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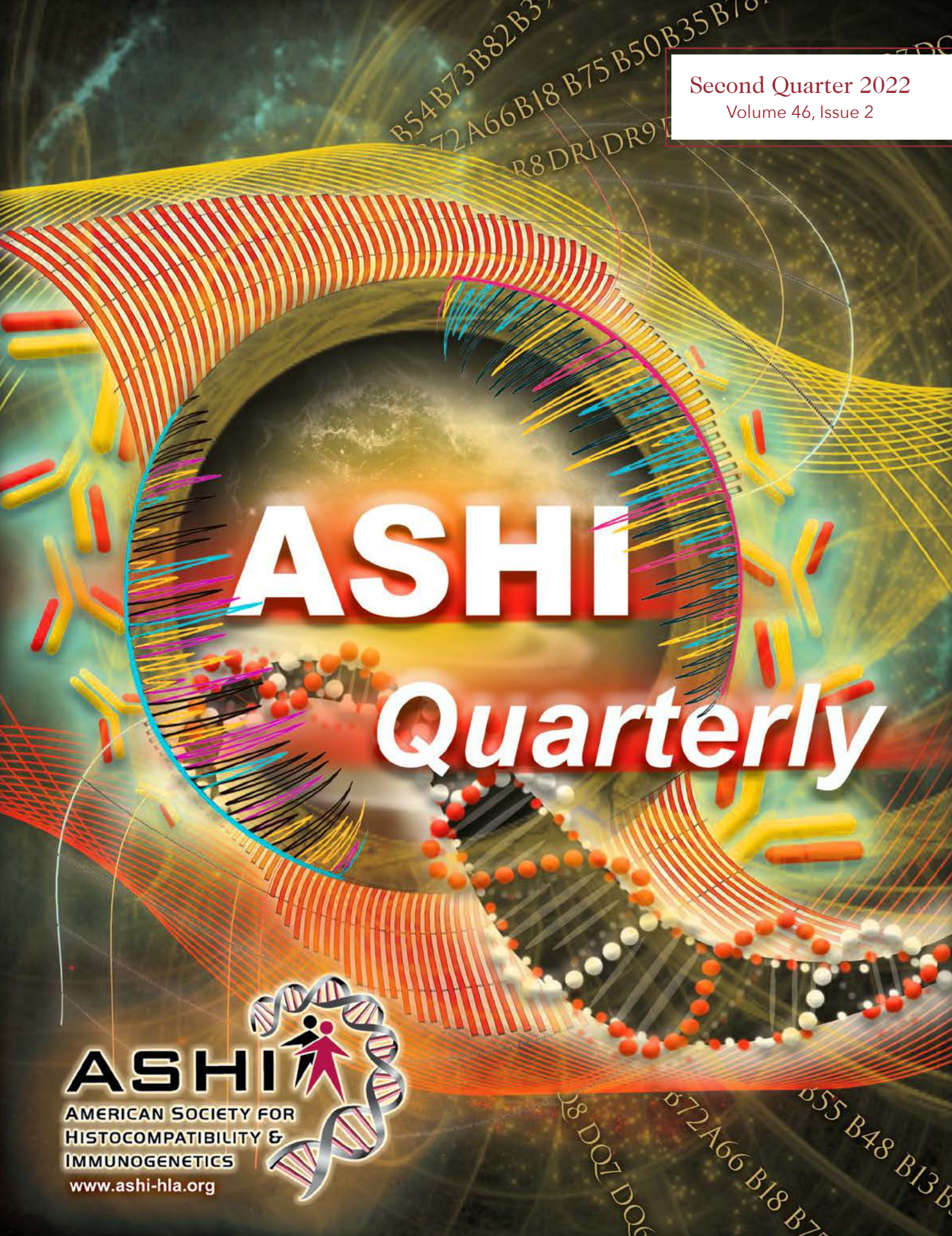

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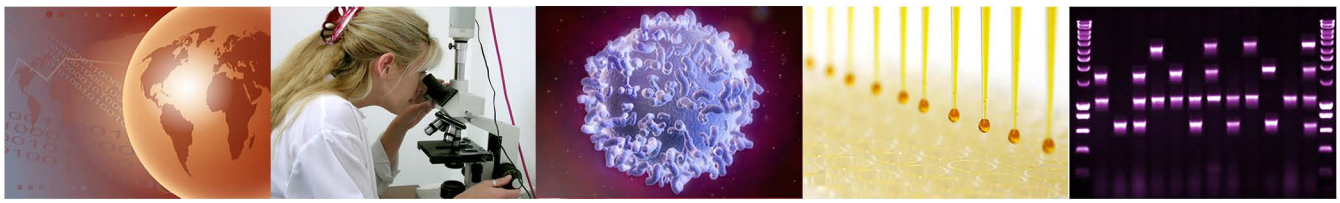

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American Society for Histocompatibility and Immunogenetics
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The *ASHI Quarterly* is published quarterly by the American Society for Histocompatibility and Immunogenetics, 1120 Route 73, Suite 200, Mount Laurel, New Jersey 08054.

EDITORIAL

From the Editor-In-Chief

Lee Ann Baxter-Lowe, *Ph.D., F(ACHI)*

Welcome to the second issue of the 2022 ASHI Quarterly. This issue contains important updates from ASHI, inspirational articles from a clinician scientist and a technologist, and scientific articles describing new transplant biomarkers. This issue also features design changes, which have been introduced by our new publisher. I am excited about this issue because it exemplifies ASHI's role in leading the field to ensure high quality clinical laboratory tests, promote basic research, and provide widespread education. I hope that you enjoy this issue.

The issue begins with **Dr. Annette Jackson's President's Column,** which provides updates on ASHI's 2022 Educational Workshops and the 48th Annual ASHI Meeting. She also describes ASHI's response to recent regulatory proposals, which included engagement with congressional leaders, CMS, the CDC, and the FDA. I am grateful for the work of the National Clinical Affairs Committee (NCAC) and the ASHI Executive Committee (EC), which have played a key role in these important endeavors.

Next, two members of our field share their personal experiences. In **"My Life in HLA," Dr. Stanley Jordan** describes his journey from humble beginnings in the Blue Ridge Mountains to his current leadership positions: Director, Nephrology & Transplant Immunology and Medical Director, Kidney Transplant Program, Cedars-Sinai Medical Center and Professor of Pediatrics & Medicine, David Geffen School of Medicine at UCLA. Dr. Jordan's pioneering work has played a key role in improving transplantation for highly sensitized patients. Throughout his journey, he reminds us of the importance of mentoring, collaborating, family, and little things we do every day. It is a truly inspiring story. **Runying Tian's article, "How Did Becoming an ASHI Certified Inspector Enrich My HLA Career?"** describes a technologist's journey from entering our field to becoming an ASHI inspector. This is another inspiring story that highlights the importance of mentoring and hard work.

The scientific section of this issue features three articles on **transplant biomarkers,** which offer exciting new opportunities for laboratories to play a key role in improving outcomes after solid organ transplantation. Dr. Siva Kanangat's **Current Literature Review** discusses HLA's role as a biomarker for cancer immunotherapy.

- In **"Quantifying Donor-Derived Cell-Free DNA in Solid Organ Transplant Recipients,"** Tracie Profaizer and Dr. Eszter Lázár-Molnár provide a sequel to their 2020 article on the clinical utility of measuring donor-derived cell-free DNA (dd cf-DNA) as a biomarker for graft injury. In this issue, the authors focus on technical aspects of measuring dd cf-DNA. They provide an excellent description of key performance characteristics for these tests and describe the technologies that are often used to measure dd cf-DNA.
- In **"Is it Prime Time for Urinary Chemokines as Clinical Biomarkers?"** Dr. Amanda Cunningham, et al., offer evidence that noninvasive measurement of chemokines in urine can reveal inflammation and rejection processes that may not be apparent using conventional approaches for post-transplant monitoring.
- In "**More than Meets the Eye: Experiences with NanoString® nCounter® Gene Expression Analysis in Transplant Biopsies,"** Dr. Benjamin Adam describes how NanoString® technology can be used to measure informative mRNA transcripts in Formalin-Fixed Paraffin-Embedded (FFPE) tissue. Other approaches for mRNA biomarkers have required fresh organ biopsies to measure transcripts that are associated with rejection, inflammation, and other processes in allografts. In contrast, NanoString® technology uses probes that are well suited to the detection of degraded RNA present in FFPE tissue. Additionally, this technology offers an affordable alternative that is technically straightforward and feasible to do at transplant centers.
- In **"HLA as Biomarkers in Cancer and Cancer Immunotherapy — Immune Checkpoint Inhibitors,"** Dr. Siva Kanangat reviews literature on the role of HLA in cancer immunotherapy. He has selected excellent publications that describe cancer genetics and immunogenetics, cancer immunotherapy, and the key role of HLA expression in these areas. These discoveries are leading to new tests that are ideally suited to HLA laboratories.

Last, but not least, I encourage you to read important updates provided by ASHI committees:

- The **ARB Update** shares the exciting news that the Centers for Medicare and Medicaid Services (CMS) has approved ASHI's deemed status as an accrediting organization (AO) for the full period of six years! The ARB Update describes some important changes and clarifications that resulted from CMS's review. Information on the review cycle is also provided.
- The **PT Committee report, "The Decision to Offer New Proficiency Testing Survey (Part I)"** describes the results of a 2020 membership survey and the Committee's thoughtful process in considering the addition of new PT surveys. Part II will follow in the Q3 issue of the ASHI Quarterly.
- The **CEMC Update, "'Real Talk' A Real Language Summary of the ASHI 2021 Annual Meeting"** describes an enormous undertaking to provide information from presentations from the 2021 Annual Meeting in a format that can be understood by patients, donors, and their families. This is a huge step forward toward meeting the CEMC's goal to engage more directly with transplant patients, donors, and caregivers.

In closing, I would like to thank everyone who contributed to this issue, including the authors of the articles and reports, the enthusiastic and talented Editorial Board, the Publications Committee, Kathy Giovetsis and the ASHI management team, and the new publisher. I am grateful for the feedback that readers have provided and encourage you to become involved in the ASHI Quarterly and other ASHI endeavors. Everyone's thoughts and expertise are important to our success.

Lee Ann Baxter-Lowe, Ph.D., F(ACHI)
Editor-in-Chief

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EDITORIAL

President's Column

Annette M. Jackson, *Ph.D., F(ACHI)*

Dear ASHI community,

At the time of this writing, spring is HERE and serves as a reminder to refresh and renew our professional goals for 2022. ASHI has been busy preparing impactful events this year. There will be TWO **ASHI Educational Workshops** (formerly ASHI Regional Education Workshops). The first will be held in person on June 23-25 in Fort Lauderdale, Florida. The second will be held virtually on consecutive Fridays, July 22 and 29. Registration, program information, and case study submissions for both workshops are OPEN. The program for both Education Workshops includes a Technologist Interactive Session to develop best practices in HLA antibody testing and analysis using csv files sent to you in advance. Want to attend, but need financial assistance? Apply for an ASHI Travel Award!

The **ASHI 48th Annual Scientific Meeting** will be held in Las Vegas from October 24-28. The Pre-Meeting Symposium will be organized by the Society for Immune Polymorphism. Annual meeting sessions will include "Gene Editing: Re-writing the future of transplantation" and "Fetal to Cancer to Transplantation — The Path Forward for Liquid Biopsy." Workshops will include "Path to Best Practices in HLA Antibody Testing" and "Virtual Crossmatching; evolution, expectation, application and limitations." Registration, program information, and the abstract submission sites are now OPEN.

ASHI continues to monitor and respond to national regulatory changes that impact our field and clinical laboratory operations. ASHI receives support and guidance from our advisors at Wheat Shroyer Government Relations. The **National Clinical Affairs Committee (NCAC)** and A**SHI Executive Committee (EC)** have engaged with congressional leaders, CMS, CDC, and FDA to facilitate informative responses to recent regulatory proposals. We shared with you our letter in response to the congressional **VALID Act,** which would create a new risk-based framework for regulating lab-developed tests and the public comment response for the **FDA proposed rule** that would classify human leukocyte antigen (HLA), human platelet antigen (HPA), and human neutrophil antigen (HNA) as a generic group of class II devices to achieve reasonable assurance for the safety and effectiveness of these devices. We also shared our letter to **CMS**, signed by numerous kidney transplant organizations, requesting clarification and an **update to section C, Histocompatibility; sub-section 1, Crossmatching 42 CFR §493.1278(e-f) to allow the use of a "virtual crossmatch" in lieu of a physical crossmatch.** We are coordinating our responses with other transplant related professional societies to strengthen our position. ASHI is prepared to expand its expertise and accreditation oversight as deemed acceptable to these regulatory agencies. To be better equipped to face these legislative challenges, ASHI introduced an ambitious Public Policy Scholarship Program. We announce two inaugural **ASHI Public Policy Scholars: Mary Philogene, Ph.D., F(ACHI)** and **Rebecca Upchurch, MPA, MLT(ASCP), CHS.** These ASHI members were selected by the NCAC and approved by the ASHI Executive Committee (EC) to undergo training with Former Congressman Alan Wheat and Former Congressional Staffer Julie Shroyer that will enable them to actively engage in public policy activities on behalf of ASHI. These scholars, together with our advisors at Wheat Shroyer, will ensure that ASHI remains informed and proactively involved in all regulatory matters that impact our profession and our ability to serve our patients.

ASHI continues to work on your behalf to drive excellence in science, education, and clinical testing. **Click here** to learn more about ASHI committees and how you can grow professionally within our society, browse the ASHI website, watch the ASHI committee update video, or join us at our upcoming educational and scientific meetings.

Sincerely,

Annette M. Jackson, Ph.D., F(ACHI)
ASHI President

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EDITORIAL

Current Literature Review

HLA as Biomarkers in Cancer and Cancer Immunotherapy — Immune Checkpoint Inhibitors

Siva Kanangat, *Ph.D., F(ACHI)*

Introduction

The focus of this issue of ASHI Quarterly is Biomarkers in Transplantation. Several biomarkers were assessed to detect and evaluate allograft injuries. There are clinically proven biomarker assays, such as cell-free DNA analysis. Some utilize blood, and others urine samples. Mixed outcomes have been obtained in terms of their value in single-organ allo-transplants. Biomarkers in solid organ transplants have been reviewed in this issue of the ASHI Quarterly. The most important limitation of cell-free DNA markers is that it does not necessarily inform if the DNA from the allograft detected in circulation is indeed due to an immunological injury or to non-immunological causes. Secondly, these tests cannot accurately distinguish between T cell and B cell mediated allograft injury compromising the decision about treatment modalities to manage allograft transplant injuries (T cell versus B cell mediated, immunological versus non-immunological). Another important scenario concerning biomarkers in our field is the potential role of Human Leukocyte Antigens (HLA) as biomarkers in cancer immunotherapy as immune checkpoint inhibitor (ICI) therapeutics. The question is if and how HLA antigen expression can be used to assess the potential benefits of ICI (i.e., whether a particular tumor is going to respond to ICI therapy or not), and how HLA expression could be used to predict the progression of cancer based on immunological surveillance and effectiveness of T cells against the tumors.

The Genetics and Immunogenetics of Cancer

Cancer is a genetic disorder with germline mutations or spontaneous somatic mutations. Epigenetic factors play a critical role in development of cancer [1-2]. The immune system plays a major role in the eradication and establishment of cancers. The innate immune system specifically, and more importantly, adaptive immune surveillance by Natural Killer (NK) cells, tumor-specific T cells, and antibodies can eliminate or control the growth of tumors in fully immunocompetent hosts [3]. However, cancers can escape the innate and adaptive immune surveillance through tumor immune escape mechanisms and immunoediting, which includes elimination, equilibrium, and escape [4]. Therefore, it is reasonable to state that the development, progression, and outcome of cancers depend on genetics, immunogenetics, and, more importantly, epigenetics. These processes will determine the outcomes in different individuals.

Immunotherapy of Cancers

Both innate and adaptive immune responses have been targeted for cancer immunotherapy. Normally, NK cells do not function in patients with hematologic malignancies. Also, their number and function are severely affected in many cases due to chemotherapy, radiation, and immunosuppressants. Adoptive transfer of NK cells has resulted in treating hematological malignancies [5]. Similarly, adaptive immune responses mediated by T cells have been and are being used to boost T cell response where the original host T cell response is not effective due to anergy, exhaustion, or other reasons, which ultimately results in the inability to eliminate the tumors. Chimeric antigen receptor T cell therapy or CAR-T cell therapy is another form of immunotherapy that can enhance the activity of T cells by providing the ability to T cells to recognize target specific tumor antigens. These antigens/peptides are presented by the HLA system.

Role of HLA pertaining to Cancer Immunotherapy

Whether innate immune response mediated by NK cells or adaptive immune response mediated by antigen-specific T and B cells, HLA molecules play important roles in mediating the NK cell and T/B cell mediated immune responses toward the tumor. While lack of certain HLA antigens on the tumor cell surface is advantageous for NK cell immune response, such absence could "blind" T cells' recognition of tumor-specific antigens since T cells can only "see" antigens expressed on the cell surface presented by the HLA antigens. It will also affect humoral response since B cells need help from T cells to induce tumor-specific antibody production. Hence, it is logical to assume that HLA molecules play pivotal roles in recognizing and eliminating tumors by innate and adaptive immune responses.

HLA as Biomarkers for Immune Checkpoint Inhibitor (ICI) Therapy

It appears that presence/absence of HLA genes, their RNA levels, or most importantly, protein levels on the cell surfaces could be used as markers for assessing innate and adaptive immune responses against tumors. From this perspective, assessing the presence or absence of certain HLA genes and their RNA as well as protein expression levels could be valuable biomarkers for assessing cancer progression and successful cancer immunotherapy by ICI.

The review by Sabbatino F et al. [6], and Naranbhai V et al. [7] address this subject in general and particularly HLA-A*03 expression. This review provides an extensive examination of all the currently known genetic and epigenetic potential mechanisms that can modulate HLA RNA and protein expression levels. The ICI based therapy assumes that the adaptive immune response by T cells is not efficient but designed to boost the T-cell-specific responses against tumors by attempting to lift the brakes on T cell actions against the tumors. This includes intercepting CTLA-4, PD-1 and PDL-1, which could, in theory, lift the ineffectiveness of the T cells in recognizing and eliminating the tumor cells. This therapy has been proven very effective in a certain subgroup of patients [8]. While this approach of ICI is effective in many instances, there are others where it is not. Knowing what biomarkers are useful could determine the effectiveness of ICI in different cases [9-10].

The tumor escape mechanisms involve the ability of tumors to avoid recognition by tumor-antigen-specific T cells and also resistance to NK cell effects. As mentioned above, HLA plays a critical role in both. Loss of heterozygosity (LOH), loss of one complete set of HLA alleles' expression or reduced expression or total loss of some HLA alleles may adversely affect the effectiveness of ICI. Hence, HLA genotyping to assess LOH, HLA expression analysis at the RNA level, HLA expression at the cell surface, and soluble HLA molecules in circulation can be evaluated as potential biomarkers to assess the efficacy of ICI. The potential role of HLA Class II expression in ICI needs further evaluation.

The more recent article by Naranbhai et al. specifically studied HLA-A*03 in response to ICI therapy in cancer. These authors conducted an epidemiological study and evaluated the clinical outcomes in terms of overall survival, progression-free survival, and objective response rate following treatments for advanced cancer. They found that HLA-A*03 was associated with reduced overall survival after ICI treatment. The mechanisms by which HLA-A*03 is linked with poor ICI response are yet to be deciphered. It has been reported that the degree of HLA heterozygosity at the HLA locus is associated with improved survival of patients with melanoma and non-small-cell lung carcinoma (NSCLC) who received ICI, although there are inconsistent validations [11-13].

Soluble HLA antigens and cancer

Several studies described associations between levels of soluble classical and non-classical HLA antigens in circulation and reduced expression on the cell surface with outcomes of various cancers [14-16].

Conclusions

As other biomarkers in transplantation are discussed, the potential use of HLA as biomarkers, especially in various forms of cancers as well as assessing the suitability and progress of ICI therapy, should be a focus, based on their immunological involvement in cancers. Immunotherapy for solid tumors is becoming increasingly relevant and feasible; however, it is being proven to be beneficial only to a subset of patients. Based on the role of HLA in presenting tumor-specific antigens to T cells and the requirement of T cells to recognize the tumor-specific antigens only in the context of HLA antigens, it is expected that the efficacy of ICI will depend on the specific set of peptides derived from the tumor that each HLA allele will present as well as the expression of HLA as a reflection of quantitative antigen-peptide presentation. The ability to perform quantitative measurements of RNA transcripts of HLA alleles, to quantify soluble HLA molecules by sensitive protein assays, and the ability to detect loss of heterozygosity can help in assessing the role of HLA in various forms as biomarkers for cancer. In solid organ transplantation, including liver transplants, research as early as 1989 has indicated the importance of soluble HLA antigens in allograft function and pathology [17]. In conclusion, as we embark on the search for biomarkers in transplantation and cancer, HLA emerges as a valuable biomarker.

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EDITORIAL

My HLA Life

Stan Jordan – 2021 Winner of the Paul I. Terasaki Clinical Science Award

Stanley C. Jordan, *MD*

I was born and raised in the Blue Ridge Mountains of North Carolina. When I was 5 years old, I was stricken by polio, two years before the polio vaccine was developed, and spent four months in a polio hospital far from my home. My mom could not stay with me, and when I was left there, the first person I met was an intern who was so kind to me. At that point, I realized I wanted to be a physician. Our family was very poor, and I was the first in my family to attend college and then medical school. It was often a hard and demanding journey, but when I graduated medical school at the University of North Carolina, Chapel Hill (UNC), I felt that I had achieved my dream! When I reflect back on my early childhood experience, one of the most important lessons I learned was you should never underestimate the impact you can have on someone else's life. I never knew who the intern was, but I will never forget him. I try to remember those lessons every day of my life and always be considerate of the pain and suffering of others.

After graduating medical school at UNC, I was not sure if I wanted to do internal medicine or pediatrics. I actually applied for residencies in both. I accepted a pediatric residency at the University of California, Los Angeles (UCLA), and really enjoyed my residency, but wanted to pursue subspecialty training and research. My first fellowship was in pediatric nephrology and focused on autoimmune kidney diseases and glomerulonephritis. I really enjoyed it and wanted to explore research in this area, and was accepted for fellowship at Scripps Clinic in La Jolla with Dr. Frank Dixon. At that time, this was the leading glomerulonephritis research center in the world. It was difficult for me as this was a very demanding and stressful time, but testing myself in an area where I had no previous experience gave me strength and direction. This was my first exposure to basic immunology, and I loved it! While at Scripps, I gained a deeper understanding of B-cell immune responses and mechanisms of antibody injury to the kidney. This has served me well during my career. When I completed my fellowship at Scripps, I was not sure what I should do. I then had the opportunity to do a fellowship in dialysis and transplantation at Children's Hospital of Los Angeles with Dr. Richard Fine. There, I really began to see how I could apply the lessons of basic immunology and antibody injury to the problems of allograft rejection. In those days, we only had prednisone and Imuran, and were just beginning to use antithymocyte globulin (ATG). One of the first manuscripts I wrote was a report on what was called delayed hyperacute rejection of kidney allografts. This intrigued me because it was clear that this was an antibody-mediated process that was not detected by standard cytotoxicity crossmatch. This began my interest in antibody-mediated rejection (AMR) and later, desensitization.

At Children's Hospital of LA, I was fortunate to meet early pioneers in transplantation including Lesley Brent, an immunologist, who was the co-discoverer, with Peter Medawar and Rupert Billingham, of acquired immunological tolerance, as well as Richard Batchelor, the internationally renowned immunologist and immunogeneticist who played a significant role in the development of kidney transplantation. Both men were inspirational and exemplary, not only for their professional expertise, but for their personal lives as well. Of course, I became acutely aware of the work and achievements of Paul Terasaki, whose work has greatly influenced my own. From these experiences, I had two more important life lessons. First, never be afraid to immerse yourself in something new where you have no "comfort zone." This is how true growth occurs. Second, always understand the importance and example of those who pioneered our understanding of transplant immunology and immunogenetics.

The next chapter of my life in Human Leukocyte Antigens (HLA) began in 1980 at UCLA where I was hired as an assistant professor with my first National Institutes of Health (NIH) grants to study the importance of anti-idiotypic (blocking antibodies) to cytotoxic HLA antibodies. This was my first venture into use of Paul's famous tray as we adapted it to look for blocking of cytotoxicity. Here, Rebecca Sakai, a research scientist, who had worked with Dr. Terasaki, came to work with me and adapt the cytotoxicity assay to determine if blocking antibodies could be detected in human serum. My first NIH grant was to examine the development of blocking antibodies to HLA after donor-specific blood transfusions

(DSTs), which were used to "accommodate" living donor recipients to donor kidneys before cyclosporine was available. I was interested in detecting anti-idiotypic (blocking) anti-HLA antibodies. We had previously identified these blocking antibodies in pregnant women and felt that they may arise to dampen or eliminate anti-HLA responses. This was in the early 1980s; by 1983-1984, the HIV epidemic was in full force, and blood transfusions for immune modulation were abandoned. We also had Epogen that substantially reduced the need for blood transfusions. This meant that my research on blood transfusions and transplantation was over. However, my previous life as a pediatrician came to the rescue! One of the diseases we dealt with during my training and early years of being a pediatric resident was Kawasaki disease, which is a childhood vasculitis of unknown cause. While at Children's Hospital, I worked with the infectious disease group to examine immune markers in these children. Of interest was the early identification of effectiveness of intravenous immune globulin (IVIg) in reducing inflammation in Kawasaki disease. IVIg is interesting since it is developed from the plasma of thousands of donors and should contain a broad and robust diversity of Immunoglobulin G (IgG) molecules, including blocking antibodies. Then one night, I woke up and had the idea that we could probably achieve the blood transfusion effect by giving IVIg without the risk of disease transmission. Here, passive immunity was the idea.

In 1986, I moved to Cedars-Sinai Medical Center to become Director of Pediatric Nephrology and Transplant Immunology. I also moved our Renal Immunology Lab to Cedars to continue this important work. Unfortunately, in 1987 my research partner and close friend, Rebecca Sakai, died suddenly. This cast doubt on our ability to continue this important work. However, this is when I began working with Dolly Tyan, who was the first HLA Lab Director at Cedars. Working with Dolly and Mikeo Toyoda, we rapidly accelerated this work to confirm anti-idiotypic blockade of specific HLA antibodies in the Terasaki assay by IVIg that led to our first clinical application of IVIg to highly HLA-sensitized patients. We then began our early treatment of patients in 1991. We now know that IVIg is one of nature's important immune modulators. It can absorb and block noxious complement mediators, regulate T-cell and B-cell immunity, and accelerate the decline of IgG (pathogenic) antibodies through saturation of the neonatal Fc receptor (FcRn). IVIg is still an important immune modulator that does not pose a risk of immunosuppression and is extensively used in autoimmune and inflammatory conditions today.

When I reflect back on my early childhood experience, one of the most important lessons I learned was you should never underestimate the impact you can have on someone else's life.

This work led to an important blinded controlled trial of IVIg vs. placebo for reduction of HLA antibodies in highly-HLA sensitized patients. In this trial, Dr. Ashley Vo was critical to the organization and implementation of this important NIH study. The results of this study demonstrated reductions in HLA antibodies and improved transplant rates for patients treated with IVIg. Data were published in The Journal of the American Society of Nephrology in 2004. This was the culmination of a decade-long journey with the NIH Clinical Trials in Organ Transplantation (CTOT) program, at that time led by Steve Rose, who was so helpful and instrumental in organizing this multicenter study.

I will never forget a young woman at Cedars-Sinai, who was highly HLA-sensitized and had been on dialysis for 12 years, and would always ask me on my rounds if she could be in the study. However, at that time, the study was not yet initiated, and I told her to hang on. Unfortunately, on a Sunday morning while making my rounds I found that she had died suddenly. I went to the morgue and sat there

looking at all the scars and failed vascular accesses on her body and knew this had to be my mission in life — to improve access to transplantation for our highly sensitized patients. At the completion of the NIH-IGO2 study, I was very proud of what we accomplished and that this early work continues to be important in opening the door for antibody reduction therapies that came to be known as desensitization.

Over the next decade, we pursued desensitization with IVIg + Rituximab (anti-CD20) and developed protocols for ABO incompatible (ABOi) transplantation. Important to this work was a better understanding of what level of antibody reduction would allow for successful transplantation. Here, the work of Nancy Reinsmoen and our current HLA lab director, Xiaohai Zhang, significantly advanced this understanding. We also had important papers in the identification of non-HLA (anti-AT1R) antibodies that could mediate antibody rejection. However, the best was yet to come.

In my career, I have evolved from a primarily NIH-funded laboratory-based researcher to a clinical trials researcher. This, I think, is because of my perceived need for new drug development in transplantation. I have always tried to create a nexus between our clinical/academic pursuits and biotechnology. I see this as an important way forward for the development of new, or repurposed, drugs in our transplant patients. Our clinical research is heavily weighted to the investigator-initiated evaluation of drugs for desensitization and treatment of AMR.

In this regard, what I consider major steps forward in desensitization therapy was our opportunity to investigate two drugs, the IgG endopeptidase (Imlifidase) and anti-IL-6 therapies for desensitization and treatment of antibody rejection. My involvement with Imlifidase began in 2014 when we developed a single-center trial of Imlifidase for desensitization. The results seen with this drug were dramatic and resulted in a New England Journal of Medicine publication. This and subsequent studies allowed the drug to receive European Medicines Agency approval as a desensitization agent in 2020. This is the first drug approved for desensitization, and FDA trials are underway for approval in the U.S. A good example of our translational research approach is the work done with interleukin (IL)-6 in mediating donor specific antibody (DSA) development and AMR. Initial data were obtained in mouse models, and we have shown that anti-IL-6R antibodies could modify anti-HLA antibody production by bone marrow and splenic plasma cells. This eventually evolved into investigator-initiated clinical trials of anti-IL-6R and anti-IL-6 for desensitization and treatment of AMR. CSL-Behring/Vitaeris now has a multinational randomized controlled trial of clazakizumab (anti-IL-6) for treatment of chronic AMR. We recently completed a study of anti-IL-6 for desensitization that showed significant reductions in HLA antibodies and prevention of DSA rebound post-transplant. We are very proud that the ideas that evolved in animal models have progressed to labeling trials in humans. We are hopeful that we can continue to engage and inspire interest in new drug development for desensitization and treatment of antibody rejection from our industry partners. I feel that we are now at a tipping point for progress in our battle against allo-sensitization.

Mentoring and teaching creative scholarship is a special obligation we all have. I have learned so much from those who believed in me and gave me a chance to be successful. I am so very grateful to Dr. David Rimoin, who hired me to come to Cedars-Sinai in 1986. He was a great role model. He would always give me good counsel and was supportive of my career. I knew I could trust him, and I also knew he wanted me to be successful. I have also worked with people who were not so kind or supportive. I would only say that you can learn from those experiences as well. I have always enjoyed being a mentor. I love seeing others obtain their goals and dreams and find a way for themselves in academics. I feel great pride in their accomplishments and that I have had a part in their development. As I have gotten older, I realize that we all lead time-constrained lives. What we do and how we do it does matter. It is not all about yourself. I feel I can take a lot of pleasure and comfort in seeing my younger colleagues take over important leadership roles and have academic achievements that ensure a bright future for transplant immunology, transplantation, and nephrology. Something I always think about is that you should take your work seriously, not yourself!

I have been extremely blessed to have had a great career, great and supportive people to work with, and important work to do. I feel so very fortunate to have been selected as the recipient of the Sir Peter Medawar Prize in 2020 and the Paul Terasaki Prize from ASHI in 2021. Both are truly the most important and impactful recognition I could ever hope to receive for my career in transplantation. To say that Dr. Paul Terasaki and Sir Peter Medawar influenced my career is an understatement. Early in my career, I was fortunate to meet Sir Richard Batchelor, Lesley Brent, and Sir Peter Morris. I was so honored to meet these distinguished individuals who laid the foundations of modern transplantation immunology and learn how they approached transplantation and research, as well as the importance of thinking translationally. The work done by Dr. Paul Terasaki and Sir Peter Medawar had an important purpose, that is, to improve our understanding of how the immune system rejects non-self. However, the other side of this is that one could use this model to develop therapeutic approaches to prevent it.

Here, I am particularly proud of our work with Imlifidase demonstrating how we can rapidly remove pathogenic IgG antibodies from sensitized patients. I would hope that Paul would have been pleased to see that extension of his work published in the New England Journal some 48 years after his seminal New England Journal paper describing cytotoxic IgG antibodies in patients rejecting allografts.

A few comments about my life outside of HLA and transplantation. I am very blessed to have a wonderful family. My wife Susan; daughters Sabrina, Ashley, and Laura; and three grandchildren, Theodore, Alexandra, and William, whom I adore. I enjoy spending time with them and being part of their lives. I am also a fairly avid golfer. I enjoy getting outdoors and playing the game without too many expectations! I also want to thank the kind people at ASHI for their recognition and collaboration over the years. I have made many great friends and look forward to many more exciting advancements in transplantation and transplant immunology!

Quantifying Donor-Derived Cell-Free DNA in Solid Organ Transplant Recipients

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Introduction

Cell-free DNA (cfDNA) and its applications in laboratory medicine were discussed in an earlier issue of ASHI Quarterly, where we presented its clinical utility as a biomarker for the detection of solid organ graft injury [1]. The goal of the current article is to compare and contrast the specific analytical methods used to quantify donor-derived cell-free DNA (dd-cfDNA) and discuss its role in diagnosing graft injury. Both immunologic and non-immunologic disease mechanisms may affect the transplanted organ, and therefore, early detection of sub-clinical injury before pathological and functional changes occur allows for treatment and prevention of long-term organ damage. The gold standard for diagnosis of rejection is performing a biopsy, but practices may vary, with some transplant centers performing protocol biopsies and others only ordering it upon suspicion of graft injury due to their cost, potential for complications, and patient discomfort [2]. Other drawbacks may include potential for limited information due to sampling errors, the subjective nature of the sample, or lack of rejection-mediated lesions in the region tested [3]. Noninvasive biomarkers such as blood creatinine, lipase or amylase, or liver enzyme levels for kidney, pancreas, and liver allografts are neither sensitive nor specific to graft rejection. For example, increases in creatinine may be due to non-rejection processes such as renal artery stenosis, recurrence of the original disease, de novo kidney disease, or even calcineurin inhibitor toxicity [3]. As previously described, the use of noninvasive techniques such as monitoring circulating dd-cfDNA has become a great interest for the early detection of graft injury. Unexpectedly, the use dd-cfDNA as a tool to monitor graft health received a boost from the COVID-19 pandemic as some centers were forced to transit from biopsies to less invasive ways of detecting graft injury [4,5].

During graft injury, the release of dd-cfDNA into the blood stream increases as the result of cell death associated with rejection. Evidence is emerging that there is a difference in the amounts released depending upon the type of rejection. Rejection may be classified as antibody mediated rejection (AMR), T cell mediated rejection (TCMR) or mixed (AMR and TCMR). Recent observation suggests that dd-cfDNA levels are higher in AMR than in TCMR, most likely because AMR has more microvascular injury, and the proximity of damaged endothelial cells to the capillaries allows for easier release of dd-cfDNA into the bloodstream. In contrast, TCMR-associated injury primarily occurs in the tubulointerstitial compartment, and dd-cfDNA is less able to readily reach the bloodstream. This observation has been strengthened by findings showing that higher dd-cfDNA levels have been correlated with C4d deposition and peritubular capillaritis, both hallmarks of AMR, whereas lower levels of dd-cfDNA have been correlated with individual elements seen in TCMR [6].

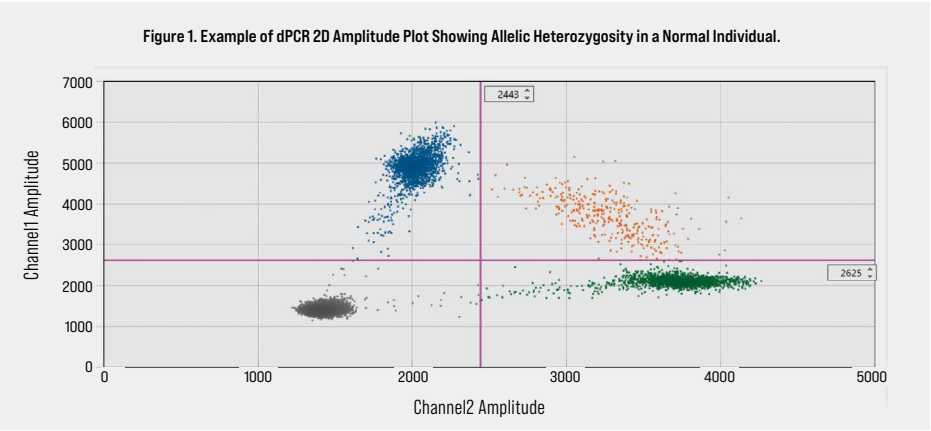

Figure 1. Example of dPCR 2D Amplitude Plot from a duplex PCR reaction containing a primer set designed to amplify rs1294331 and two hydrolysis probes. One probe is FAM labeled and will hybridize to the C allele, while the other probe is VIC labeled and will hybridize the T allele. Channel 1 fluorescence (FAM) is plotted against channel 2 fluorescence (either VIC or HEX). See text for further explanation. The DNA used here is Coriell Institute repository DNA (HG03846) which is heterozygous for rs1294331.

Analytical considerations for measuring dd-cfDNA

Detection and quantification of dd-cfDNA requires sensitive diagnostic techniques that can reliably detect and quantify small DNA amounts against the much larger background of recipient DNA. For example, when testing for chimerism in haematopoietic stem cell transplantation (HSCT) patients, short-tandem repeat (STR) testing is used to quantify and distinguish donor from recipient DNA. Conventional STR methods have a limit of sensitivity of 2%. However, recent research suggests that being able to detect even smaller amounts of recipient DNA or microchimerism may be clinically significant since it may suggest potential for relapse or graft failure [7]. For quantification of dd-cfDNA in kidney transplantation, it has been established that serum levels above 1% are indicative of graft injury but values less than 1% typically rule out the need for an invasive biopsy [2]. Higher actionable levels may be seen in liver transplant patients, most likely due to the liver's greater mass [8]. Detection of dd-cfDNA may have greater value when serum level changes are serially monitored post-transplant [9].

For purposes of quantification, values on the lower end of the analytical measurement range are of more interest than values on the upper end, especially when detection of extremely small amounts of analyte are necessary to define disease states. Therefore, attention must be paid to performance characteristics such as limit of blank (LoB), limit of detection (LoD), and limit of quantification (LoQ) as outlined by the Clinical and Laboratory Standards Institute (CLSI) document EP-17-A2 [10]. The LoB is the highest measurement result that is likely to be observed for a blank sample and is essentially the background noise that might be present in a sample. In evaluating a dd-cfDNA assay, the LoB would be determined by running a set number of cfDNA samples from individuals who were never transplanted. The LoD is the lowest concentration of an analyte that can be consistently detected with a 95% confidence. One possible approach to determine the LoD for a dd-cfDNA assay would be to serially dilute a surrogate donor DNA sample and spike it into a surrogate recipient DNA sample. Repeat testing would be performed to discover the lowest percentage of surrogate donor DNA that could be detected 95% of the time. Finally, the LoQ is the lowest amount of analyte that can be quantitatively determined with stated accuracy. Both the LoB and LoD are statistical constructs, but for the LoQ, the assay developer must set a preestablished accuracy goal that may vary depending upon the application of the assay. Similar to the evaluation of the LoD, LoQ may be determined by spiking known percentages of surrogate DNA into surrogate recipient DNA at varying concentrations and finding the lowest percentage that matches the predetermined coefficient of variation, which is often 20%. More detailed descriptions of how the LoB, LoD, and LoQ can be determined for dd-cfDNA assays can be found in references by Altuğ et al., Grskovic et al., and Beck et al. [11-13].

The LoB will always be lower than the LoD, and typically, the LoD is less than the LoQ. Regardless, since dd-cfDNA values greater than 1% are actionable, it is imperative that the methods used to quantify dd-cfDNA have an analytical measurement range that well-encompass the 1% value. An accurate LoQ is essential because a false positive result or Type I error would result in an unnecessary biopsy and its attendant risks, while a false negative or Type II error could result in a missed diagnosis of graft rejection. For dd-cfDNA assays, the negative predictive value (NPV) is more important than the positive predictive value (PPV). If the dd-cfDNA value is less than 1%, the need for a biopsy is ruled out, whereas any value over 1% suggests possible graft injury due to rejection and the need for a biopsy.

Finally, another metric frequently used to evaluate clinical sensitivity of these assays is the area under curve (AUC). This is a component of the receiver operating characteristic curve (ROC), a graphical plot that compares the true positive rate (individuals with graft injury) against the false positive rate (individual who test positive but do not have graft injury). The true positive rate is also known as the assay's clinical sensitivity and the AUC is the probability of the

Figure 2. Example of dPCR 2D Amplitude Plot Showing 1% Spike-in Surrogate Donor DNA into a Surrogate Recipient.

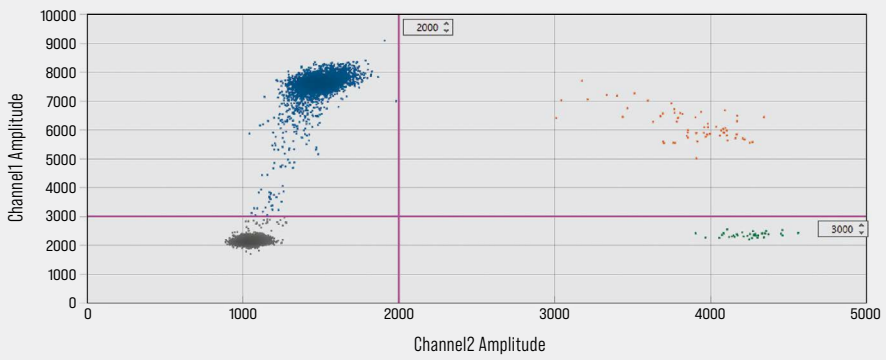

Figure 2. In this example, 1% surrogate donor DNA was spiked into a surrogate recipient DNA. The surrogate recipient (FAM-blue droplets) DNA is readily identified and is the abundant fraction. The donor (HEX-green droplets) DNA makes up 1.04% of the total DNA fraction.

assay's true positivity rate. AUC values less than 0.5 are considered uninformative, while values closest to 1 have the highest discriminatory ability for a diagnostic test.

What to quantify?

The core of using dd-cfDNA to measure graft injury is the ability to distinguish between donor and recipient DNA. An obvious choice would be HLA alleles since HLA genotyping is almost always performed prior to transplant, and most recipient/donor pairs will have some differences at the allele level. But this doesn't lend itself to HLA-matched pairs or situations where the recipient was transplanted at another center, and the donor HLA genotype is not obtainable, or the donor DNA is unavailable. A more pragmatic approach is the use of genome-wide polymorphisms such as single-nucleotide polymorphisms (SNPs). Ideally, the most useful SNPs have a minor allele frequency (MAF) between 0.4-0.5. With Hardy-Weinberg equilibrium, a SNP with a MAF in this range would be homozygous in both donor and recipient 23%-25% of the time [13]. In the event that no donor DNA is available, Beck et al. [14] uses a clever work-around to identify informative SNPs. They first screen the recipient DNA using SNP hydrolysis primer/probes sets on a real-time qPCR platform. Sets that are heterozygous are excluded, and the remaining SNP sets are used to set up a second screening step with dPCR, described below. Typically, at least 3-4 informative SNPs can be identified, allowing for the monitoring of the recipient over time.

Because MAFs differ between different ethnic groups, consideration must be given to select SNPs that cover as many populations as possible. Additionally, these SNPs should not be associated with human disease and, ideally, more than one informative SNP should be identified for distinguishing between donor and recipient. Although SNPs are most utilized, alternatively, other genomic polymorphisms such as copy number variants (CNVs) based on genetic imbalances may also be used [15].

For this article, we will discuss the two most commonly used assay platforms for quantification of dd-cfDNA: digital PCR and next-generation sequencing (NGS).

Quantifying dd-cfDNA • Digital PCR (dPCR)

The concept behind dPCR is not a new idea. Previously known as single molecule PCR or limiting dilution PCR, the idea is to dilute or partition the PCR reaction to the point where each well or reaction has an all-or-none, or digital, signal: Amplified DNA is either present or not present [16]. It is by diluting out the abundant recipient DNA that the rarer donor-derived DNA can be more readily distinguished. Poisson distribution is then used to calculate the concentration. The process of diluting the reaction has been simplified by the advent of microfluidics and emulsion chemistries [17]. Some instruments use a chip digital format (cdPCR) while others use a droplet digital format. Many publications refer to droplet digital PCR (ddPCR) because the droplet-based platform is most common. It is unnecessary to make this distinction between the two platforms due to their concordance, and therefore dPCR is sufficient [17].

The dPCR reaction consists of forward and reverse primers, master mix, and Taqman probes, also known as hydrolysis probes. These probes have a fluorochrome attached at the 5' end (typically FAM and either VIC or HEX) and a quencher molecule at the 3' end. If the 5' and 3' ends are together, any fluorescent signal is quenched. But upon hybridization to the amplicon, the probe is exposed to the Taq polymerase's 5'-3' exonuclease activity. The enzyme separates the fluorochrome and quencher, and the fluorochrome can become excited. After setting up the PCR reaction, a droplet generation step is required. Here, the BioRad droplet generator instrument (currently, the most commonly used dPCR system), will partition the 22 µl sample reaction into ~20,000 uniform nano-sized water-in-oil droplets. Each droplet, which contains all the PCR reaction components, is essentially its own PCR reaction chamber. The plate is then placed on a thermocycler. After PCR amplification, the entire

plate is read on a fluorescent plate reader. Positive reaction wells will have an increased fluorescent intensity compared to negative reaction wells. **Figure 1** shows the 2D amplitude plot of a reaction designed to amplify both alleles of rs1294331 SNP.

In this example, the blue droplets in the upper left quadrant are droplets containing at least one copy of the FAM labeled DNA (C allele). The green droplets in the lower right corner are the droplets containing at least one copy of the VIC labeled DNA (T allele). The orange droplets in the upper right corner contain copies of both FAM and VIC labeled DNA. The gray droplets in the lower left corner have no DNA present. These "null" droplets are essential in the Poisson statistical calculation. The sample concentration is calculated (typically, an automated process with the instrument software) using this formula:

$$\lambda = -\ln(1-\kappa/n)$$

where λ is the copies per droplet, κ is the number of positive droplets, and n is the total number of droplets.

The power of dPCR lies in its ability to measure low levels of target DNA because each droplet or partition is either positive or negative [17]. Because the target molecule is quantified absolutely, there is no need to include a standard curve. In addition, it has a good dynamic range (five logs) and is considered more reproducible than qPCR.

Figure 2 is an example of a 2D amplitude plot showing 1% spike-in DNA, representing a surrogate donor, into surrogate recipient DNA. The blue droplets represent the abundant fraction (recipient) against the much smaller donor fraction shown in green. Increases in the donor fraction may suggest that rejection is occurring.

Quantifying dd-cfDNA • Next-generation sequencing (NGS)

SNP allelic variants can also be detected by NGS where targeted amplification and sequencing of SNPs is used to quantify the donor and recipient DNA contributions. The number of times a certain nucleotide is sequenced, also known as the read depth, or depth of coverage, is used to calculate the percentage of dd-cfDNA. While with dPCR, only a few SNPs can realistically be used, NGS is readily able to genotype and quantify hundreds or thousands of informative markers resulting in the likelihood of a more accurate percentage of dd-cfDNA. However, large number of SNPs is not necessarily helpful if the coverage is low; therefore, a higher read depth is more important for assay sensitivity than the actual number of SNPs sequenced.

The transplant community has been slow to adopt dPCR technology to quantify dd-cfDNA largely because of the availability of two commercial NGS testing services for dd-cfDNA quantification in CLIA (Clinical Laboratory Improvement Amendments)-certified laboratories: AlloSure® offered by CareDx [18] and Prospera™ offered by Natera [19]. Notably, both assays have CMS approval for Medicare reimbursement and are offered along with blood drawing and consulting services.

The AlloSure NGS assay uses 266 SNPs selected from across all 22 somatic chromosomes [12]. In a recent large, multicenter study (ADMIRAL Study), 1,092 kidney transplant patients had their dd-cfDNA levels followed for a three-year period using this assay [20]. Of these patients, dd-cfDNA results were paired with 200 biopsies. A 62% improvement (AUC: 0.80) was found over serum creatinine (AUC: 0.49) in identifying subclinical and clinical rejection. Specifically, the Allosure assay had a median dd-cfDNA of 1.6% for patients undergoing rejection in contrast to 0.23% ($p < 0.0001$) in patients with no rejection. In comparison, serum creatinine had a median 1.57 mg/dl in rejection patients and 1.38 mg/dl in non-rejection patients ($p = 0.096$), clearly showing the superiority of dd-cfDNA over creatinine in identifying rejection. Both TCMR and AMR can be detected, although individuals with AMR had a higher median dd-cfDNA serum concentration at 1.8% versus 0.7% in TCMR affected individuals. These results correlate well with the initial clinical validation study (Circulating Donor-Derived Cell-Free DNA in Blood for Diagnosing Active Rejection in Kidney Transplant Recipients (DART)) [2]. In the DART study, a 1% threshold was established to discriminate rejection from non-rejection. In the new ADMIRAL study, this threshold is confirmed. In addition, they found that serial changes in dd-cfDNA level are important in the interpretation of graft injury. Specifically, increases of 149% from baseline signal suggest a change from quiescence to potential allograft injury. In most patients, this translates to an absolute elevation in baseline signal of ≥0.24%. Based on these findings, monthly testing is recommended by the manufacturer for the first four months after transplant, and then quarterly thereafter.

Natera's Prospera test offers dd-cfDNA quantification testing for kidney, heart, and lung transplant patients [19]. Natera originally began offering NGS testing services for noninvasive prenatal testing (NIPT) and has since expanded into post-transplant monitoring and oncology (circulating tumor DNA) testing. The Prospera NGS platform interrogates over 13,000 SNPs covering chromosomes 13, 18, 21, X, and Y [21]. The published analytical validation in kidney transplant patients [11] claims that the assay has an LoQ of 0.15% for unrelated donors and 0.29% for related donors. A low LoQ is desirable as it is an indication that dd-cfDNA levels can be readily tracked from low, non-actionable levels to values that require clinical intervention, i.e., >1%. In the clinical validation study, 217 biopsy-matched cfDNA samples were tested for dd-cfDNA [22] and the assay discriminated active rejection from non-rejection status with an AUC of 0.87%, offering an 88.7% sensitivity and 78% specificity at a clinical cutoff of 1% dd-cfDNA. Ultimately, this study reported that the median dd-cfDNA was significantly higher in the acute rejection group (2.32%) versus the non-rejection group (0.47%; $p < 0.0001$). Moreover, when dd-cfDNA was compared to eGFR measurements, it was found that eGFR had a clinical sensitivity of 67.7% and a specificity of 65.3%, demonstrating the superiority of dd-cfDNA for detection of graft injury. Finally, in this limited dataset, no significant difference was found in dd-cfDNA fractions between AMR and TCMR patients, in contrast to earlier studies showing higher increase of dd-cfDNA in AMR compared to TCMR [6].

Correlation of donor specific antibodies (DSA) to dd-cfDNA results

Before the development of dPCR or NGS assays for dd-cfDNA quantification, transplant clinicians had been relying on the detection of de novo donor-specific antibodies (dnDSA) in conjunction with biopsy results to predict risk for ABMR [23]. However, it has been observed that not all dnDSAs result in allograft injury. How do >1% levels of dd-cfDNA correlate with the presence of dnDSA? The DART study has found that in patients with dnDSA and ABMR, the average dd-cfDNA was 2.9%, compared to 0.34% in patients with dnDSA but no ABMR, and 0.29% in patients without dnDSA [24]. The ADMIRAL study did not correlate the role of dnDSA with dd-cfDNA, but a few other studies have demonstrated increased diagnostic value of dd-cfDNA testing in conjunction with DSA detection as useful noninvasive post-transplant monitoring tool [24, 25]. How might the small events that cause molecular injury as seen by increases in dd-cfDNA drive antibody formation, or does antibody formation drive molecular injury and subsequent increases in dd-cfDNA? Are the antibodies being absorbed by the allograft before being detected by DSA testing [20]? Unfortunately, no data is yet available about the correlation of dnDSA and dd-cfDNA levels detected using the Prospera assay.

Conclusions

New technologies and discoveries have ushered in a new era of noninvasive post-transplant monitoring. But many questions remain to be answered. dd-cfDNA testing by both dPCR and NGS techniques has the capability to revolutionize the way transplant recipients are monitored post-transplant, but it remains to be seen if testing a few markers (dPCR) is as good as testing many markers (NGS). How often should patients be tested for dd-cfDNA levels? The companies providing the testing services have specific recommendations, but professional society guidelines are not yet available. Finally, correlation of dd-cfDNA levels with existing post-transplant monitoring approaches such as dnDSAs and infectious disease status await further studies.

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Is It Prime Time for Urinary Chemokines as Clinical Biomarkers?

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Despite advances in immunosuppressive therapy, T-cell-mediated rejection (TCMR) continues to confer significant morbidity to renal transplant recipients and is an important contributor to premature allograft loss [1]. Early rejection is also associated with subsequent chronic cellular and antibody-mediated rejection, and inflammation can persist well after treatment of a rejection episode [2]. Current gold standards rely on the use of biopsies to diagnose rejection, and whilst biopsies have become safer and the classification more refined, the procedure remains invasive with risks to patients and is impractical for serial sampling. There is a paucity of evidence and recommendations on best practices regarding monitoring, management, and ongoing follow-up of these patients and an urgent need to implement new diagnostic tools into the clinic for routine serial, noninvasive monitoring, which could improve outcome by risk stratifying patients based on immune activation or quiescence status.

Early rejection is defined as within the first post-transplant year. Early subclinical acute rejection requires surveillance biopsy for diagnosis and is common, with year-one detection rates increasing (up to 58%) with more frequent biopsies [3-6]. Earlier diagnosis and treatment are therefore critical to prevent damage and preserve long-term function [7], which is greatly limited by reliance on infrequent surveillance biopsy monitoring. Outcomes are much worse in late rejection (>1 year post-transplant), where more entrenched inflammation leads to 2 to 10-fold higher risk of chronic damage [8] and graft failure compared to early rejection [2, 9]. Identification of late subclinical acute rejection is currently impossible, since diagnosis until now has relied of functional deterioration to indicate a biopsy [8]. Early identification, with new noninvasive tools, could dramatically improve the outlook in these high-risk populations. Evidence of persistent but sub-clinical inflammation/rejection is an underappreciated risk. In Canada, 70% of transplant specialists relied exclusively on functional monitoring after treatment [10], but data evaluating utility of creatinine (Cr) monitoring suggests this approach is nonsensical with subclinical rejection [11, 12] and the use of anti-HLA donor-specific antibodies (DSA) has limited predictive value in up to 60% of patients who exhibit clinically silent antibody mediated rejection (ABMR) [13, 14].

Efforts to improve identification of rejection using noninvasive methods has led to the search for appropriately sensitive and specific biomarkers that improve upon the characteristics of serum creatinine alone. There has been increasing evidence over the last decade of the role of chemokines in the immunological events around the time of allograft rejection [15, 16] and their detection in the urine is likely more representative of the allograft microenvironment than peripheral blood. Chemokines are a family of proteins that induce directed chemotaxis in nearby responsive cells [17-20]. Elevated urinary CXC-receptor 3 chemokines, such as CXC chemokine ligand CXCL10, are an early prognostic marker for graft loss, late acute rejection, and graft dysfunction [21]. Low urinary CXCL10 levels have been correlated with better graft prognosis [22] whilst elevated levels are observed in the setting of subclinical and clinical TCMR in adult and pediatric renal transplants [23, 24]. Jackson, et al. [25] have shown that the CXCL10 chemokine in urine identifies early renal allograft inflammation and renal injury with better sensitivity and predictability than serum CXCL10. In acute rejection, CXCL10 is induced by IFNγ [26, 27], binds the chemokine receptor CXCR3 on activated T cells and natural killer cells [28], and is highly expressed by infiltrating leukocytes and renal tubules [29, 30]. CXCL9 and CXCL10 have also been evaluated as promising noninvasive biomarkers for subclinical and clinical tubulointerstitial inflammation in renal transplantation [25]. The clinical validity of urinary CXCL10 as a rejection biomarker has been consistently supported by observational trials [6, 29]. In the PROBE study, we established the clinical validity of urinary CXCL10 for detection of subclinical rejection in children, and proposed relevant diagnostic thresholds for CXCL10 use in clinical monitoring [31]. The CTOT-01 trial obtained serial urinary CXCL9 protein and found CXCL9 protein best identified acute rejection (PPV 67.6%, NPV 92%) [32]. Monocyte chemoattractant protein-1 (MCP-1/CCL2) also belongs to the chemokine C–C family [33] and is an early response inflammatory mediator with a strong chemoattractant potency for immune cells [34] produced by renal tubular and glomerular epithelial cells. Fractalkine (CX3CL1) is an unusual chemokine that can act as either a soluble or membrane-bound mediator and is expressed on monocytes, natural killer cells, T cells, and smooth muscle cells. Accumulating evidence suggests that fractalkine, in addition to its role in chemotaxis and adhesion of leukocytes, supports the survival of multiple cell types during homeostasis and inflammation [35]. A prospective cohort study, published in 2021, showed urine MCP-1 elevation one week post LDKT can predict the potential risk of developing AR within three months post KT. It also showed elevation in urinary MCP-1, fractalkine and IL-10 correlates with intrarenal total leukocytes including T & B lymphocytes [36-39]. Consistent association of early urinary CCL2:Cr with poor long-term outcomes strongly supports a role for CCL2 and monocytes/macrophages in the pathogenesis of IF/TA, graft dysfunction, and loss [40]. Taken together, urinary chemokines likely provide differing but complementary information on the allograft/immune milieu. Advances in bioinformatics now also permit researchers to evaluate the power of multiple targets and targets types, strengthening the diagnostic accuracy of any individual biomarker, to provide more comprehensive information. The future of clinical biomarkers will likely be panels of multiomic targets, able to discern the nuances of complex disease and patient heterogeneity.

We now understand that sustained inflammation and persistent rejection lead to chronic forms of rejection [29, 30, 41-44] and progressive allograft failure [11, 45-52]. Conventional post-transplant monitoring relies heavily on tracking renal function with serial measurements of serum creatinine. This approach is neither sensitive nor specific for detecting acute rejection as many factors influence allograft function, and significant immune injury must take place

prior to detectable changes in creatinine. Detecting DSA by solid phase assay can also be helpful in diagnosing rejection. Unfortunately, patients with immune rejection and newly evolved DSA are often on an inexorable path to allograft failure due to the paucity of treatments for antibody mediated rejection. Furthermore, immune rejection can occur in the absence of new DSA [22, 51-54]. This highlights the need to discover new biomarkers that facilitate the diagnosis of emerging immune rejection prior to allograft injury. In a study of 281 consecutive biopsies, indicated by an increase in serum creatinine levels, only 27.8% revealed any sign of acute rejection (AR) [55]. Conversely, subclinical AR was found in over 40% of patients with normal renal function in the presence of anti-HLA de novo DSA [14, 56]. Taken together, there is an urgent need to implement clinically diagnostic tools for routine serial, noninvasive monitoring to detect early onset of rejection. Several recent reviews describe how we might optimally incorporate biomarkers into our decision-making process [57-60]. And although a number of biomarkers have shown promise in preliminary studies, most have yet to be incorporated into routine clinical decision-making. The current literature highlights heterogeneity in clinical practice and the need for further study to establish optimal pathways for identification and management of persistent inflammation and subclinical rejection. This kind of subtle, subclinical inflammation is certainly linked with future poor outcomes, whether a patient has undergone biopsy or not. Certain biomarkers can be followed after the treatment of rejection including those that indicate persistent rejection, which are backed by data where patients have also had repeat biopsies, and those where there was only a measurement of the noninvasive marker. Following these episodes, some recover fully, others only partially or not at all.

While kidney biopsy remains the clinical gold standard to identify rejection, incorporating biomarkers into noninvasive monitoring may enable clinicians to use surveillance biopsies in a directed manner. Biomarkers show a significant correlation with evolving TCMR histology from sequential renal biopsies and are thus promising noninvasive surveillance tools. Serial monitoring of these indicators following rejection treatment may reveal which patients have persisting allograft inflammation and prognosticate risk for long-term outcomes. Further studies are needed to study these tools in a larger cohort of renal transplant recipients with a variety of clinical phenotypes.

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More than Meets the Eye: Experiences with NanoString® nCounter® Gene Expression Analysis in Transplant Biopsies

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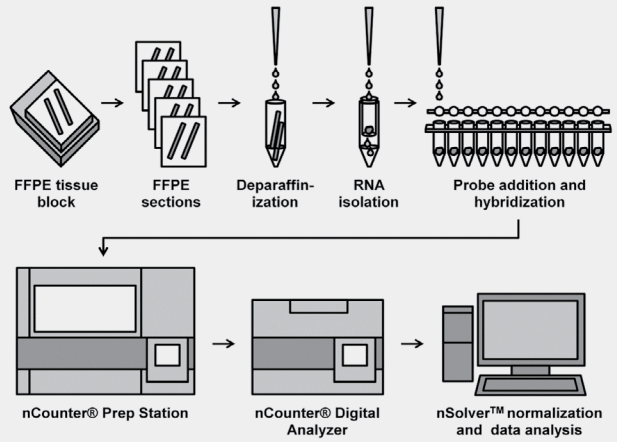
Molecular Transplant Pathology: Expanding the Toolbox

As transplant pathologists, arriving at the final diagnosis for a particular case requires integration of multiple sources of information, and we have various tools available to us, including the standard pathology methods of light microscopy, immunohistochemistry, immunofluorescence, and electron microscopy. We also rely heavily on clinical history and other laboratory results, including donor specific antibody testing. More recently, molecular diagnostics have emerged as another important ancillary tool for the transplant pathologist's toolbox. The molecular methodologies currently available to us in the field of transplantation include tissue, blood, urine, and other fluid-based approaches. Focusing on biopsy-based tools, the primary modalities include gene expression technologies such as RT-PCR, microarrays, RNA sequencing, and NanoString® nCounter®, which will be the focus of this article.

Philip Halloran and his colleagues have arguably led the field of molecular transplant pathology up to this point, with many novel insights derived from their molecular microscope project over the past two decades. Some of the most important learnings from their work include the understanding that molecular disturbances associated with allograft inflammation and injury are highly stereotyped, the description of specific transcripts and classifiers associated with antibody-mediated rejection (ABMR) and T-cell mediated rejection (TCMR), confirmation of the relevance of Banff histology lesions, identification of C4d-negative ABMR, and characterization of the significance of inflammation in scarred tissue [1].

Given that we now know that many molecular disturbances in allograft inflammation and injury are stereotyped (i.e., many of the genes travel in "herds"), this raises the possibility of being able to translate microarray-based discoveries to other more convenient and/or affordable methodologies by focusing our efforts on a few of the most representative genes in the pack. This is where the NanoString® nCounter® platform comes into play. This platform uses a unique color-coded probe-based technology, which allows for direct digital quantification of specific nucleic acid targets of interest. The color-coded reporter tags are hybridized to a biological sample, fixed in place on a cartridge, and then visualized and counted by an automated digital camera without the need for amplification, providing a direct count of the number of transcripts of interest in the sample. The lack of amplification, along with the relatively short probe lengths (100 base pairs), is what makes it appropriate for the highly degraded RNA that we tend to get from formalin-fixed paraffin-embedded (FFPE) tissue, which is the processing standard for clinical pathology specimens around the world (**Figure 1**).

Figure 1. Overview of NanoString® nCounter® Workflow Using Formalin-Fixed Paraffin-Embedded (FFPE) Tissue.



The primary benefit of being able to reliably analyze FFPE samples with NanoString® is that it opens a massive worldwide archive of historical clinical samples for retrospective gene expression testing. This allows for immediate access to long-term clinical follow-up data, testing of rare samples that are difficult to collect in a prospective manner, and better correlation with histology because you can perform molecular testing on the exact same piece of tissue. Although it is not as multiplexed as microarrays or RNA sequencing, it still allows simultaneous analysis of up to 800 transcripts with either off-the-shelf or custom probe sets. Other benefits include relatively minimal RNA input (100 ng), technical simplicity, relative affordability, reproducibility, and suitability for decentralized testing [2].

Experiences with NanoString® in Transplantation

Since the development of NanoString® nCounter® in 2008 [3], there have been numerous publications demonstrating its technical robustness, including a 2016 report from our group describing our initial experience with the platform in kidney transplant samples [2]. In this study, we used NanoString® to measure the expression of 34

previously reported ABMR-related genes in 112 archival FFPE renal allograft samples and evaluated the following: correlation between different RNA input quantities, inter-observer and inter-laboratory reproducibility, and NanoString vs. RT-PCR.

We were pleased to find that sufficient mRNA was able to be obtained for NanoString® from more than 90% of the samples attempted, including biopsies with as little as 1 mm^2 of tissue remaining in the FFPE block. The correlation between replicates performed with different RNA input amounts (i.e., 50, 100, 200, 400 ng) was almost perfect ($r=0.99$, $p<0.001$). Replicates performed in the hands of three different operators of varying experience, including a qualified molecular pathology technologist, an undergraduate summer student, and a physician researcher, also demonstrated near-perfect reproducibility ($r=0.99$, $p<0.001$). To simulate inter-laboratory reproducibility, we also performed replicates using three different lots of reagents, and again observed excellent correlation between runs ($r=0.98$, $p<0.001$). Comparing the results generated by NanoString® from archival FFPE tissue with RT-PCR from corresponding fresh tissue samples, we found that there was relatively poor correlation between these methodologies ($r=0.49$, $p<0.001$); however, Nano String® results demonstrated much stronger correlation with Banff histology scores, suggesting that the data generated by this platform were more accurate [2].

Our group has since used NanoString® to validate previous microarray-based gene expression signatures in additional FFPE kidney [4-6], heart [7], lung [8-9], and pancreas [10] transplant biopsies. Although the details of these studies are beyond the scope of this article, one point worth highlighting is that they included FFPE samples that had been in storage for more than 20 years, further confirming the robustness of the platform and the benefits of being able to utilize such previously inaccessible historical tissue for retrospective molecular analysis. Encouragingly, the NanoString® platform has since been adopted by several other research groups within the field of transplantation, and has undergone further technological developments, including the ability to perform spatially resolved transcriptomics (i.e., NanoString® GeoMx® Digital Spatial Profiler).

Conclusion

Microarray analysis has revolutionized molecular transplant pathology over the last two decades, but novel technologies such as NanoString® now provide the opportunity to translate and extend these discoveries to FFPE tissue, the benefits of which include retrospective analysis of archival tissue, immediate access to long-term clinical follow-up data, direct correlation with histology on the same sample, the opportunity for more decentralized testing and multicenter research, and a relatively simple and more affordable methodology. It will be exciting to see how these game-changing platforms contribute to further discovery and implementation of clinical molecular diagnostics within the field of transplantation over the coming years.

Disclosure

The author of this article has no conflicts of interest to disclose, including no financial relationship with NanoString®.

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THE NEXT GENERATION IN GENETIC MATCHING WITH INNOVATIVE HYBRID-CAPTURE TECHNOLOGY

Margot D.,
Stem cell transplant recipient

Expandable Gene Content
without Affecting Lab Workflow

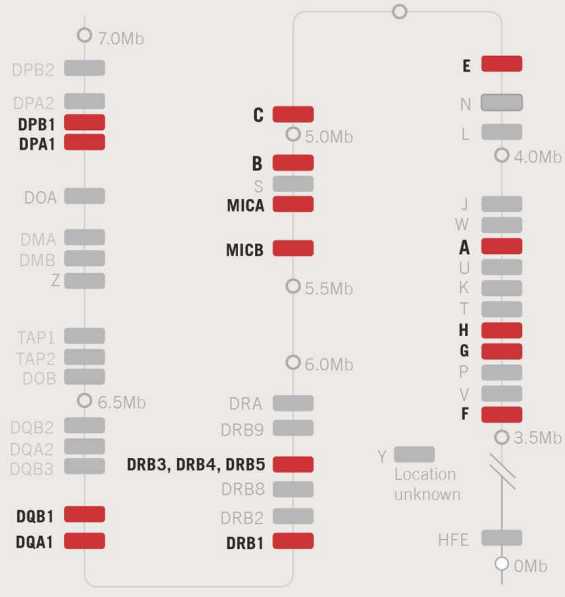
Easy Single Tube Workflow
with Early Indexing Step

No Long-range PCR =
No Amplification Inefficiencies

Fast Software Analysis
with Data Upload at 1 min/sample*

*as per internal testing

AlloSeq Tx17 is CE/IVD in European Union.
For research use only in the rest of the world.



AlloSeq Tx 17 moves beyond the traditional transplant related loci to consider more transplant associated genes.

For more information visit **www.caredx.com/alloseq-tx17** or reach out to your local CareDx representative.

ASHI QUARTERLY CONTINUING EDUCATION QUIZ

Quiz may be taken online at ashi-u.com.

Quiz Instructions: The multiple-choice quiz below is provided as an alternative method of earning continuing education (CE) credit (CEC) hours. Read each article then select the ONE best answer to each question. The questions are based solely on the content of the article. Answer all of the questions and send the quiz answer sheet and fee (if applicable) to the ASHI Executive Office before the processing date listed on the answer sheet. To be eligible for 3.0 credit/contact hours or 0.45 CECs, ALL questions must be answered, a passing score of 70% must be obtained, and the answer sheet must be submitted with fee (if applicable) before the deadline. Quizzes may not be retaken, nor can ASHI staff respond to questions. Allow six weeks for paper processing following the submission deadline to receive return notification of your completion of the CE process. The CEC will be dated when it is submitted for grading. That date will determine the CE cycle year.

Scientific Article 1 — Quantifying cfDNA for Post-Transplantation Monitoring

1. What level of dd-cfDNA may be indicative of kidney graft injury?

A. Any level that is picked up by a sensitive assay.
B. Any level that is above 1%.
C. Any level that is above 0.5%.
D. dd-cfDNA is not a reliable marker for graft injury.

2. Which statement below is correct regarding the analytical parameters of a clinical assay designed to detect dd-cfDNA?

A. LoB stands for limit of binding to single antigen beads.
B. LoD or limit of detection is determined by repeated measurements of blank samples.
C. LoQ is the lowest amount of analyte that can be quantitatively determined.
D. There is no practical difference between LoD and LoQ when measuring dd-cfDNA.

3. Which of the methods below is NOT commonly used to measure serum dd-cfDNA?

A. Digital polymerase chain reaction (dPCR).
B. SNP detection using next generation sequencing (NGS).
C. CNV detection using dPCR.
D. Detection of HLA allele differences using qPCR.

4. Which of the following statements is correct about digital PCR (dPCR)?

A. It is based on partitioning of the PCR reaction to droplets that have an all or none signal.
B. The endpoint is determined by measuring the cycle threshold (Ct) value.
C. It calculates dd-cfDNA using a standard curve measured within the same assay.
D. It can be run using the existing qPCR instrument in the lab.

5. What is the most important determinant of assay sensitivity for a next generation sequencing (NGS) based assay designed to detect dd-cfDNA in transplant recipients?

A. The higher the number of SNPs analyzed, the more sensitive the assay is.
B. Read depth is more important for assay sensitivity than the number of SNPs sequenced.
C. SNP coverage must include regions from all chromosomes.
D. To be used only in conjunction with donor specific antibody (DSA) measurements.

Scientific Article 2 — Is it Prime Time for Urinary Chemokines as Clinical Biomarkers?

6. What percentage of Canadian transplant specialists rely exclusively on functional monitoring after treatment of a rejection episode?

A. 15%.
B. 70%.
C. 65%.

7. What is a way noninvasive biomarkers could benefit patients?

A. Save on travel time to testing site.
B. No requirement for general anesthetic.
C. Stratification of active versus quiescence to modulate immune suppression.

8. In terms of rejection onset, which one carries worse outcome (acute or chronic)?

A. Acute.
B. Chronic (2-10 fold higher risk of graft loss).

9. Name a noninvasive chemokine biomarker that plays role as an early prognostic marker for graft loss.

A. MCP-1/CCL2.
B. CX5.
C. Kryptonite.

10. What percentage of patients with normal graft function had demonstrable anti-HLA antibodies?

A. 40%.
B. 27.8%.
C. 70%.

Scientific Article 3 — More than Meets the Eye: Experiences with NanoString® nCounter® Gene Expression Analysis in Transplant Biopsies

11. Which is not a standard histopathology method?

A. Immunohistochemistry.
B. Light microscopy.
C. RT-PCR.
D. Electron microscopy.

12. Molecular testing can be performed on which sample types in transplantation?

A. Tissue.
B. Blood.
C. Urine.
D. Other fluids.
E. All of the above.

13. Step(s) of the NanoString® nCounter® platform assay for FFPE biopsies include:

A. Probe hybridization.
B. De-paraffinization.
C. Cryopreservation.
D. a and b.
E. a, b, and c.

14. Advantages of using FFPE tissue for molecular testing include:

A. Use of the same tissue used to perform standard pathology tissue analysis.
B. Ability to test stored/retrospective and rare samples.
C. a and b.
D. None of the above.

15. A 2016 study evaluating the use of NanoString® nCounter® in renal transplantation showed:

A. Strong correlation between reagent lot numbers.
B. The ability to use tissue samples as small as $1mm^2$.
C. Strong correlation between replicate tests.
D. All of the above.

CONTINUING EDUCATION QUIZ

ANSWER SHEET

Please print clearly in black ballpoint pen

First Name | M.I. | Last Name

Institution

Address

Address

City | State/Province | Zip Code/Postal Code

Country

ASHI Membership Number (**MUST** complete if requesting fee waiver)

Instructions: Mark your answers clearly by filling in the correct answer, like this ■, not like this ☒. 3.0 credit/contact hours or 0.45 CECs earned with passing score (70%).

Please use black ballpoint pen.

1	A	B	C	D	
2	A	B	C	D	
3	A	B	C	D	
4	A	B	C	D	
5	A	B	C	D	
6	A	B	C		
7	A	B	C		
8	A	B			
9	A	B	C		
10	A	B	C		
11	A	B	C	D	
12	A	B	C	D	E
13	A	B	C	D	E
14	A	B	C	D	
15	A	B	C	D	

The quiz answer sheet will not be graded, no CEC will be awarded and the processing fee will be forfeited unless postmarked by:

August 31, 2022

Quiz Identification Number:

46-2-2022

ACHI Program Number:	CA Provider Number:	FL Provider Number:
ACHI 2022-19	0085	50-2203

❑ Processing Fee: **ASHI Member – no fee** **Nonmember – $30**

❑ Payment is due with submission of answer sheet. Make check or money order payable to ASHI (U.S. currency only). Do not send cash. No refund under any circumstances. Please allow six weeks following the submission deadline for paper processing.

❑ Individuals paying by credit card may fax the answer sheet to 856-439-0525.

Please check one:

❑ Enclosed is check #_____ (payable to ASHI)

❑ Charge to the following card:

❑ MasterCard (16 digits) ❑ Visa (13 or 16 digits) ❑ Amex

Card Number_____ Exp. Date _____

Signature _____

Print Cardholder's Name _____

For Internal Use Only

Date Received: _____

Amount Received: _____

Notification Mailed: _____

Answers to the 1st Quarter 2022 *ASHI Quarterly* Quiz:

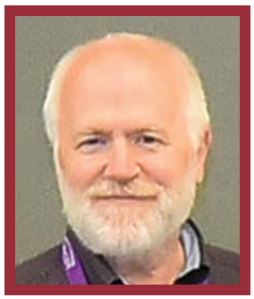
1. B, 2. D, 3. A, 4. C, 5. E, 6. C, 7. A, 8. B, 9. D, 10. D, 11. C, 12. A, 13. A, 14. C, 15. D

PT Update

The Decision to Offer
New Proficiency Testing Survey (Part I)

Taba Kheradmand

Idoia Gimferrer



Danny Youngs

Mary Carmelle Philogene

Introduction

The results of the 2020 ASHI PT satisfaction survey were previously discussed in detail (2020 ASHI Quarterly Q2, PT Committee Report). As part of the satisfaction survey, the proficiency testing committee sought information from the survey participants regarding the addition of new analytes to the ASHI PT program. Respondents indicated interest in surveys that assess analytes linked to drug hypersensitivity and disease associations, lineage-specific myeloid and lymphoid chimerism, killer cell immunoglobulin-like receptor (KIR) genotyping, non-Human Leukocyte Antigens (non-HLA) and COVID antibodies, and ABO blood grouping/subgrouping. In this report, the Executive Committee (EC) sought to respond to these requests by summarizing parameters that must be considered in determining the feasibility of additional PT surveys. We also outline some of the limitations in evaluation of new analytes as part of a PT survey. We use data and insights gathered from the addition of ungraded surveys over the last five years to inform future practices. The first four factors will be presented in this ASHI Quarterly (Part I). The impact of factors 5 and 6 mentioned below will be discussed in Part II (next ASHI Quarterly).

Factors Involved in Providing a New Survey

The following components are considered when assessing feasibility of providing a new survey or adding a new analyte to an existing survey:

1. Availability of appropriate samples.

2. Effort from vendors who provide access to samples:

- **Access to samples with analyte of interest.**
- **Multiple shipments.**

3. Requirements from accrediting agencies:

- **Is there enough participation from labs to allow grading?**

4. Grading strategy:

- **What parameters are gradable?**

5. Cost to the survey participants:

- **Are survey participants willing to pay for new surveys?**

6. Software accommodations and updates.

Many of these challenges can be illustrated using examples from the ungraded surveys (AT1RAb and virtual crossmatch).

1. Availability of Appropriate Samples

Vendors obtain samples from blood collection institutions, typically collected from donors who are randomly selected for the PT survey. Blood draws are obtained from altruistic donors that are willing to donate blood for research purposes. As one would imagine, the vendor must make sure that there are enough donors available for donation and account for unforeseen circumstances that can render the donors ineligible or that may result in donor no-show. The amount of blood that can be drawn from each donor and the frequency in which each donor can donate is also limited. In addition, the PT committee requests variability in the donors' demographics and requires certain serum criteria to make each survey challenging.

Analytes that can be evaluated using existing surveys are easier to incorporate into the ASHI PT Program. Therefore, additional donors do not need to be recruited for each additional survey. This approach reduces the cost and complexity of providing a new survey. For example, detection of HLA antigens or alleles for disease association or pharmacogenetics can be incorporated into the high-resolution or low-resolution HLA typing survey (HT surveys). The HT survey has been used for HLA-B27 detection. Similarly, detection of antibody against angiotensin II type I receptor (AT1R Ab) was incorporated into the AC survey. The virtual crossmatch educational challenge takes advantage of the already available data for HLA typing and antibody identification results from each AC survey, and with slight modifications, that primarily require effort from the members of the PT committee, cells were swapped among different groups. This allowed the virtual crossmatch predictions to be performed independently from the physical crossmatch data obtained by the same AC group. Despite the advantages offered with the use of existing surveys, there remain some challenges in collecting, analyzing, and potentially grading these additional analytes. These challenges will be discussed in more detail in Part II of this article, in the next ASHI Quarterly, under software updates.

2. Effort From Vendors Who Provide Access to Samples

Each analyte must be tested using samples that contain or do not contain the analyte of interest. Therefore, it is ideal that both positive and negative samples be provided. This creates a challenge for

rarely positive analytes. For example, surveys that would be designed for disease association and pharmacogenetics may require that donor typing with the analyte of interest is identified prior to selection of samples to submit for PT. This can limit the number of donors with the HLA type that can be used in each survey every year. This issue can also be illustrated with the ungraded survey for detection of AT1R Ab. Since the samples used for detection of this analyte are collected from donors at blood centers who are considered healthy, identifying sera that are positive for AT1R Ab has been difficult (Table 1).

The AT1R Ab PT has been provided as an ungraded analyte since 2018. Serum samples included in the AC survey were used for this assessment. Between 2018 and 2021, a total of 40 sera were used for AT1R Ab detection. A consensus result was reached for 90% of the samples by 12 to 14 participating laboratories. The consensus response was primarily among samples that were "negative" for the analyte. Thirty-one of 40 samples (77.5%) reached consensus negative results. Only 5% (n=2) of samples were found to be consensus positive. Therefore, this analyte would not be detected in every survey (Table 1). Obtaining the required samples that would include a proportion of samples that are either negative or positive for an analyte requires screening of a category of serum donor that may not be accessible to the program. Alternatively, spiking samples has been an acceptable mechanism for obtaining positive samples for survey. This option has been considered and discussed by the PT committee and requires coordination with the vendor providing the samples. Nevertheless, a negative consensus, whether in case of disease association, or rare non-HLA analytes, can still be informative and measure the ability of participating laboratories to identify the correct results.

3. Requirements From Accrediting Agencies

Several ASHI survey participants utilize data from the ASHI survey to satisfy requirement for accreditation by the College of American Pathologists (CAP). A CAP accepted analyte must include 10 or more participants to be graded. Consequently, one of the oldest analytes, the HLA class II antibody identification by serology, is no longer an accepted CAP analyte due to a low number of participants. If fewer than 10 participants submit results for an analyte, the results would be ungraded. ASHI and Centers for Medicare & Medicaid Services (CMS) accept ungraded analytes if the participants can compare data with the consensus results, even graded PT surveys are unavailable (2022 PT Operations Manual).

In the PT satisfaction survey, the analytes that were ranked first-choice for additional offerings were ABO antibody titers and ABO genotyping (performed by 9.8% of labs; n=8). Offering this analyte may result in an ungraded PT survey if only 8 laboratories participated in the survey.

Analytes such as disease association/drug hypersensitivity (performed by 64% of labs; n=24), and KIR genotyping (performed by 22% of labs; n=19) could be considered in the future as potential ASHI PT surveys since the number of interested participants exceeds the required 10 participants. However, since these analytes are provided by other PT providers, it would be important to determine how many participants would subscribe to the new ASHI offering versus similar surveys from other providers.

Interestingly, 16 laboratories (19.28%) reported an interest in non-HLA antibodies other than AT1R Ab, although the majority commented on

Table 1: Summary of Ungraded AT1R Ab Surveys (2018-2021)

SHIPMENT	SAMPLE #S	CONSENSUS NEGATIVE	CONSENSUS AT-RISK	CONSENSUS POSITIVE	NO CONSENSUS	TOTAL SAMPLES PER SURVEY	# OF PARTICIPANTS
2021 AC2 Survey	535-539	4	1	0	0	5	13
2021 AC1 Survey	530-534	1	1	1	2	5	14
2020 AC2 Survey	525-529	5	0	0	0	5	14
2020 AC1 Survey	520-524	4	1	0	0	5	14
2019 AC2 Survey	515-519	4	0	0	1	5	12
2019 AC1 Survey	510-514	4	0	1	0	5	12
2018 AC2 Survey	505-509	4	0	0	1	5	11
2018 AC1 Survey	501-504	5	0	0	0	5	11
Total # of Samples	501-539	31	3	2	4	40	
Percent Consensus		77.50%	7.50%	5%	10%	100%	

lack of standardization of existing assays. In the case of the AT1R Ab, there have been a stable number of participants (11-14 participants), which would meet the requirements for grading of this analyte. Of the laboratories that responded to the PT survey in 2020, only 10% (n=6) showed interest or indicated that they would continue to participate in the survey if it was offered as a graded analyte.

4. Grading strategy

Reaching consensus: For some analytes, it is important to determine what parameters should be graded. As an example, for the AC survey, presence or absence of HLA antibody is not dependent on the relative strength of the antibody, but rather on what the individual centers consider to be a reportable antibody. For AT1R Ab, there are several possible results to consider for grading: a qualitative determination of detection (negative, at risk, positive) which is independent of antibody level or, a quantitative value (units/ml) detected by ELISA (which ranges from 0 to >40 units/ml as indicated by the provider). Table 2 illustrates how different grading outcomes will be reached depending on the grading schema selected.

This table shows the mean (M) and the standard deviation (SD) per each analyte on survey, AC-1 2021 as an example. Using M and SD values, a low and a high cut off can be calculated. If using M+3SD (less restrictive) all analyte reached consensus, but when using M+2SD, one result was discrepant per each analyte, except AC-532. On the

Table 2: Example 2021 AC1 survey AT1R Ab Proposed Grading

	AC-530	AC-531	AC-532	AC-533	AC-534
AC-1	18.00	11.00	7.00	40.00	7.00
	13.67	11.27	6.70	40.00	8.54
	12.80	9.88	5.50	40.00	7.09
	14.00	10.00	5.00	40.00	9.00
	12.00	9.80	6.10	32.70	8.60
	14.00	10.00	6.00	40.00	7.00
	13.00	7.00	4.00	40.00	7.00
	13.52	7.55	6.48	40.00	11.81
	15.74	12.78	6.80	40.00	10.01
	10.90	9.90	5.80	40.00	11.10
	12.00	10.10	7.50	30.00	11.00
	14.98	11.55	6.88	51.36	7.49
	13.00	10.90	6.90	25.60	6.70
	14.00	12.00	4.00	47.00	4.00
MEAN	13.69	10.27	6.05	39.05	8.31
SD	1.754	1.560	1.091	6.361	2.135
>M+2SD	17.19	13.39	8.23	51.77	12.58
>M+3SD	18.95	14.95	9.32	58.13	14.71
<M+2SDn	10.18	7.15	3.87	26.33	4.04
<M+3SDn	**8.42**	**5.59**	**2.77**	**19.96**	**1.91**
Consensus Qualitative	**At Risk**	**No Consensus**	**NEG**	**POS**	**No Consensus**
Consensus Quantitative 3D	**YES**	**YES**	**YES**	**YES**	**YES**
Consensus Quantitative 2D	**1 Discrepant**	**1 Discrepant**	**YES**	**1 Discrepant**	**1 Discrepant**

contrary, if grading using qualitative determination, AC-531 and AC-534 did not reached consensus. The reason is because the quantitative values were close to the negative-to-at-risk cut off. Deciding which parameter will be used clearly influences the grading.

For the virtual crossmatch educational challenge, the number of participating laboratories for each of the three groups has been sufficient to reach a grading agreement; however, one of the main concerns in grading this challenge is that the virtual crossmatch is not considered a CMS-approved analyte. Additionally, the virtual crossmatch challenge was designed to capture differences in practice across HLA laboratories, from serum and cell treatment to lab-specific cutoff threshold for both the antibody and crossmatch assays, and ultimately, the decision process for risk assessment. This creates a challenge to assess grading in a meaningful way, as the cumulative differences in these components may result to lack of consensus. Therefore, at this time, the virtual crossmatch challenge will remain an educational tool.

Future Directions

Lineage-specific chimerism testing: Almost 30% of respondents (n=24) to the ASHI PT satisfaction survey showed interest in participating in the lineage-specific survey. Although it may not be possible to offer all cell types as proficiency testing challenges, the committee is currently evaluating incorporation of the most common — lymphoid and myeloid — chimerism testing into the currently available EMO survey. As part of this evaluation, the committee is working with the vendor to determine if enough blood will be available for these additional challenges. The committee is also working with the IT team and the software engineers to incorporate the data collection and analysis into the PT software. The committee is hopeful that the lineage-specific survey can be offered soon.

The mission of the ASHI Proficiency Testing Program is to promote high quality testing practices in clinical histocompatibility and immunogenetics. This is accomplished through consistent and objective evaluation of competency as well as exposure to educational opportunities. Toward that end, the Proficiency Testing Program supplies specimens and materials that challenge laboratory procedures and testing personnel. As we have illustrated in Part I of this series, several factors must be considered when offering a PT survey. The impact of cost to participants and software updates will be discussed in the next ASHI Quarterly (Part II). As a reminder, when an analyte cannot be offered as part of the ASHI survey, the laboratories are required to perform an alternative assessment that may include biannual verification or splitting a patient's specimen with another laboratory that offers the same test(s) (ASHI Standard C.1.1.5).

ARB Update

John Schmitz, *Ph.D., F(ACHI), ARB Program Director*

It is my pleasure to announce here that ASHI's deemed status as an accrediting organization (AO) has been approved by the Centers for Medicare and Medicaid Services (CMS) for the full period of six years. With this, ASHI remains one of seven organizations that accredit clinical laboratories for CMS. This was a long process that took place over nearly a year and involved not only the Accreditation Review Board (ARB) but also the leadership of the American College of Histocompatibility & Immunogenetics (ACHI) Portfolio Committee (former DTRC). The process started in April 2021 with a request for our application that was submitted in June 2021 after a complete crosswalk between ASHI and the Clinical Laboratory Improvement Amendments (CLIA) regulations. After CMS' review of the application, a four-day virtual inspection occurred in August. Three CMS staff members, Melissa Weeks, the ARB Executive Committee, and PC leadership took part in some or all of the virtual inspections. Over the ensuing months, three different requests for clarification were received by ASHI, reviewed, and responded to. An additional virtual meeting took place to clarify a handful of items. Ultimately, ASHI was reaccredited for the maximum six-year period. I would like to personally thank Melissa Weeks for another outstanding job organizing data and responses to questions, and shepherding us through this process. In addition, I would like to recognize the ARB Executive Committee (Sam Ho, Cal Stone, Julie Houp, and David Kiger) for their efforts on this project. Also, Chantale Lacelle and Paula Arnold from the DTRC provided critical input and clarification on laboratory director certification questions.

Things clarified or changed from the process:

- ARB Operations Manual: added more detail about immediate jeopardy/sentinel events — and added a template for our labs to report such events.
- ARB Inspections: removed "partial" and "complete" designations for deficiencies. CMS recommended this change backed by their "a deficiency is a deficiency, period" perspective, and the ARB agreed. Going forward, inspectors will not see an option to mark a deficiency partial or complete and will instead be encouraged to be as detailed as possible in the "comments" box for each citation — completeness, severity, frequency, etc. can be explained there.
- There was significant discussion about ASHI's practice of accepting certification boards other than the American Board of Pathology and the American Osteopathic Board of Pathology for MD/D0 directors as it does deviate from CLIA. However, ASHI's explanation of the suitability of alternative boards was accepted by CMS.

A copy of the letter approving ASHI is provided on the right.

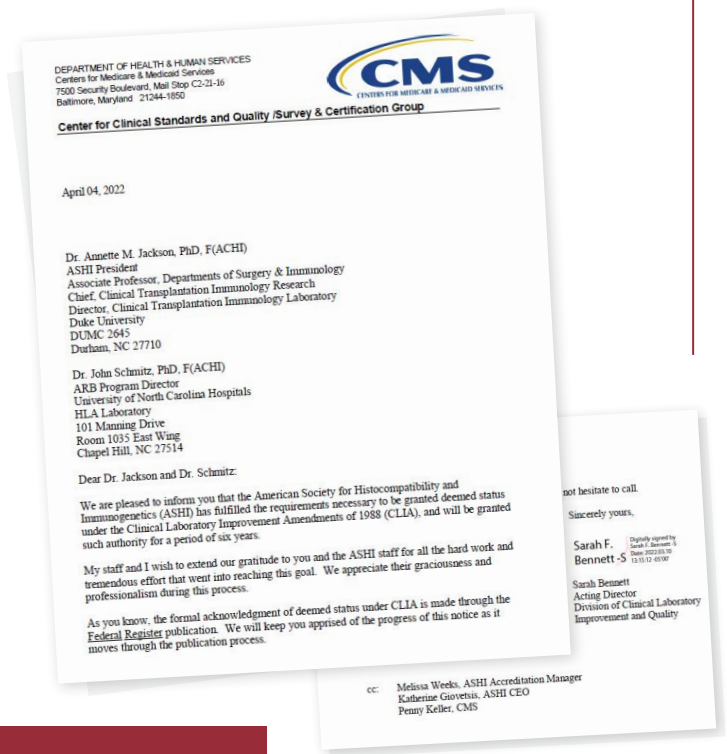
Other news: Invoicing for the annual accreditation fees ($2,060) is changing — for the better! Starting with cycle 2 in May 2022 laboratories will receive accreditation invoices through ASHI's YM database — similar to the way ASHI PT subscribers are billed, this will all be streamlined.

Laboratory Cycle Updates:

CYCLE 1: Laboratories in cycle 1 were sent links to their accreditation applications on Jan. 1, 2022 due back on March 1, 2022. Onsite laboratories in this cycle will be inspected between the dates of April 15 and June 15, 2022. Laboratories in this cycle will receive their new accreditation letters and/or certificates at the end of August 2022.

CYCLE 2: Laboratories in cycle 2 were sent links to their accreditation applications on May 1, 2022 due back on July 1, 2022. Onsite laboratories in this cycle will be inspected between the dates of Aug. 15 and Oct. 15, 2022. Laboratories in this cycle will receive their new accreditation letters and/or certificates at the end of December 2022.

CYCLE 3: Laboratories in cycle 3 will receive their accreditation letters & certificates in April 2022. The ARB met in person in Louisville, Kentucky to discuss this cycle & other business. Cycle 3 will be inactive until September of 2022 when the application & inspection process starts up again.



DEPARTMENT OF HEALTH & HUMAN SERVICES
Centers for Medicare & Medicaid Services
7500 Security Boulevard, Mail Stop C2-21-16
Baltimore, Maryland 21244-1850

CMS CENTERS FOR MEDICARE & MEDICAID SERVICES

Center for Clinical Standards and Quality /Survey & Certification Group

April 04, 2022

Dr. Annette M. Jackson, PhD, F(ACHI)
ASHI President
Associate Professor, Departments of Surgery & Immunology
Chief, Clinical Transplantation Immunology Research
Director, Clinical Transplantation Immunology Laboratory
Duke University
DUMC 2645
Durham, NC 27710

Dr. John Schmitz, PhD, F(ACHI)
ARB Program Director
University of North Carolina Hospitals
HLA Laboratory
101 Manning Drive
Room 1035 East Wing
Chapel Hill, NC 27514

Dear Dr. Jackson and Dr. Schmitz:

We are pleased to inform you that the American Society for Histocompatibility and Immunogenetics (ASHI) has fulfilled the requirements necessary to be granted deemed status under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), and will be granted such authority for a period of six years.

My staff and I wish to extend our gratitude to you and the ASHI staff for all the hard work and tremendous effort that went into reaching this goal. We appreciate their graciousness and professionalism during this process.

As you know, the formal acknowledgment of deemed status under CLIA is made through the Federal Register publication. We will keep you apprised of the progress of this notice as it moves through the publication process.

not hesitate to call.

Sincerely yours,

Sarah F. Bennett -S

Sarah Bennett
Acting Director
Division of Clinical Laboratory Improvement and Quality

cc: Melissa Weeks, ASHI Accreditation Manager
Katherine Giovetsis, ASHI CEO
Penny Keller, CMS

COMMITTEE REPORT

How Did Becoming an ASHI Certified Inspector Enrich My HLA Career?

Runying Tian, *MS, CHS(ACHI)*

Dr. Hildebrand became ASHI's president-elect in 2019. I sent him an email to congratulate him, and he encouraged me to volunteer in the ASHI community. I took his suggestion and joined the Technologists' Affairs Committee.

When I joined the committee, I wasn't sure what I could bring to the table. ASHI's chief executive officer, Katherine Giovetsis, gave a presentation about volunteer committees and opportunities, and one key thing that I learned was to attend committee meetings regularly. I thought to myself, "I can do that." I attended every meeting and learned about the committee's purpose: to provide histocompatibility technologists with the necessary information and resources to be successful in their careers.

I love the column "My Way to HLA" in the ASHI Quarterly. Those stories gave me a sense of belonging, so I shared my HLA story in the first quarter of the 2020 ASHI Quarterly. As I continued to attend more meetings, I became more aware of what ASHI was trying to do to fulfill this committee's purpose. I reflected on my HLA career path and realized that becoming an ASHI certified inspector was an important step in achieving my career goals.

As I shared in "My way to HLA," when I worked in Dr. Hildebrand's HLA lab at Oklahoma University Health Science Center, Dr. Marilyn Pollack came to inspect our lab in 2005. It did not go as smoothly as we expected; however, I remember vividly how it triggered my curiosity, and I knew at that moment that I wanted to become an inspector.

I attended the inspection training at an ASHI Annual Meeting that year and found that the information was way over my head. Two years in HLA did not give me enough experience to tread on that water. Over the next few years, I expanded my HLA career and moved to Dr. Dongfeng Chen's lab at Duke University Health System in 2011. Six years later, I became an ASHI inspector.

My first experience as a trainee was not that great. I did not feel I really learned anything, and it was discouraging. I thought of quitting, but at the same time I wanted to give myself one more chance. Then, I was paired with Dr. Neng Yu for my second inspection. She reignited my passion and helped me to see the bigger picture. It was an experience of learning, exchanging knowledge, and being an eye for ASHI.

I started to pay more attention to ASHI standards and attended every inspector training possible. Those common deficiencies shared on our annual inspector training session were super helpful. I would go back and read those related standards and try to understand the interpretation in real situations. Since every lab was different, I would print out everything from the lab's application online and make a list of things I would focus on during the inspection. The journey has been very rewarding and has positioned me to view our daily routines in the lab from a unique perspective.

During the last five years, I have encountered traveling challenges and found that I cannot solely depend on GPS. I have also experienced people who have very different personalities from mine. These experiences have sharpened my communication skills. I also learned that the Chief Learning and Accreditation Officer, Melissa Weeks, and the commissioner would cover my back when I needed them.

"The journey has been very rewarding and has positioned me to view our daily routines in the lab from a unique perspective."

I moved to Albany New York and joined Dr. Amy Hahn's lab in 2021. I was assigned to a lab in New York City for inspection in January of this year. As the inspection date was getting closer, the weather was getting worse. The broadcast was saying there would be a snowstorm and expected more than 10 inches of snow. I was getting nervous, so I emailed Melissa, my commissioner, the lab director, and my trainee to let them know the situation. Melissa was very confident and believed that it would not be that bad. She called me and asked me, "Do you want to cancel it?" I answered, "No, but what if it gets so bad that I'm unable to drive?" She assured me that the weather would be okay based on all her experiences with weather situations in New York. It turned out that she was right, and it was my best inspection experience.

If you are a technologist and have interest in becoming an ASHI certified inspector, I encourage you to move forward. You will not regret the decision, and you will find the experience rewarding.

COMMITTEE REPORT

Communication, Engagement, and Marketing Committee (CEMC) Update

"Real Talk" A Real Language Summary of the ASHI 2021 Annual Meeting

An update from the CEMC: Kelley Hitchman, Carey Killion, and Anne Halpin

From its inception, the ASHI Communications, Engagement, and Marketing Committee (CEMC) has had a goal to engage more directly with transplant patients, donors, and caregivers. In September of 2020, Dr. Kelley Hitchman managed to invite themselves to an American Society of Transplantation Transplant Community Advisory Council (AST TCAC) call. A special thank you is extended to Dr. Howie Gebel, an AST Board member who assisted with that ASHI invasion to the TCAC. The goal was to find a patient partner who would be willing to join our newly formed CEMC team. Amy Silverstein put her hand up, and the ASHI had found our first patient partner. We didn't know it at the time, but we had really hit the jackpot. Amy's voice is honest, real, and enlightening. We have been slowly expanding our number of ASHI patient partners (more on that to follow, so stay tuned!).

Patient partnership is relatively new to ASHI, and we have much to learn. One of our goals is to better explain what we do to patients, donors, and families. The 2021 ASHI annual meeting program planning committee was willing to help! Our 2021 ASHI past-president and the 2021 program planning chair, Dr. Medhat Askar agreed that we would ask speakers to provide lay, in other words, "real life," descriptions of their talks and research. Carey Killion, Anne Halpin, and Kelley Hitchman are working on a summary of all these descriptions that will be posted on the ASHI website on our patient resources page.

One thing that we have learned so far is that many transplant patients do not know much about the work that is done by histocompatibility and immunogenetics clinical laboratories. By sharing more digestible summaries of the topics of our meetings, we aim to create awareness of our field and to provide information for patients, donors, and families who seek information related to transplantation.

We are very grateful to every ASHI 2021 program planning committee member as well as each speaker who assisted by submitting a description of their talk for us to summarize. This will be a hefty document, but there will be a few content teasers. We will also include a list of commonly used histocompatibility and immunogenetics acronyms as, like all specialists, we love to speak in code language. Stay tuned for the full meeting summary. We will let you know when it is posted on our patient page and shared on our ASHI social media accounts.

Immune Memory

This all-day pre-meeting session focused on how our immune system "remembers" things such as the virus that causes COVID-19 infections, vaccines, or transplanted organs and tissues. We reviewed not only antibody responses to SARS-CoV-2 but also how different immune cells respond to the virus. It is possible that if our immune cells have seen something before that looks a bit like a new virus, they can help us respond faster due to memory of this previous encounter. Our individual Human Leukocyte Antigens (HLA) markers may determine how we respond to infections. We also discussed that, because of immunosuppressive medication taken to prevent organ rejection, transplant recipients don't respond as well to vaccines as a person who is not taking these medications. We learned about the studies that are taking place in transplanted people to track their vaccine responses and ways that we can assist their immune system to better respond to COVID vaccines. Measuring antibodies to the vaccine target is one way to determine how well a vaccine worked, but there are many different ways to measure COVID-19 immune responses.

Transplanted patients have a harder time recovering from COVID-19 infections, and we discussed how different organ transplant groups handled this virus.

But we didn't *just* talk about COVID! We also covered topics such as how we might train the immune system by loading it with tiny

targets that could deliver drugs or messages to our immune cells. We also discussed ways we might be able to better study immune responses in the hard-to-see, tucked away areas of our immune systems where many important steps of the immune response occur. It is much easier to look at immune cells in the blood, but they only tell part of the story. Inflammation can be part of the immune memory response, and in transplantation we would like to reduce inflammation to try to decrease the risk of rejection. Last but not least, we discussed how immunological memory may affect transplanted organs and cells and discussed new ways that we might be able to measure this memory so that we know how to avoid rejection or see it coming.

Breaking Barriers to Patient Engagement

Patient, donor, and family engagement and partnership are important parts of the Canadian Donation and Transplantation Research Program. We learned about how this organization works with patients, donors, and caregivers to prioritize research projects as well as to plan, conduct, and summarize transplant research. We heard from a heart-transplant patient who candidly described barriers and successes in her transplant journey. (We can't refrain from bragging — this was our very own Amy Silverstein.) We also discussed some of the racism barriers that exist in stem cell (bone marrow) transplantation and how addressing these barriers can lead to more donors being available when patients need them.

New Frontiers in Histocompatibility Testing

We have immune cells in our bodies called "Natural Killer Cells." These cells could potentially be used and trained to kill cancer cells without a lot of accidental collateral healthy cell damage. Another new area of transplantation is the transplant of hands. Hand transplants improve the quality of life of the patients, and we discussed immune responses following these life-changing operations. Another exciting new frontier is 3D printing of organs and tissues. A major barrier in this field is the creation of blood vessels, and we were treated to presentations that talked about how this is being done in the laboratory.

We are looking forward to sharing more of these summaries from the ASHI 2021 meeting!

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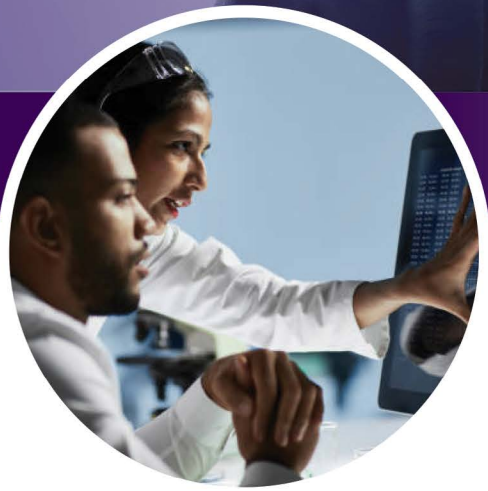

CLICK HERE to find out what your peers experienced by adapting to change.

Change can be intimidating, but without change there is limited growth. Differences in MFI values between vendors can be challenging to explain, but they should not be seen as a determent. There are many ways to help overcome MFI differences between vendors and with the right partner and tools you can overcome this barrier. As Darwin said, “It is not the strongest that survive, nor the most intelligent, but the ones that are most adaptable to change.”

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