[44], the red box represents the target AA sequence of the primary antibody used in this investigation (AA 32-76).

A-5. RhCE

Full Amino Acid Sequence: (www.uniprot.org/uniprotkb/P18577)

MSSKYPRSVR RCLPLCALTL EAALILLFYF FTHYDASLED QKGLVASYQV GQDLTVMAAL GLGFLTSNFR RHSWSSVAFN LFMLALGVQW AILLDGFLSQ FPPGKVVITL FSIRLATMSA MSVLISAGAV LGKVNLAQLV VMVLVEVTAL GTLRMVISNI FNTDYHMNLR HFYVFAAYFG LTVAWCLPKP LPKGTEDNDQ RATIPSLSAM LGALFLWMFW PSVNSALLRS PIQRKNAMFN TYYALAVSVV TAISGSSLAH PQRKISMTYV HSAVLAGGVA VGTSCHLIPS PWLAMVLGLV AGLISIGGAK CLPVCCNRVL GIHHISVMHS IFSLLGLLGE ITYIVLLVLH TVWNGNGMIG FQVLLSIGEL SLAIVIALTS GLLTGLLLNL KIWKAPHVAK YFDDQVFWKF PHLAVGF

Polyclonal Antibody Immunogen Target Sequence: AA 388-417

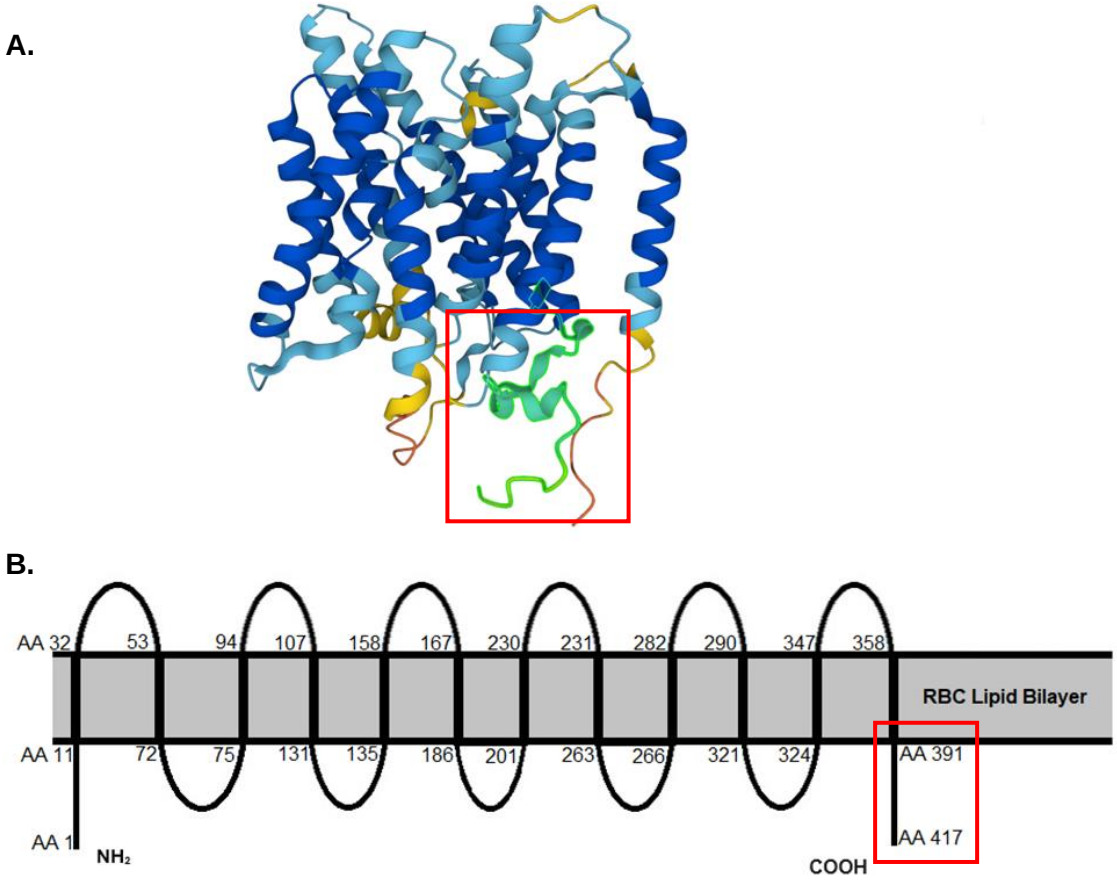
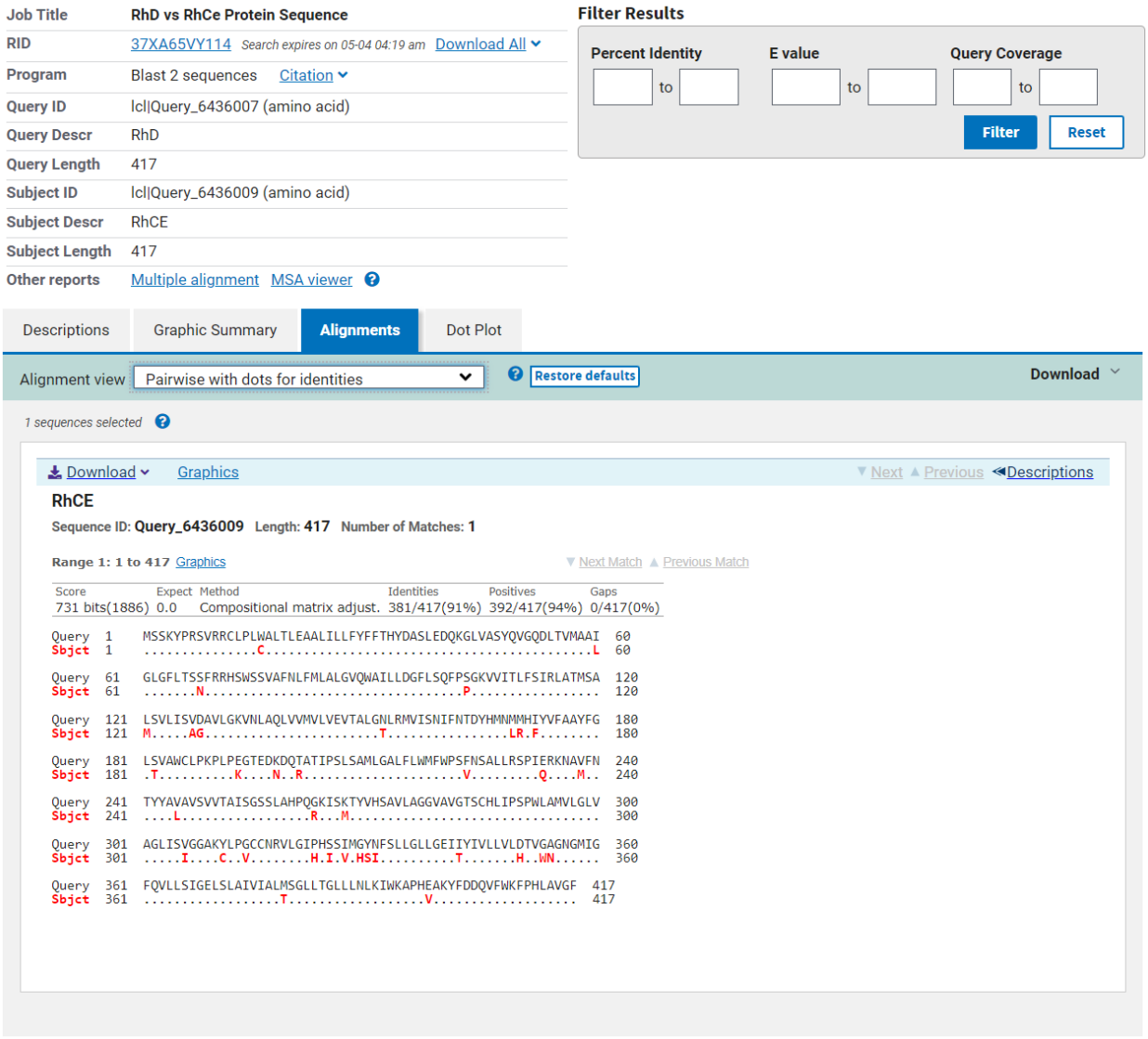


Figure A-5.1. RhCE Protein Structure
(A.) 3D model of the RhCE protein from www.alphafold.ebi.ac.uk/entry/P18577. Green highlighting and red box represent the target AA sequence of the primary antibody used in this investigation (AA 388-417). **(B.)** Simple diagram of the RhCE protein adopted from: Cohn CS, et al. Technical Manual. AABB (2023)[9]; Reid ME, et al. The Blood Group Antigen Facts Book (2012)[44], the red box represents the target AA sequence of the primary antibody used in this investigation (AA 388-417).

A-6. RhD and RhCE BLAST Sequence Data



Job Title	RhD vs RhCe Protein Sequence
RID	37XA65VY114 *Search expires on 05-04 04:19 am* Download All
Program	Blast 2 sequences Citation
Query ID	lcl|Query_6436007 (amino acid)
Query Descr	RhD
Query Length	417
Subject ID	lcl|Query_6436009 (amino acid)
Subject Descr	RhCE
Subject Length	417
Other reports	Multiple alignment MSA viewer

Filter Results

Percent Identity to E value to Query Coverage to Filter Reset

Descriptions | Graphic Summary | Alignments | Dot Plot

Alignment view: Pairwise with dots for identities | Restore defaults | Download

1 sequences selected

Download Graphics ▼Next ▲Previous ◀◀Descriptions

RhCE

Sequence ID: **Query_6436009** Length: **417** Number of Matches: **1**

Range 1: 1 to 417 Graphics ▼Next Match ▲Previous Match

Score	Expect	Method	Identities	Positives	Gaps
731 bits(1886)	0.0	Compositional matrix adjust.	381/417(91%)	392/417(94%)	0/417(0%)

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Query  61   GLGFLTSSFRRHSWSSVAFNLFMLALGVQWAILLDGFLSQFPSGKVVITLFSIRLATMSA  120
Sbjct  61   .......N.....................................P.................  120

Query  121  LSVLISVDAVLGKVNLAQLVVMVLVEVTALGNLRMVISNIFNTDYHMNMMHIYVFAAYFG  180
Sbjct  121  M.....AG.......................T.................LR.F........  180

Query  181  LSVAWCLPKPLPEGTEDKDQTATIPSLSAMLGALFLWMFWPSFNSALLRSPIERKNAVFN  240
Sbjct  181  .T..........K....N..R.....................V.........Q....M..  240

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Sbjct  241  ....L.................R...M..............................  300

Query  301  AGLISVGGAKYLPGCCNRVLGIPHSSIMGYNFSLLGLLGEIIYIVLLVLDTVGAGNGMIG  360
Sbjct  301  .....I....C..V........H.I.V.HSI..........T.......H..WN......  360

Query  361  FQVLLSIGELSLAIVIALMSGLLTGLLLNLKIWKAPHEAKYFDDQVFWKFPHLAVGF  417
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Figure A-6.1. RhD and RhCE BLAST protein sequence comparison
RhD and RhCE protein sequence comparison adapted from:
https://blast.ncbi.nlm.nih.gov/Blast.cgi
Query Sequence = RhD, Subject Sequence = RhCE

A-7. Kell

Full Amino Acid Sequence: (www.uniprot.org/uniprotkb/P23276)

MEGGDQSEEE PRERSQAGGM GTLWSQESTP EERLPVEGSR PWAVARRVLT AILILGLLLC FSVLLFYNFQ NCGPRPCETS VCLDLRDHYL ASGNTSVAPC TDFFSFACGR AKETNNSFQE LATKNKNRLR RILEVQNSWH PGSGEEKAFQ FYNSCMDTLA IEAAGTGPLR QVIEELGGWR ISGKWTSLNF NRTLRLLMSQ YGHFPFFRAY LGPHPASPHT PVIQIDQPEF DVPLKQDQEQ KIYAQIFREY LTYLNQLGTL LGGDPSKVQE HSSLSISITS RLFQFLRPLE QRRAQGKLFQ MVTIDQLKEM APAIDWLSCL QATFTPMSLS PSQSLVVHDV EYLKNMSQLV EEMLLKQRDF LQSHMILGLV VTLSPALDSQ FQEARRKLSQ KLRELTEQPP MPARPRWMKC VEETGTFFEP TLAALFVREA FGPSTRSAAM KLFTAIRDAL ITRLRNLPWM NEETQNMAQD KVAQLQVEMG ASEWALKPEL ARQEYNDIQL GSSFLQSVLS CVRSLRARIV QSFLQPHPQH RWKVSPWDVN AYYSVSDHVV VFPAGLLQPP FFHPGYPRAV NFGAAGSIMA HELLHIFYQL LLPGGCLACD NHALQEAHLC LKRHYAAFPL PSRTSFNDSL TFLENAADVG GLAIALQAYS KRLLRHHGET VLPSLDLSPQ QIFFRSYAQV MCRKPSPQDS HDTHSPPHLR VHGPLSSTPA FARYFRCARG ALLNPSSRCQ LW

Polyclonal Antibody Immunogen Target Sequence: AA 201-500

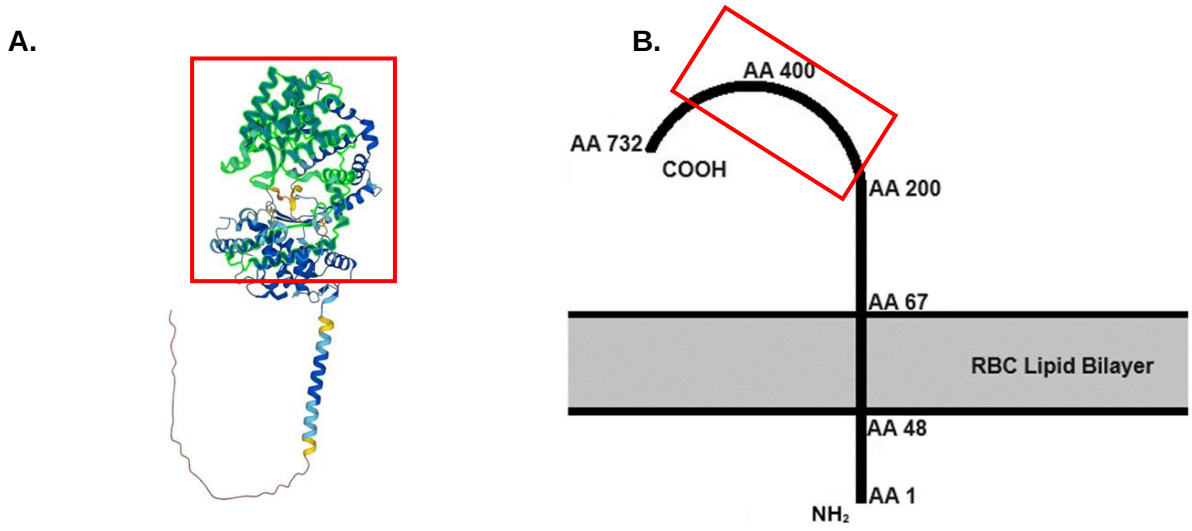

Figure A-7.1. Kell Protein Structure
(A.) 3D model of the Kell glycoprotein from www.alphafold.ebi.ac.uk/entry/P23276. Green highlighting and red box represent the target AA sequence of the primary antibody used in this investigation (AA 201-500). **(B.)** Simple diagram of the Kell protein adopted from: Cohn CS, et al. Technical Manual. AABB (2023)[9]; Reid ME, et al. The Blood Group Antigen Facts Book (2012)[44], the red box represents the target AA sequence of the primary antibody used in this investigation (AA 201-500).

A-8. Lewis (FUT3)

Full Amino Acid Sequence: (www.uniprot.org/uniprotkb/P21217)

MDPLGAAKPQ WPWRRCLAAL LFQLLVAVCF FSYLRVSRDD ATGSPRAPSG SSRQDTTPTR PTLLILLRTW PFHIPVALSR CSEMVPGTAD CHITADRKVY PQADMVIVHH WDIMSNPKSR LPPSPRPQGQ RWIWFNLEPP PNCQHLEALD RYFNLTMSYR SDSDIFTPYG WLEPWSGQPA HPPLNLSAKT ELVAWAVSNW KPDSARVRYY QSLQAHLKVD VYGRSHKPLP KGTMMETLSR YKFYLAFENS LHPDYITEKL WRNALEAWAV PVVLGPSRSN YERFLPPDAF IHVDDFQSPK DLARYLQELD KDHARYLSYF RWRETLRPRS FSWALDFCKA CWKLQQESRY QTVRSIAAWF T

Polyclonal Antibody Immunogen Target Sequence: AA 94-144

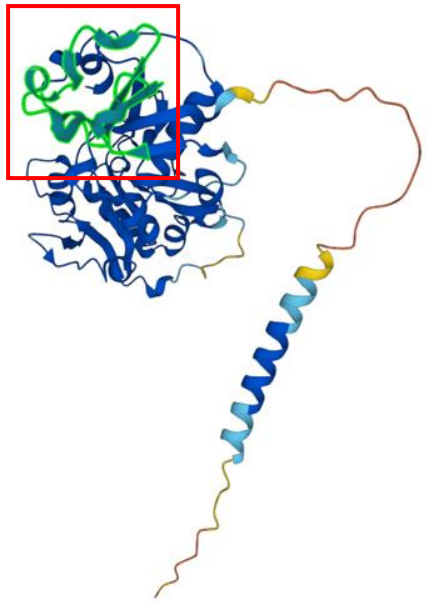

Figure A-8.1. Lewis (FUT3) Protein Structure
3D model of the LE antigen from www.alphafold.ebi.ac.uk/entry/P21217. Green highlighting and red box represent the target AA sequence of the primary antibody used in this investigation (AA 94-144).

A-9. Duffy (ACKR1)

Full Amino Acid Sequence: (www.uniprot.org/uniprotkb/Q16570)

MGNCLHRAEL SPSTENSSQL DFEDVWNSSY GVNDSFPDGD YGANLEAAAP CHSCNLLDDS ALPFFILTSV LGILASSTVL FMLFRPLFRW QLCPGWPVLA QLAVGSALFS IVVPVLAPGL GSTRSSALCS LGYCVWYGSA FAQALLLGCH ASLGHRLGAG QVPGLTLGLT VGIWGVAALL TLPVTLASGA SGGLCTLIYS TELKALQATH TVACLAIFVL LPLGLFGAKG LKKALGMGPG PWMNILWAWF IFWWPHGVVL GLDFLVRSKL LLLSTCLAQQ ALDLLLNLAE ALAILHCVAT PLLLALFCHQ ATRTLLPSLP LPEGWSSHLD TLGSKS

Polyclonal Antibody Immunogen Target Sequence: AA 308-336

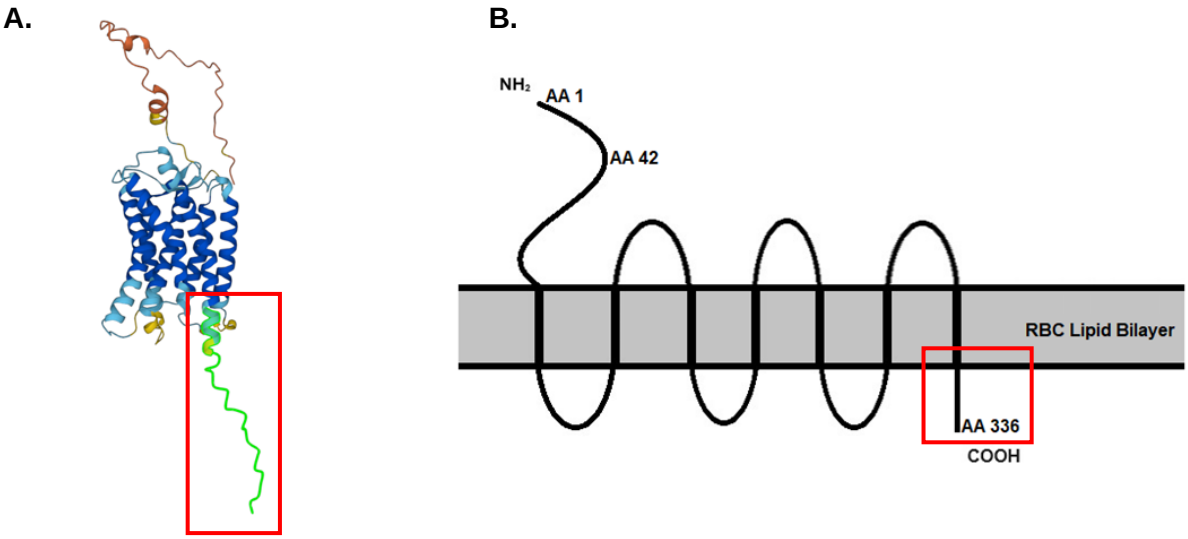

Figure A-9.1. Duffy (ACKR1) Protein Structure
(A.) 3D model of the FY glycoprotein from www.alphafold.ebi.ac.uk/entry/Q16570. Green highlighting and red box represent the target AA sequence of the primary antibody used in this investigation (AA 308-336). **(B.)** Simple diagram of the FY protein adopted from: Cohn CS, et al. Technical Manual. AABB (2023)[9]; Reid ME, et al. The Blood Group Antigen Facts Book (2012)[44], the red box represents the target AA sequence of the primary antibody used in this investigation (AA 308-336).

A-10. Kidd (SLC14A1)

Full Amino Acid Sequence: (www.uniprot.org/uniprotkb/Q13336)

MEDSPTMVRV DSPTMVRGEN QVSPCQGRRC FPKALGYVTG DMKELANQLK DKPVVLQFID WILRGISQVV FVNNPVSGIL ILVGLLVQNP WWALTGWLGT VVSTLMALLL SQDRSLIASG LYGYNATLVG VLMAVFSDKG DYFWWLLLPV CAMSMTCPIF SSALNSMLSK WDLPVFTLPF NMALSMYLSA TGHYNPFFPA KLVIPITTAP NISWSDLSAL ELLKSIPVGV GQIYGCDNPW TGGIFLGAILL SSPLMCLHAA IGSLLGIAAG LSLSAPFEDI YFGLWGFNSS LACIAMGGMF MALTWQTHLL ALGCALFTAY LGVGMANFMA EVGLPACTWP FCLATLLFLI MTTKNSNIYK MPLSKVTYPE ENRIFYLQAK KRMVESPL

Polyclonal Antibody Immunogen Target Sequence: AA 376-389

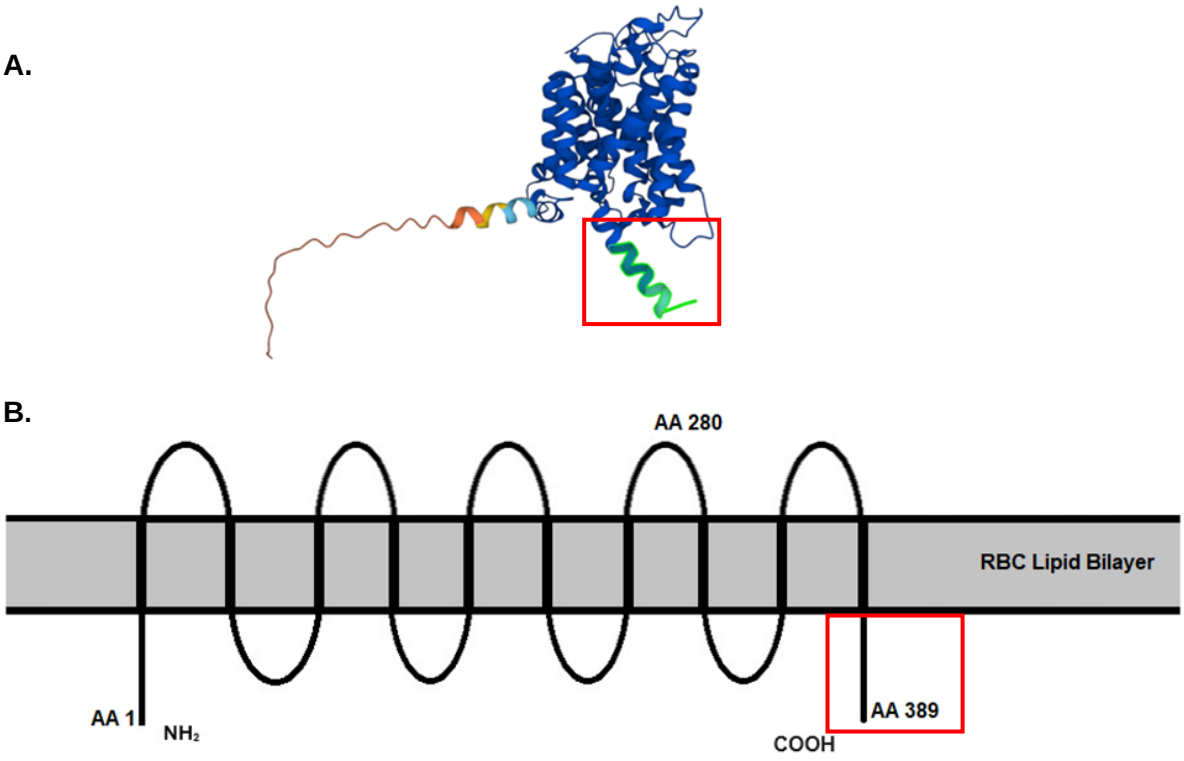

Figure A-10.1. Kidd (SLC14A1) Protein Structure
(A.) 3D model of the JK glycoprotein from www.alphafold.ebi.ac.uk/entry/Q13336. Green highlighting and red box represent the target AA sequence of the primary antibody used in this investigation (AA 376-389). **(B.)** Simple diagram of the JK protein adopted from: Cohn CS, et al. Technical Manual. AABB (2023)[9]; Reid ME, et al. The Blood Group Antigen Facts Book (2012)[44], the red box represents the target AA sequence of the primary antibody used in this investigation (AA 376-389).

A-11. XG (CD99)

Full Amino Acid Sequence: (www.uniprot.org/uniprotkb/P14209)

MARGAALALL LFGLLGVLVA APDGGFDLSD ALPDNENKKP TAIPKKPSAG DDFDLGDAVV DGENDDPRPP NPPKPMPNPN PNHPSSSGSF SDADLADGVS GGEGKGGSDG GGSHRKEGEE ADAPGVIPGI VGAVVVAVAG AISSFIAYQK KKLCFKENAE QGEVDMESHR NANAEPAVQR TLLEK

Polyclonal Antibody Immunogen Target Sequence: AA 1-185

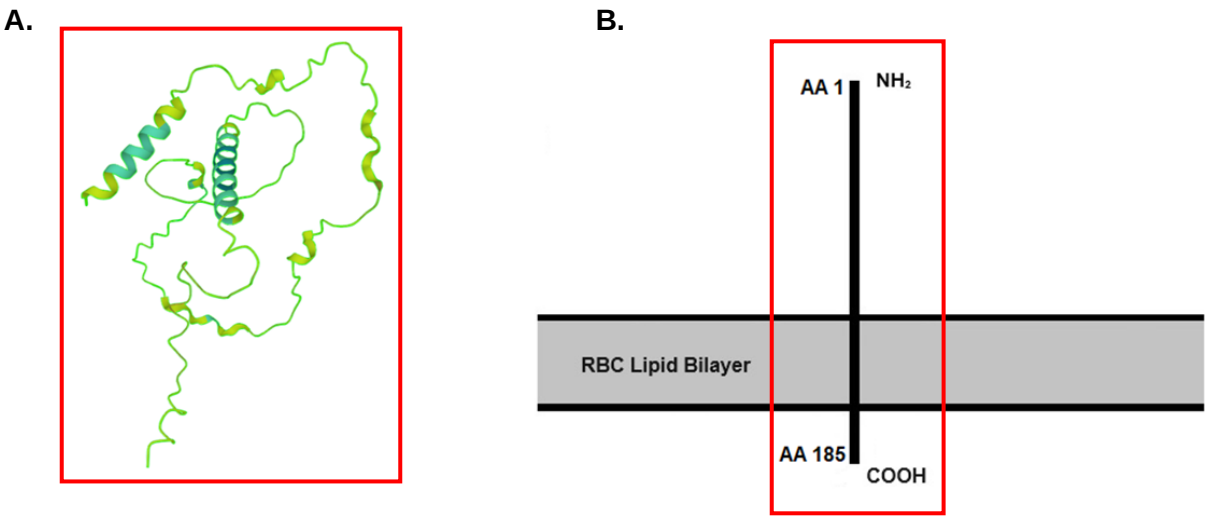

Figure A-11.1. XG (CD99) Protein Structure
(A.) 3D model of the CD99 glycoprotein from www.alphafold.ebi.ac.uk/entry/P14209. Green highlighting and red box represent the target AA sequence of the primary antibody used in this investigation (AA 1-185). **(B.)** Simple diagram of the CD99 protein adopted from: Cohn CS, et al. Technical Manual. AABB (2023)[9]; Reid ME, et al. The Blood Group Antigen Facts Book (2012)[44], the red box represents the target AA sequence of the primary antibody used in this investigation (AA 1-185).

A-12. Chido/Rodgers (C4)

Full Amino Acid Sequence: (www.uniprot.org/uniprotkb/P0C0L4)

MRLLWGLIWA SSFFTLSLQK PRLLLFSPSV VHLGVPLSVG VQLQDVPRGQ VVKGSVFLRN PSRNNVPCSP KVDFTLSSER DFALLSLQVP LKDAKSCGLH QLLRGPEVQL VAHSPWLKDS LSRTTNIQGI NLLFSSRRGH LFLQTDQPIY NPGQRVRYRV FALDQKMRPS TDTITVMVEN SHGLRVRKKE VYMPSSIFQD DFVIPDISEP GTWKISARFS DGLESNSSTQ FEVKKYVLPN FEVKITPGKP YILTVPGHLD EMQLDIQARY IYGKPVQGVA YVRFGLLDED GKKTFFRGLE SQTKLVNGQS HISLSKAEFQ DALEKLNMGI TDLQGLRLYV AAAIIESPGG EMEEAELTSW YFVSSPFSLD LSKTKRHLVP GAPFLLQALV REMSGSPASG IPVKVSATVS SPGSVPEVQD IQQNTDGSGQ VSIPIIIPQT ISELQLSVSA GSPHPAIARL TVAAPPSGGP GFLSIERPDS RPPRVGDTLN LNLRAVGSGA TFSHYYYMIL SRGQIVFMNR EPKRTLTSVS VFVDHHLAPS FYFVAFYYHG DHPVANSLRV DVQAGACEGK LELSVDGAKQ YRNGESVKLH LETDSLALVA LGALDTALYA AGSKSHKPLN MGKVFEAMNS YDLGCGPGGG DSALQVFQAA GLAFSDGDQW TLSRKRLSCP KEKTTRKKRN VNFQKAINEK LGQYASPTAK RCCQDGVTRL PMMRSCEQRA ARVQQPDCRE PFLSCCQFAE SLRKKSRDKG QAGLQRALEI LQEEDLIDED DIPVRSFFPE NWLWRVETVD RFQILTLWLP DSLTTWEIHG LSLSKTKGLC VATPVQLRVF REFHLHLRLP MSVRRFEQLE LRPVLYNYLD KNLTVSVHVS PVEGLCLAGG GGLAQQVLVP AGSARPVAFS VVPTAAAAVS LKVVARGSFE FPVGDAVSKV LQIEKEGAIH REELVYELNP LDHRGRTLEI PGNSDPNMIP DGDFNSYVRV TASDPLDTLG SEGALSPGGV ASLLRLPRGC GEQTMIYLAP TLAASRYLDK TEQWSTLPPE TKDHAVDLIQ KGYMRIQQFR KADGSYAAWL SRDSSTWLTA FVLKVLSLAQ EQVGGSPEKL QETSNWLLSQ QQADGSFQDP CPVLDRSMQG GLVGNDETVA LTAFVTIALH HGLAVFQDEG AEPLKQRVEA SISKANSFLG EKASAGLLGA HAAAITAYAL TLTKAPVDLL GVAHNNLMAM AQETGDNLYW GSVTGSQSNA VSPTPAPRNP SDPMPQAPAL WIETTAYALL HLLLHEGKAE MADQASAWLT RQGSFQGGFR STQDTVIALD ALSAYWIASH TTEERGLNVT LSSTGRNGFK SHALQLNNRQ IRGLEEELQF SLGSKINVKV GGNSKGTLKV LRTYNVLDMK NTTCQDLQIE VTVKGHVEYT MEANEDYEDY EYDELPAKDD PDAPLQPVTP LQLFEGRRNR RRREAPKVVE EQESRVHYTV CIWRNGKVGL SGMAIADVTL LSGFHALRAD LEKLTSLSDR YVSHFETEGP HVLLYFDSVP TSRECVGFEA VQEVPVGLVQ PASATLYDYY NPERRCSVFY GAPSKSRLLA TLCSAEVCQC AEGKCPRQRR ALERGLQDED GYRMKFACYY PRVEYGFQVK VLREDSRAAF RLFETKITQV LHFTKDVKAA ANQMRNFLVR ASCRLRLEPG KEYLIMGLDG ATYDLEGHPQ YLLDSNSWIE EMPSERLCRS TRQRAACAQL NDFLQEYGTQ GCQV

Polyclonal Antibody Immunogen Target Sequence: AA 23-302

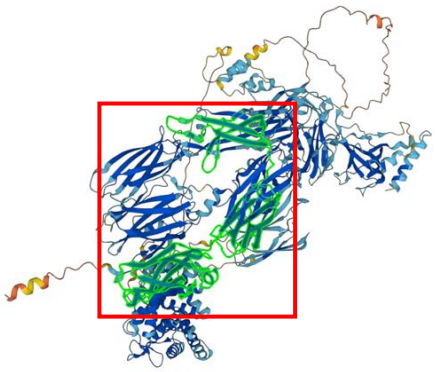

Figure A-12.1. Chido/Rodgers (C4) Protein Structure
3D model of the C4 glycoprotein from www.alphafold.ebi.ac.uk/entry/P0C0L4. Green highlighting and red box represent the target AA sequence of the primary antibody used in this investigation (AA 23-302).

A-13. CH and RG BLAST Sequence Data

Job Title	**C4a vs C4b Protein Sequence**
RID	37YXWXH2114 *Search expires on 05-04 04:46 am* Download All
Program	Blast 2 sequences Citation
Query ID	lcl|Query_7661701 (amino acid)
Query Descr	C4a
Query Length	1744
Subject ID	lcl|Query_7661703 (amino acid)
Subject Descr	C4b
Subject Length	1744
Other reports	Multiple alignment MSA viewer

Filter Results

Percent Identity ___ to ___ E value ___ to ___ Query Coverage ___ to ___ Filter Reset

Descriptions | Graphic Summary | **Alignments** | Dot Plot

Alignment view: Pairwise with dots for identities | Restore defaults | Download

1 sequences selected

Download Graphics ▼Next ▲Previous ◀◀Descriptions

C4b

Sequence ID: Query_7661703 Length: 1744 Number of Matches: 1

Range 1: 1 to 1744 Graphics ▼Next Match ▲Previous Match

Score	Expect	Method	Identities	Positives	Gaps
3584 bits(9294)	0.0	Compositional matrix adjust.	1734/1744(99%)	1737/1744(99%)	0/1744(0%)

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  Query  901   VVPTAAAAVSLKVVARGSFEFPVGDAVSKVLQIEKEGAIHREELVYELNPLDHRGRTLEI  960
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  Query  1021  TLAASRYLDKTEQWSTLPPETKDHAVDLIQKGYMRIQQFRKADGSYAAWLSRDSSTWLTA  1080
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Figure A-13.1. CH and RG BLAST protein sequence comparison
CH and RG protein sequence comparison adapted from: https://blast.ncbi.nlm.nih.gov/Blast.cgi
Query Sequence = C4a, Subject Sequence = C4b ***** AA1-840 (not pictured) are identical.

A-14. Knops (CD35)

Full Amino Acid Sequence: (www.uniprot.org/uniprotkb/P17927)

MGASSPRSPE PVGPPAPGLP FCCGGSLLAV VVLLALPVAW GQCNAPEWLP FARPTNLTDE FEFPIGTYLN YECRPGYSGR PFSIICLKNS VWTGAKDRCR RKSCRNPPDP VNGMVHVIKG IQFGSQIKYS CTKGYRLIGS SSATCIISGD TVIWDNETPI CDRIPCGLPP TITNGDFIST NRENFHYGSV VTYRCNPGSG GRKVFELVGE PSIYCTSNDD QVGIWSGPAP QCIIPNKCTP PNVENGILVS DNRSLFSLNE VVEFRCQPGF VMKGPRRVKC QALNKWEPEL PSCSRVCQPP PDVLHAERTQ RDKDNFSPGQ EVFYSCEPGY DLRGAASMRC TPQGDWSPAA PTCEVKSCDD FMGQLLNGRV LFPVNLQLGA KVDFVCDEGF QLKGSSASYC VLAGMESLWN SSVPVCEQIF CPSPPVIPNG RHTGKPLEVF PFGKTVNYTC DPHPDRGTSF DLIGESTIRC TSDPQGNGVW SSPAPRCGIL GHCQAPDHFL FAKLKTQTNA SDFPIGTSLK YECRPEYYGR PFSITCLDNL VWSSPKDVCK RKSCKTPPDP VNGMVHVITD IQVGSRINYS CTTGHRLIGH SSAECILSGN AAHWSTKPPI CQRIPCGLPP TIANGDFIST NRENFHYGSV VTYRCNPGSG GRKVFELVGE PSIYCTSNDD QVGIWSGPAP QCIIPNKCTP PNVENGILVS DNRSLFSLNE VVEFRCQPGF VMKGPRRVKC QALNKWEPEL PSCSRVCQPP PDVLHAERTQ RDKDNFSPGQ EVFYSCEPGY DLRGAASMRC TPQGDWSPAA PTCEVKSCDD FMGQLLNGRV LFPVNLQLGA KVDFVCDEGF QLKGSSASYC VLAGMESLWN SSVPVCEQIF CPSPPVIPNG RHTGKPLEVF PFGKAVNYTC DPHPDRGTSF DLIGESTIRC TSDPQGNGVW SSPAPRCGIL GHCQAPDHFL FAKLKTQTNA SDFPIGTSLK YECRPEYYGR PFSITCLDNL VWSSPKDVCK RKSCKTPPDP VNGMVHVITD IQVGSRINYS CTTGHRLIGH SSAECILSGN TAHWSTKPPI CQRIPCGLPP TIANGDFIST NRENFHYGSV VTYRCNLGSR GRKVFELVGE PSIYCTSNDD QVGIWSGPAP QCIIPNKCTP PNVENGILVS DNRSLFSLNE VVEFRCQPGF VMKGPRRVKC QALNKWEPEL PSCSRVCQPP PEILHGEHTP SHQDNFSPGQ EVFYSCEPGY DLRGAASLHC TPQGDWSPEA PRCAVKSCDD FLGQLPHGRV LFPLNLQLGA KVSFVCDEGF RLKGSSVSHC VLVGMRSLWN NSVPVCEHIF CPNPPAILNG RHTGTPSGDI PYGKEISYTC DPHPDRGMTF NLIGESTIRC TSDPHGNGVW SSPAPRCELS VRAGHCKTPE QFPFASPTIP INDFEFPVGT SLNYECRPGY FGKMFSISCL ENLVWSSVED NCRRKSCGPP PEPFNGMVHI NTDTQFGSTV NYSCNEGFRL IGSPSTTCLV SGNNVTWDKK APICEIISCE PPPTISNGDF YSNNRTSFHN GTVVTYQCHT GPDGEQLFEL VGERSIYCTS KDDQVGVWSS PPPRCISTNK CTAPEVENAI RVPGNRSFFS LTEIIRFRCQ PGFVMVGSHT VQCQTNGRWG PKLPHCSRVC QPPPEILHGE HTLSHQDNFS PGQEVFYSCE PSYDLRGAAS LHCTPQGDWS PEAPRCTVKS CDDFLGQLPH GRVLLPLNLQ LGAKVSFVCD EGFRLKGRSA SHCVLAGMKA LWNSSVPVCE QIFCPNPPAI LNGRHTGTPF GDIPYGKEIS YACDTHPDRG MTFNLIGESS IRCTSDPQGN GVWSSPAPRC ELSVPAACPH PPKIQNGHYI GGHVSLYLPG MTISYICDPG YLLVGKGFIF CTDQGIWSQL DHYCKEVNCS FPLFMNGISK ELEMKKVYHY GDYVTLKCED G YTLEGSPWSQ CQADDRWDPP LAKCTSRTHD ALIVGTLSGT IFFILLIIFL SWIILKHRKG NNAHENPKE VAIHLHSQGG SSVHPRTLQT NEENSRVLP

Polyclonal Antibody Immunogen Target Sequence: AA 420-550

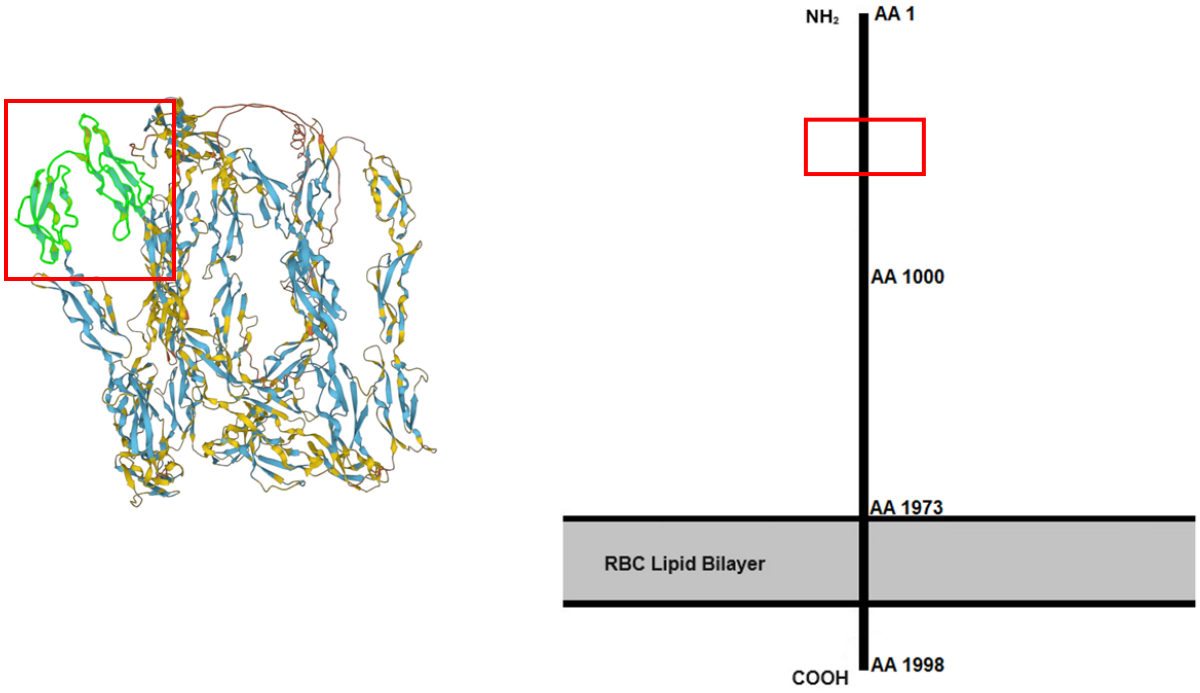

Figure A-14.1. Knops (CD35) Protein Structure
(A.) 3D model of the KN glycoprotein from www.alphafold.ebi.ac.uk/entry/P17927. Green highlighting and red box represent the target AA sequence of the primary antibody used in this investigation (AA 420-550). **(B.)** Simple diagram of the KN protein adopted from: Cohn CS, et al. Technical Manual. AABB (2023)[9]; Reid ME, et al. The Blood Group Antigen Facts Book (2012)[44], the red box represents the target AA sequence of the primary antibody used in this investigation (AA 420-550).