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REVIEW

Applying genomics in heart transplantation

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SUMMARY

While advances in patient care and immunosuppressive pharmacotherapies have increased the lifespan of heart allograft recipients, there are still significant comorbidities post-transplantation and 5-year survival rates are still significant, at approximately 70%. The last decade has seen massive strides in genomics and other omics fields, including transcriptomics, with many of these advances now starting to impact heart transplant clinical care. This review summarizes a number of the key advances in genomics which are relevant for heart transplant outcomes, and we highlight the translational potential that such knowledge may bring to patient care within the next decade.

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Introduction

Heart transplantation is often the only available treatment for patients with significant congenital cardiac disease and/or end-stage heart failure [1,2]. Advances in immunosuppressive therapies (IST), surgical techniques, and preoperative and postoperative patient management have yielded substantial gains in short- and long-term post-transplant outcomes over the last few decades [2]. Despite these advances, the 5-year heart allograft survival rates are ~71%, because of an interplay of immune related as well as nonimmune comorbidities including hypertension, diabetes, dyslipidemia, a range of coronary diseases, infection, renal insufficiency, and malignancies [3]. Acute rejection (AR) is most likely to occur in the first three to twelve months post-transplant, with at least one rejection episode occurring in upwards of 50% of cardiac transplant recipients. AR remains a frequent and life-threatening complication increasing the risk of acute and downstream graft damage [1,4] and greatly impacts the progression of chronic allograft vasculopathy (CAV), which is the leading cause of diminished cardiac allograft survival [1]. Acute and chronic rejection pathogeneses are complex, affected by many established factors such as human leukocyte antigen (HLA) mismatches, immunosuppression regimens, compliance, and recipient age [5].

Patients who receive renal allograft from HLA-identical donors can undergo acute or chronic rejection, indicating a role for non-HLA factors in alloimmunity [6].

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Large-scale retrospective analyses of 10-year national registry data show that ~18% of allograft failures are attributable to donor–recipient (D-R) *HLA* genetic factors, with 38% of the failures reported to be caused by immunological reactions against non-HLA factors (as observed in HLA-identical sibling grafts) [7]. There is a clear lack of knowledge of the genetic underpinnings of allograft rejection and other complications of transplantation, and understanding these processes may advance clinical management of individual heart transplant recipients and impact short- and long-term allograft survival. While assessment of HLA compatibility for heart transplant donor–recipient (D-R) selection is an important factor for graft survival outcomes, it is not always predicated in all transplant centers, because of limited pools of available organs and time constraints with recovery of thoracic organs from deceased donors, but it is becoming increasingly implemented and standardized across national programs [8].

The last decade has seen staggering progress in genomic and other omic technologies, and their application in the Mendelian and complex disease arena. Since the initial draft sequences of the first human genomes nearly 15 years ago, genetic single nucleotide polymorphism (SNP) maps have been generated across the major human populations using genome-wide genotyping panels of typically >500 000 to several million SNP markers. This has facilitated the advent of genome-wide association studies (GWAS) which have led to the elucidation of robust genetic associations for the vast majority of polygenic traits and diseases with heritable components [9]. Subsequent advances in sequencing chemistries and advanced engineering allowed the capture and sequencing of the known gene-coding regions, termed whole-exome sequencing (WES) as well as whole-genome sequencing (WGS). These processes have become more affordable in the last 2-3 years, and sequencing of thousands of large reference populations has facilitated characterization of common and rarer genetic variants [10], leading to the broad observation that two unrelated human genomes differ by ~3.5 million to 10 million polymorphisms, depending on their respective ancestral backgrounds. WGS of human populations also shows that an average genome contains ~100 genuine loss-of-function (LoF) variants (defined as variants ablating all of part of a gene product/function), with ~20 genes having LoFs in both copies [11]. Two copy LoFs may cause graft rejection through the LoF gene product in the donor being treated as an allogenic epitope [12-16]. Additional sources of neo-antigens include stop-loss mutations where the conventional stop codon is disrupted resulting in novel amino acids being synthesized. Indeed, recent WES in >15 500 human protein-coding genes in >2 000 individuals of diverse ancestry identified more than 500 000 variants < 1% frequency with an average of >13 500 low-frequency variants observed per individual, of which ~2% was predicted to impact the function of > 300 genes, per genome assessed [11,17]. While it is clear that a broad spectrum of genetic differences could represent significant reservoirs of potential mHA differences which could potentially contribute to allogenicity and thus acute rejection pathology, there have been limited efforts to date to look at the global mismatches of amino acids between donors and recipients.

Studies of genetic polymorphisms in association studies using large well-characterized heart transplant cohorts are currently lacking. We also discuss how advances in genome-wide tools can be used to unveil sources of potential alloimmunity in, and beyond, conventional HLA regions, and we outline a number of key genomic studies in pharmacological genes (pharmacogenes) relevant in the transplant setting and where such knowledge may begin to be implemented in broader clinical care in pre- and postcardiac transplant clinical management. We discuss a recently formed transplant genomic consortium whose aims are to discover and validate genome-wide associations for a number of complications post-transplant. We also outline recent advances in the development of molecular characterization of allograft biopsies, as well as noninvasive or minimally invasive biofluids, for diagnoses and prognostication of acute rejection. This is particularly relevant in the post-transplant cardiac allograft setting where standard-of-care in many post-transplant clinics necessitates frequent highly invasive protocol biopsies to assess rejection at a histopathology level.

Genetics and genome-wide studies in heart transplantation

The human major histocompatibility complex (MHC) and natural killer cell immunoglobulin-like receptor (KIR) regions

The Human *MHC* region, located on the short arm of chromosome 6, comprising ~200 coding genes including the *HLA Class I* (*HLA-A*,*-B,* and-*C*), *II* (*HLA-DPA1*,-*DPB1*,-*DQA1*,-*DQB1*,-*DRA,* and *-DRB1*), and *III* gene families, with *HLA Class I* and *II* exons being the most polymorphic regions observed across the human genome. The *MHC* region consistently shows the strongest

associations for a wide range of diseases and phenotypes [9], and associations of HLA polymorphisms with transplant outcomes are well-established [18–21]. *HLA Class I/II* molecules are key proteins responsible for the presentation of endogenously and exogenously derived peptides to T cells.

HLA Class I and *Class II* matching is well established in graft outcomes in renal and hematopoietic cell transplantation (HCT). While there is a clear role for HLA compatibility in transplantation outcomes of other solid organs, currently, the use of HLA matching is not performed in all heart transplant regions, because oflimited availability of organs and time constraints with HLA typing and recovery of organs. *De novo* antibody production has a clear impact on cardiac allograft recipient survival (Hazard Ratio > 3), with HLA Class II DQ-specific donor-specific antibody (DSA) being observed with poorer outcomes [22,23]. There is also an increasing body of evidence that nonclassical HLA molecules, such as HLA-G, also impact transplant outcomes [24,25,26]. There is a major need for more comprehensive catalogs of polymorphisms across the *HLA Class I*, *II,* and wider *MHC* regions such as the Immuno Polymorphism Database (IPD) which contains a wealth of HLA alleles [27], although there is still a limited amount of HLA datasets from non-EA populations. There is an ever-growing body of clinical data showing that epitope-based HLA matching is superior to conventional HLA antigen matching for a range of post-transplant clinical outcomes, and it is likely that these approaches will become a major consideration in clinical D-R matching in the next few years [28,29].

The *KIR* region, the second most polymorphic region in the genome after the *MHC*, is comprised of a family of 13 genes on chromosome 19 [30]. KIRs play essential roles in educating and regulating the ability of NK cells to sense and respond to *HLA Class I* surface expression and have been shown to have critical roles in human health and are implicated in multiple immune-related diseases [31–33], and clinical studies show associations of combinatorial diversity of *KIR* and *HLA* alleles with multiple diseases, including infections and autoimmune disorders [18,20,21]. *KIR/HLA Class I* incompatibility exemplifies how interactions may negatively impact histocompatibility and while the impact of *KIR-HLA* mismatch in transplantation is controversial [21,34], recent studies do show evidence of *KIR* genotype associations with kidney and HCT transplant-related outcomes [35–37], although no well-powered studies to date have been performed in heart transplantation. There is some recent intriguing data that indicate that surveillance of *CD28* and *KIR2D* receptor expression on T lymphocytes correlate with immune status of both heart and liver recipients [38]. As *KIR* and *HLA Class I* genes are located on different chromosomes, the statistical power to assess potential SNP–SNP interactions becomes very constrained. Where HLA and KIR have not been directly sequenced, it is possible to infer or "impute" HLA and KIR amino acid status from GWAS, WES, and WGS datasets using a number of different open-source algorithms [39–42]. Such approaches may add significant insight into additional HLA/KIR associations with transplant outcome particularly where samples were not typed at high resolution or at all, which is the case for most liver transplant centers.

Genetic association studies in transplantation across the rest of the human genome

It is also becoming increasingly evident that non-HLA variants, often termed minor histocompatibility antigen (mHA), impact rejection risk in transplantation [6,7,43,44]. In females receiving a male kidney allografts, worst survival outcomes were observed versus all other gender–gender D-R combinations [45]. This has been attributed in part to the H-Y antigen, against the Y-chromosome male-enhanced antigen *MEA1* gene which has been associated with acute renal rejection [46]. D-R genetic differences in mHA across the entire human genome have yet to be investigated at large-scale in the solid organ transplant setting let alone in the cardiac transplant setting.

Association studies of polymorphisms in *a priori* candidate genes are notorious for publication bias and spurious and inconsistent results, and there are often confounding issues across sites such as adjustment for ancestry of the study participants [47]. The majority of genetic studies in transplantation outcomes published to date have mostly been limited to *a priori* candidate gene regions, suffer from small study sample sizes, and lack of replication in independent studies. The clinical and demographic covariates of recipient and donors in transplantation are extremely complex relative to most common genetic disease studies, and it is thus not surprising that apart from pharmacogenetic/genomic studies with large effect sizes in genes known to impact that replication of initial findings is limited.

To date, only a handful of solid organ transplant GWA studies have been performed in modest numbers of patients and have focused mostly on renal transplantation (reviewed in an accompanying review article in this edition [48]) with very few significant findings. The

only GWA study using heart transplant subjects was performed in relation to skin cancer outcomes [49]. There have been several dozen candidate genes studies in the heart transplant field, but only two studies used over 500 DNA samples. Four beta-adrenergic receptor (βAR) polymorphisms were screened by Khush *et al* in donor hearts to assess left ventricular (LV) dysfunction after brain death in 1 043 heart transplant donors from 2001 to 2006 [50]. The β2AR-46 SNP was significantly associated with LV systolic dysfunction in multivariable regression analyses, with carriage of the less common variants significantly impacting LV ejection fraction. The β1AR1165 and β2AR46 SNPs were associated with increased inotropic dopamine requirement during procurement of the allograft (OR of 2.6 for requiring >10 μg/kg/min of dopamine compared to those with the homozygous wild-type genotypes). Gallardo and colleagues examined the impact of common mitochondrial variants and contiguous stretches of variants or "haplotypes," on end-stage heart failure in patients undergoing heart transplantation, in relation to CAV and graft survival, in 450 recipients, 248 donors, and 206 healthy controls [51]. Carriage of mitochondrial haplogroup H was significantly higher in recipients versus controls [OR: 1.86 (95% CI: 1.27-2.74), $P = 0.014$, and in recipients versus donors (OR: 1.47 [95% CI: 0.99-2.19), $P = 0.032$]) after adjustment for age and sex. In CAV patients versus non-CAV patients, the haplogroup Uk was observed to be significantly more frequent (OR: 4.1 [95% CI: 1.51-11.42], $P = 0.042$). Additionally, haplogroups in the heart donor were observed to have no impact on the morbidity or graft survival after heart transplantation.

There is an ever-growing catalog of specific variants that impact drug uptake, metabolism, clearance, efficacy, and severe adverse events [52,53]. Large consortia, such as the Clinical Pharmacogenetics Implementation Consortium (CPIC) [54] and the Pharmacogenomics Research Network (PGRN) [53], are making significant advances in discovery and systematic documentation of a number of these key pharmacogenes and specific polymorphisms of major clinical value. Transplant patients are exposed to large number of pharmacotherapies over extensive periods of time including, immunosuppressants, inotropic, anti-hypertensive, and dyslipidemia agents as well as anti-fungal, anti-viral and antibiotic treatments, and chemotherapies. Table 1 outlines the most commonly prescribed drugs pre- and postcardiac transplant, and known genes and variants which impact patient responses to these drugs. Table 1 also outlines the current CPIC and PGRN guidelines along with the current FDA recommendation for patient monitoring/testing for these drugs. There has been much focus to date on the pharmacogenetics/pharmacogenomics of tacrolimus, the most commonly used immunosuppressant, as there is high interindividual variability in dosing required to reach, and to maintain, optimal therapeutic trough levels [55]. The narrow therapeutic range requires close monitoring of plasma drug concentrations especially during the initial period post-transplant. Trying to balance avoidance of overimmunosuppression, which can lead to nephrotoxicity and increased risks of opportunistic infections, against undersuppression of the allograft recipient, which can lead to increased risk of acute rejection, can be challenging in many patients [56]. While gender, age, BMI, type 2 diabetes status and exposure to calcium channel blockers influence tacrolimus blood levels and clearance, an intronic LoF variant *CYP3A5**3 (rs776746), in cytochrome P450 3A5 *(CYP3A5)*, the main enzyme that metabolized tacrolimus, explains ~45% of drug level and 30% of clearance. *CYP3A5**1 classically referred to a functional gene copy of *CYP3A5*, but without ascertainment of all variants in the gene-coding regions, as well as in the intronic and untranslated regions, then a "*CYP3A5**1 functional gene status" cannot truly be derived. Additional variants, including *CYP3A5 *2,* *5, *6, *7, *10, and *CYP3A4* 22*, cause LoF or reduced expression of these key enzymes and have been found to explain an additional 20% of the genetic variance in tacrolimus blood levels. The allele frequency of *CYP3A5**3 is ~82–95% in European ancestral populations and ranges from 33% in African to 75-85% in Asian and 75% in Mexican populations [57]. Higher carriage of LoF SNPs in *CYP3A5*, as observed in European, Hispanic, and Asian populations, invariably requires that less tacrolimus dosing be administered to reach and maintain optimal trough level, and indeed, less nephrotoxicity and side effects are observed because of lower cumulative tacrolimus exposure. African Americans are known to have higher rates of rejection following kidney transplantation, which may be caused in part from failure to reach therapeutic immunosuppression dosing of tacrolimus [58,59].

Ancestry has also been shown to play an important role in heart transplantation. A retrospective analysis of over 20 000 adult heart allograft recipients transplanted from 1997 through 2007 assessed the impact of D-R race-matching, on mortality using 23 variables and D-R interaction terms. African Americans recipients were shown to have an 11.4% absolute decrease in 10-year survival and a 46% proportional increase in the risk of cumulative mortality (HR, 1.46; 95% CI, 1.24 to 1.72;

Table 1. Commonly used drugs in transplantation and their pharmacogenes of importance.

Drug class(es)	Drug	Gene(s)	FDA Label for PGx testing	PharmGKB level of evidence	CPIC level (s)	CPIC publications (PMID)
Immunosuppression	Azathioprine	*TPMT, NUDT15*	Testing recommended (TPMT)	1A, 1B	A, A/B	21270794;23422873 (TPMT)
	Cyclophosphamide	*TP53, SOD2, GSTP1, MTHFR*		2B, 2B, 2A, 2A	D, D, C/D, C	
	Cyclosporine	*CYP3A4/CYP3A5*		2B	C	
	Mercaptopurine	*TPMT, NUDT15*	Testing recommended (TPMT)	1A, 1B	A, A/B	21270794;23422873 (TPMT)
	Methotrexate	*MTRR, ATIC, ABCB1, SLCO1B1, MTHFR*		2B, 2B, 2A, 2A, 2A	D, D, C/D, C, C	
	Mycophenolic acid	*HPRT1*	Actionable PGx		B	
	Sirolimus	*CYP3A5*		2A	C	
	Tacrolimus	*CYP3A4/CYP3A5*		2A, 1A	C, A	25801146 (CYP3A5)
Statins	Atorvastatin	*LDLR, KIF6, COQ2, APOE*	Actionable PGx (LDLR)	-, 2B, 2B, 2A	D, D, D, C	
	Cerivastatin	*SLCO1B1*		2A	B	
	Pravastatin	*KIF6, SLCO1B1*		2B, 2A	D, C	
	Simvastatin	*ABCB1, SLCO1B1*		2A, 1A	C/D, A	22617227;24918167 (SLCO1B1)
Anti-Diabetic	Metformin	*SLC47A2, C11orf65*		2B, 2B	D, D	
Anti-arrhythmic	Propafenone	*CYP2D6*	Actionable PGx	2A	C	
Anti-hypertensive	Ace Inhibitors	*KCNIP4, ACE*		2A	D	
	Digoxin	*ABCB1*		2A	C/D	
Anti-hypertensive/ Beta blocker	Propranolol	*CYP2D6*	Informative PGx	4	C	
Diuretic	Spironolactone	*ADD1*		2B	D	
Blood thinner	Acenocoumarol	*CYP4F2, CYP2C9*		2B, 2A	B, B	
	Aspirin	*PTGS1, LTC4S, GP1BA, HLA-DPB1*		2B, 2B, 2B, 2B	D, D, D, C	
	Clopidogrel	*CYP2C19, CES1*	Actionable PGx (CYP2C19)	1A, 2B	A, C/D	21716271;23698643 (CYP2C19)
	Phenprocoumon	*CYP4F2*		2A	B	
	Warfarin	*PROS1, PROC, VKORC1, CYP2C9, GGCX, CALU, CYP4F2*	Actionable PGx (PROS1, PROC, VKORC1, CYP2C9)	-, -, 1A, 1A, 2B, 2B, 1B, 2B	D, D, A, A, D, D, A	21900891;28198005 (VKORC1, CYP2C9, CYP4F2)
Osteoporosis	Bisphosphonates	*FDPS*		2B	D	
Antibiotic	Nalidixic acid	*G6PD*	Actionable PGx		B	
	Nitrofurantoin	*G6PD*	Actionable PGx	3	B	
	Norfloxacin	*G6PD*	Actionable PGx		B	
	Sulfadiazine	*G6PD*	Actionable PGx		B	

Table 1. Continued.

Drug class(es)	Drug	Gene(s)	FDA Label for PGx testing	PharmGKB level of evidence	CPIC level (s)	CPIC publications (PMID)
Anti-Viral	Abacavir	*HLA-B*	Testing required	1A	A	22378157;24561393
	Atazanavir	*CYP3A5, UGT1A1*		2B, 1A	C, A	26417955 (UGT1A1)
	Interferon alfa-2b	*ITPA*		2B	C/D	
	Peginterferon alfa-2a	*IFNL4, IFNL3*		1A, 1A	D, A	24096968 (IFNL4)
	Peginterferon alfa-2b	*IFNL4, IFNL3, VDR*	Actionable PGx (IFNL3)	1A, 1A, 2A	D, A, D	
	Ribavirin	*VDR, IFNL3*	24096968 (IFNL3)	2A, 1A	D, A	24096968 (IFNL3)
	Tenofovir	*ABCC4*		2B	D	
Chemotherapy (skin cancer)	Fluorouracil	*DPYD, UMPS, TYMS, NQO1, GSTP1, MTHFR*	Actionable PGx (DPYD)	1A, 2B, 2A, 2A, 2A, 3	A, D, D, D, C/D, C	23988873 (DPYD)
Chemotherapy (other)	Anthracycline	*CBR3, SLC28A3, NQO1, HAS3*		2B, 2B, 2A, 2B	D, D, D, D	
	Belinostat	*UGT1A1*	Actionable PGx	3	B	
	Cisplatin	*TP53, TMEM43, GSTM1, COMT, XPC*		2B, -, 2B, 3, 1B	D, D, D, C/D, D	
	Irinotecan	*UGT1A1, SEMA3C, C8orf34*	Actionable PGx (UGT1A1)	2A, 2B, 2B	A, D, D	
	Nilotinib	*UGT1A1*	Actionable PGx	3	C	
	Sunitinib	*ABCB1*		2B	C/D	

The major classes of drugs prescribed to patients post-transplantation are shown along with specific drugs and the genes of known interaction.

For the Pharmacogenomics Knowledgebase (PharmGKB), the following describes the different degrees of evidence: Level 1A: clinical annotation for a variant–drug combination in a Clinical Pharmacogenetics Implementation Consortium (CPIC) or medical society-endorsed PGx guideline, or implemented at a PGRN site or in another major health system; Level 1B: clinical annotation for a variant–drug combination where the preponderance of evidence shows an association. The association must be replicated in >1 cohort with significant *P* -values, and preferably with a strong effect size; Level 2A: clinical annotation for a variant–drug combination that qualifies for level 2B where the variant is within a Very Important Pharmacogene (VIP) as defined by PharmGKB. Variants in level 2A are in known pharmacogenes, so functional significance is more likely. Level 2B: clinical annotation for a variant–drug combination with moderate evidence of an association. The association must be replicated, but there may be smaller studies where statistical significance is not shown, and/or the effect size may be small; Level 3: annotation for a variant–drug combination based on a single significant (but not yet replicated), or annotation for a variant–drug combination evaluated in multiple studies but lacking clear evidence of an association; Level 4: annotation based on a case report, nonsignificant study or *in vitro*, molecular or functional assay evidence only.

For the CPIC Grading: Level 1 indicates that the evidence includes consistent results from well-designed and well-conducted studies; Level 2 indicates that the evidence is sufficient to determine the effects, but the strength of the evidence is limited by the number, quality, or consistency of the individual studies, by the inability to generalize to routine practice or by the indirect nature of the evidence; Level 3: the evidence is insufficient to assess the effects on health outcomes because of the limited number of studies, insufficient power of the studies, or important flaws in the study design or in the way in which they were conducted. A three-tier rating scheme is used for evaluating the strength of the recommendation where: A is a strong recommendation for the statement, B is a moderate recommendation for the statement, and where C is an optional recommendation for the statement.

$P < 0.001$) versus European recipients [60]. Decreased survival in recipients from African American allograft donors or any other racial groups was not observed to be improved with race-matched transplantation in this study. African Americans have been shown to exhibit poorer transplant outcomes versus those of European ancestry, even after adjusting for clinical and socioeconomic covariates [61]. Using United Network for Organ Sharing (UNOS) registry data for 14 265 heart transplant patients, a 13-point risk score incorporating age, race, sex, HLA matching showed high predictive ability for clinically important rejection episodes within 1 year [62]. Race was observed to impact one-year rejection rates; when excluding individuals of European ancestry, individuals of non-European ancestry had comparable rejection rates, with the exception of cardiac allograft recipients of Asian descent, who had reduced rates of rejection.

The international genetics & translational research in transplantation network (iGeneTRAiN)

Large well-characterized numbers of genome-wide datasets are needed for D-R pairs or for recipient-only samples, to accrue sufficient numbers of transplant-related phenotypes/events [63]. This was one of the main considerations for establishing iGeneTRAiN, whose initial aims are to generate and harmonize genome-wide genotyping and phenotypic datasets across transethnic heart, kidney, liver, and lung transplant studies, and integrating analyses and risk models to increased statistical power to detect transplant-related outcomes ([63] and www.igenetrain.org).

iGeneTRAiN has now aggregated GWAS and phenotypic datasets from >48 000 DNAs from transplant subjects and controls (with >12 000 D-R pairs), collected from 1989 to present, including >1 800 heart transplant recipients and >1 000 of their respective deceased donors [49,63–67]. A dedicated GWAS array, the "TxArray," with 780 000 markers, designed for the transplant community by iGeneTRAiN, provides robust genome-wide coverage using conventional genome-wide mapping content, but with dense coverage of variants in key transplant-related regions, such as *MHC*, *KIR* and is enriched for recent pharmacogenomic and CKD related-findings [27]. Furthermore, a deep collation of all published cardiac allograft genetic association studies (and all other solid organs) up to 2015 was performed, and probes for these genetic variants were directly captured on the array to allow for meta-analyses with previous publications. A dedicated pipeline for quality control and processing of the GWAS data has been developed (see Fig. 1) for the transplant community. A number of clinically relevant transplant outcomes, including graft and patient survival, acute and chronic rejection, new-onset of diabetes after transplant (NODAT), cause of transplant, and various malignancies, are being investigated using recipient-only, donor-only, and various D-R models.

Diagnostics & prognostication biomarker studies of post-transplant complications

Most transplant centers currently diagnose cardiac allograft rejection through histological evaluation of endomyocardial biopsy (EMB) from surveillance standard-of-care visits or from "for-cause" biopsies after the onset of clinically observed allograft dysfunction. EMBs are costly, highly invasive and are subject to interobserver variability and sampling errors at the histopathology level [68]. Furthermore, surveillance biopsies may detect allograft rejection after irreversible damage has already occurred. Early identification of biological markers of subclinical allograft rejection and/or injury using highly sensitive and specific assays may allow more timely intervention to preserve graft function and thus increase allograft lifespan. The development of diagnostic and prognostic biomarkers of allograft dysfunction has been a major endeavor of many groups over the last two decades, with most of the focus being on the transcriptome (mRNA and miRNA studies) and donor-derived cell-free DNA (dd-cfDNA) [69].

Messenger RNA (mRNA) studies

The Cardiac Allograft Rejection Gene Expression Observational (CARGO) study, which began in 2001 [70], collected blood and EMBs from the corresponding timepoints of heart allograft recipients across eight sites and identified altered expression of 11 genes which discriminates acute cellular rejection (ACR) from immunologically quiescence timepoints. The CARGO investigators also developed an expression-based algorithm with a score from 0 to 40, where higher scores (34 or higher) are indicative of an acute rejection episode. This assay was developed into an FDA approved *in vitro* diagnostic (IVD) and is available for clinical use in stable heart allograft recipients. A number of additional studies including the Invasive Monitoring Attenuation through Gene Expression (IMAGE) Study compared AlloMap to protocol EMB as the primary means of ACR surveillance assessing primary outcomes

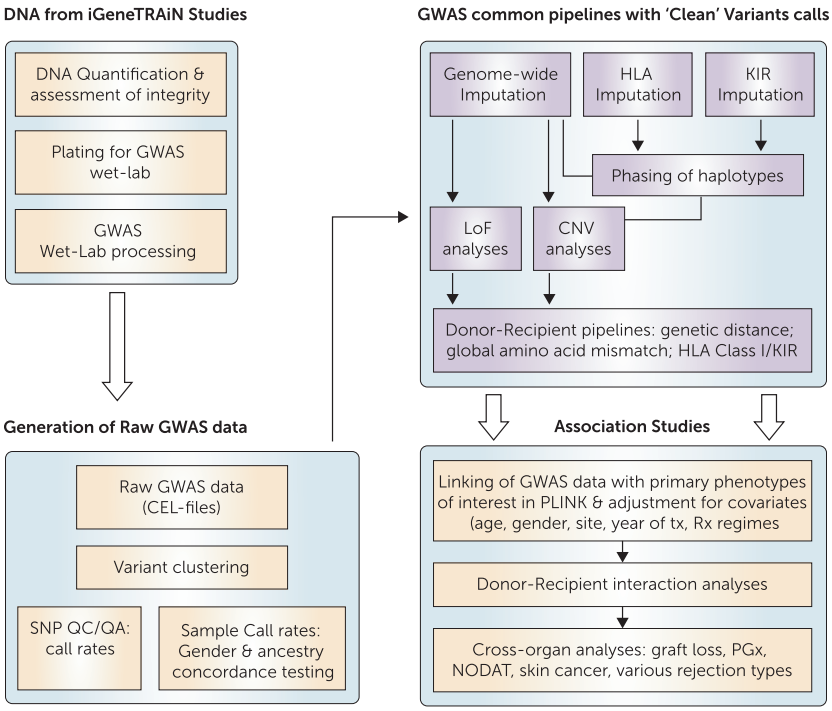

Figure 1 iGeneTRAiN Genome-wide association study analyses (GWAS) pipelines. The genome-wide association study analyses (GWAS) for The International Genetics & Translational Research in Transplantation Network (iGeneTRAiN) is illustrated from assessment of the DNA quality for the different studies, through to the wet-laboratory processing of the genome-wide genotyping plates to generate several hundreds of thousands of SNP/SNV genotype calls. The pipelines for genome-wide imputation (IMPUTE2 and ShapeIT) HLA (SNP2HLA, HLA*IMP) and KIR (KIR*IMP) are generated and phased. The loss-of-function (LoF) pipeline using VEP and LOFTEE utilizes the phased imputed GWAS data (typically 15 million variants) and copy number variant (CNV) is generated from the raw image files using standard Affymetrix pipelines or PennCNV [98]. The donor–recipient interaction analyses utilized the imputed LoF and CNV datasets, using ancestry data derived from the GWAS data and other means including genome-wide amino acid mismatches. Finally, the phenotypes and covariates of interest are integrated with the various GWAS-derived datasets primarily using PLINK [99].

of first rejection episodes with hemodynamic compromise allograft dysfunction, retransplantation, or death [71]. The IMAGE study observed similar outcomes in the AlloMap-alone versus EMB groups for primary outcome incidences (14.5% vs. 15.3%). The CARGO II study found that the negative predictive value (NPV) for a graft failure, retransplantation, or death was 97% where patients had an AlloMap score variability (AMV) of 0.6 (defined as the standard deviation of four AlloMap scores collected at least 315 days post-transplantation with a 95% CI of 91.4–100) [72]. As of the middle of 2017, over 100 000 blood samples from heart transplant recipients had been subjected to AlloMap assays.

A recent cardiac allograft rejection mRNA diagnostic study examined EMBs from four French transplant centers for antibody-mediated rejection (AbMR) ($n = 55$) with a control group of 55 biopsies without AbMR, and a Canadian validation cohort of 27 AbMR cases and 71 non-AbMR controls using ISHLT 2013 histopathology grading [73]. Genome-wide expression microarrays were used to molecularly characterize the entire 240 biopsies and demonstrated molecular pathways within the AbMR samples characterized by endothelial activation with microcirculatory inflammation from monocytes–macrophages and NK cells. They also showed changes in endothelial, angiogenesis, and NK cell mRNA expression profiles, including *CD16A* signaling and mRNAs, influenced by interferon-γ. Panels of AbMR-related transcripts demonstrated decent discrimination for AbMR biopsies versus non-AbMR: NK-related (AUC = 0.87), endothelial activation-related (AUC = 0.80), macrophage-related (AUC = 0.86), and interferon-γ-related (AUC = 0.84) (with $P < 0.0001$ for all four sets). These four gene panels showed increased expression with increasing ISHLT grading of AbMR pathology ($P < 0.001$) and association with DSA levels. These samples are part of a major international effort called *Molecular Microscope Diagnostic System* (MMDx) examining AbMR and T-cell mediated rejection (TCMR) and other post-transplant compilations across a range of solid organ allograft biopsies. These are, and will undoubtedly continue to be, a significant reference resource for characterization of subtypes of

rejection as well as other complications of rejection such as CAV. Indeed, subsets of the expression classifiers of AbMR in the study showed association with CAV [73]. Array-based expression platforms have a number of limitations compared to more recent methods which include sequencing RNA transcripts (RNA-Seq). When comparing human T-cell activation using RNA-Seq against microarray-based-expression, RNA-Seq demonstrates superiority in dynamic range, as well as for detection of low abundance transcripts, and differentially expressed mRNA isoforms [74].

MicroRNA (miRNA) studies

Noncoding RNAs include microRNAs (miRNAs), which are typically 22 nucleotides in length, are potent regulators of transcriptional and post-transcriptional gene expression. They have been shown to disperse into the periphery circulatory system from cells within solid organs, and their small sizes make them less susceptible to RNase enzymatic degradation. Furthermore, they are generally stable in blood at room temperature for up to 48 hours, and thus, they are an attractive target to assess patterns of injury or recovery in disease processes [75]. In one of the most recent and largest cardiac allograft miRNA study conducted to date, EMBs from 113 heart transplant recipients from four French transplant sites (discovery component $n = 60$, validation cohort, $n = 53$) [76] were screened for miRNA levels. In the discovery arm, miRNA expression was compared between EMBs and sera from patients with acute biopsy-proven allograft rejection ($n = 30$) versus controls subjects without rejection ($n = 30$). Seven miRNAs were observed to be differentially expressed between allograft rejection timepoints versus nonrejection biopsies ($P < 0.0001$). Of these seven miRNAs, four were observed to be detectable and exhibited differential expression in sera. The ROC analyses showed that these four circulating miRNAs strongly discriminated allograft rejection versus those without rejection (all had AUC ranging from >0.93 to >0.99 with $P < 0.0001$), and these signals were confirmed with an additional replication set of cardiac allograft patient sample sets. Furthermore, the discrimination capability of the four miRNAs remained significant when stratified by TCMR versus AbMR diagnoses, and time post-transplant.

Cell-free circulating DNA (cfDNA) studies

Stemming from seminal noninvasive prenatal diagnoses (NIPDs) research which assesses fetal DNA in maternal blood, the cfDNA approaches in transplantation take advantage of donor-derived circulating cfDNA (dd-cfDNA) which has been shown to increase in ratio when compared with recipient DNA after necrosis/apoptosis of donor allograft cells/tissue [77]. Panels of several hundred SNPs across the genome, whose frequencies are high in the most common human populations, can be used to discriminate donor and recipient DNA ratios in the blood of kidney, lung, and heart recipients. The dd-cfDNA method was first successfully applied in heart transplantation in a retrospective study where increased levels of dd-cfDNA were shown to correlate with ACR episodes using EMB as the reference pathological standard [78]. The clinical utility of dd-cfDNA in monitoring acute rejection was subsequently tested in a prospective heart transplant recipient study [79]. dd-cfDNA was shown to be highly elevated from day 1 post-transplant (indicative of early ischemia–reperfusion injury postsurgery), followed by a quick decline to <0.1% within a week, and remained low until a rejection event. The performance of dd-cfDNA in distinguishing ISHLT Grade 2 or 3 rejections from immunological quiescence had an observed AUC of 0.83, with a sensitivity of 58% and a specificity of 93%. The authors also outlined the use of dd-cfDNA monitoring as a prognostic monitoring assay for rejection as levels of dd-cfDNA were observed to be significantly elevated weeks to months preceding a rejection episode. As dd-cfDNA can be assessed at defined periods post-transplant, in a minimally invasive manner, and as it is essentially a quantitative read-out of donor versus recipient cfDNA, then it can also be used in a prognostic manner to monitor heart allograft status. Cell-specific dd-cfDNA approaches, using methylation and/or histone mapping, to identify the cell(s) or tissue of origin of the cfDNA, are now emerging as powerful tools to delineate the underlying cause of increased dd-cfDNA [80,81].

Discussion and future directions

To date, genetic association studies in heart transplant studies have mostly been limited to the HLA and pharmacogenomic setting, although a number of large-scale GWA studies including iGeneTRAiN are now underway with GWAS from >1 800 heart allograft recipients and >1 000 donors. There are still significant challenges that have to be overcome though and greater numbers of samples are needed, as well as collaboration between sites for more comprehensive phenotype harmonization, as adjusting for clinical and demographic recipient and donor covariates across sites can be very challenging. A wealth of existing DNA already exists for donor and

recipient DNA samples from organ procurement organizations (OPOs) and HLA reference laboratories. With appropriate regulatory approval, these D-R genomic and outcome datasets can be linked with medical records (EMRs) and national-level databases such as the Scientific Registry of Transplant Recipients (SRTR), the most powerful registry in the United States, for assessment of long-term post-transplant outcomes [82]. Knowledge gained from how *MHC, KIR,* and mHA variants impact outcomes will facilitate greater insight into the potential biology of genomic incompatibility of D-R pairings, which may lead to better patient care through more regular monitoring of recipients paired with a higher genetic-risk donor. With the increased use of LVADs and rapid-HLA genotyping, more appropriate D-R matching prior to transplantation may be possible based on *MHC/HLA*, *KIR,* and mHA genotype combinations.

In the last decade, there have been significant advances in the development of molecular tools for the diagnoses and prognoses of acute and chronic rejection, as well as other complications post-transplant. While there are better international classifications of acute rejection, there are still significant issues with histopathology grading, and there is an increasingly clear case for molecular characterization of the EMB for diagnoses and prognostication of various outcomes. Furthermore, blood samples from the same timepoints will likely have value for immune surveillance in a minimally invasive manner as we move toward miRNA, mRNA, and dd-cfDNA laboratory developed tests (LDT) and IVD assays with better sensitivity and specificity [71,83–85]. Ultimately, with robust enough biomarkers, such approaches could lead to personalization of immunosuppression therapy to limit side effect, but great caution is needed in this area [86].

Comparing biomarker signatures across different solid organ allograft studies is also an area of major value as while there are clearly organ-specific signals in post-transplant outcomes, there is also biological overlap in a number of processes related to rejection and other post-transplant complications across all solid organ transplants. Levels of miR-21 were observed to be associated with AR, fibrosis, and CAV in heart recipients [76,87] but were also associated with ischemia–reperfusion injury, fibrosis AbMR, and other complications in kidney [88–91] and with graft dysfunction in lung allografts [92]. Furthermore, downstream miRNAs derived from miR-142 (miR-142-5p and miR-142-3p) are associated with AR, chronic rejection, and/or fibrosis across all four major solid organ transplantations [92-96]. Levels of miR-223p-3p and miR-93-5p were shown to be present in CKD stages [97], which may also have broad utility for routine monitoring of kidney function in cardiac and other allograft patients especially when combined with miRNAs that are known to have clinical utility in post-transplant surveillance. Furthermore, assessment of 10 genes expressed in blood that are diagnostic of kidney acute rejection was shown to have utility in a study of 250 blood samples from heart transplant recipients with and without acute rejection, indicating common pathways of immune activation [65].

Genomic and other omic applications will undoubtedly start to play a more significant role in the personalization of patient management in the heart transplant setting. Ascertaining the genetic underpinnings of various types of cardiac allograft rejection and complications post-transplantation will yield significant advances in our understanding of fundamental molecular processes involved in such processes. Identification of potential new genomic biomarkers for diagnoses and prognostication of post-transplant outcomes, as well as risk stratification of transplant patients, is also likely to result from such studies.

Conflict of interests

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