Gene expression on FFPE EMB for the diagnosis of rejection in Heart Transplantation

Bahman Afzali[1,2], Erin Chapman[1], Benjamin Adam[1], Fernando Gil[2], Daniel Kim[3], Luis Hidalgo[1], Patricia Campbell[1], Banu Sis[1], Michael Mengel[1]

1 Dept. of Laboratory Medicine & Pathology, University of Alberta

2 Dept. of Pathology, University Hospital, University Duisburg-Essen

3 Div. of Cardiology, University of Alberta

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Faculty / Presenter Disclosure

- Faculty: Michael Mengel

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  - Other: none

Disclosure of Commercial Support

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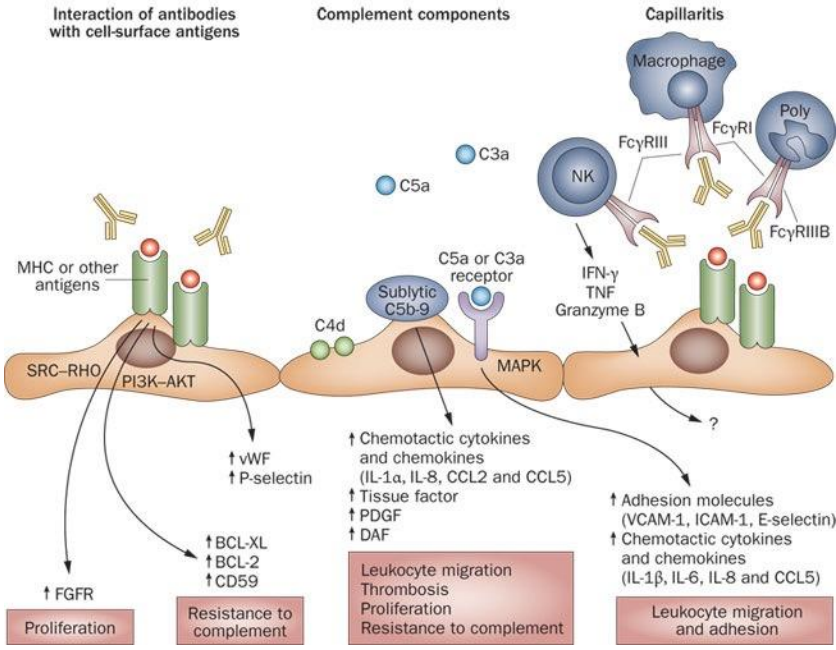
Diagnosis of Antibody-mediated Rejection

Clinical symptoms are vague, thus routine testing by means of endomyocardial biopsy are necessary

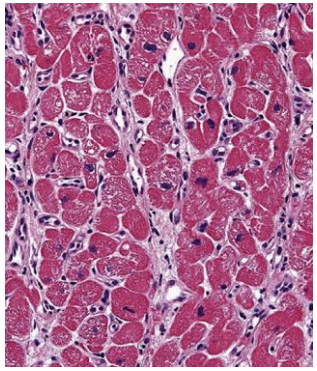
In contrast to acute cellular rejection (ACR) Diagnosis of AMR is more challenging

Pathological grading based on the presence of **histologic and immunologic findings only**

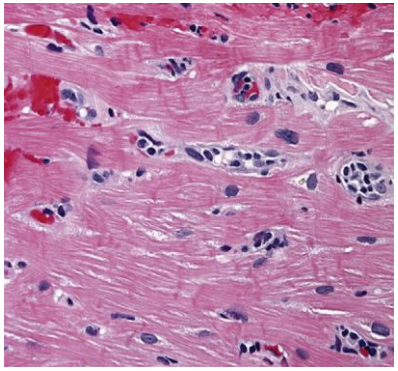


Farkash, E. A. & Colvin, R. B. Diagnostic challenges in chronic antibody-mediated rejection. Nat. Rev. Nephrol. 8, 255–7 (2012).

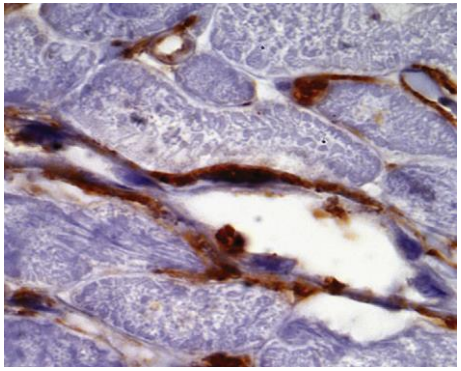
Berry, G. J. et al. The 2013 International Society for Heart and Lung Transplantation Working Formulation for the standardization of nomenclature in the pathologic diagnosis of antibody-mediated rejection in heart transplantation. J. Heart Lung Transplant. 32, 1147–62 (2013).



Hematoxylin and eosin (HE) stain (Paraffin)
increased cellularity & intravascular macrophages



Hematoxylin and eosin (HE) stain (Paraffin)
Activated mononuclear cells fill and expand vessels



Immunohistochemistry shows C4d (Paraffin)
Uniform sub-endothelial localization of C4d is seen in capillaries

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Challenges in Antibody-mediated Rejection

- C4d staining is of limited sensitivity, specificity and reproducibility
- Donor-specific anti-HLA antibodies are found in only 2/3 of biopsy-proven cardiac AMR cases
- Pathological grading lack clinical utility regarding graft survival
- Treatment protocols differ substantially by centers in terms of who and how to treat

1. Mengel, M. et al. Banff initiative for quality assurance in transplantation (BIFQUIT): reproducibility of C4d immunohistochemistry in kidney allografts. Am. J. Transplant 13, 1235–45 (2013).
2. Sis, B. & Halloran, P. F. Endothelial transcripts uncover a previously unknown phenotype: C4d-negative antibody-mediated rejection. Curr. Opin. Organ Transplant. 15, 42–8 (2010).
3. Fine, N. M. et al. The Role of Donor-Specific Antibodies in Acute Cardiac Allograft Dysfunction in the Absence of Cellular Rejection. Transplantation 00, 1–10 (2014).
4. Berry, G. J. Antibody-mediated rejection of the cardiac allograft: where do we stand in 2012? Curr. Opin. Organ Transplant. 17, 303–8 (2012).

Research Approach

- Endothelial, NK cell transcripts are associated with DSA and histological features of microcirculation inflammation in renal transplants.

- NanoString nCounter technology allows multiplex gene expression quantification in FFPE biopsies

A literature-based gene expression profile potentially identifies antibody-mediated tissue injury accurately.

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1. Hidalgo, L. G. et al. NK cell transcripts and NK cells in kidney biopsies from patients with donor-specific antibodies: evidence for NK cell involvement in antibody-mediated rejection. Am. J. Transplant 10, 1812–22 (2010).
2. Sis, B. et al. Endothelial gene expression in kidney transplants with alloantibody indicates antibody-mediated damage despite lack of C4d staining. Am. J. Transplant 9, 2312–23 (2009).
3. Geiss, G. K. et al. Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat. Biotechnol. 26, 317–25 (2008).

Integrated diagnostics in heart rejection

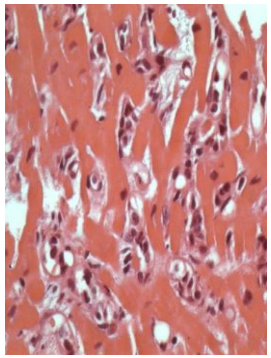
Gene Expression Profiling for the Identification and Classification of Antibody-Mediated Heart Rejection

- Multicentric study (5 French referal centers) 2004 – 2011
- 617 patients screened
- Cases: Heart ABMR (ISHLT)
- Matched controls: Non ABMR (Normal EMB – TCMR EMB)

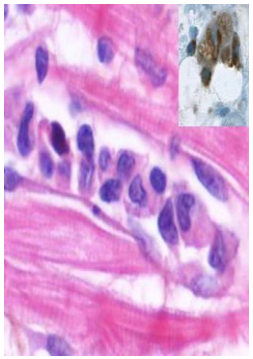
Histological phenotype

DSA phenotype

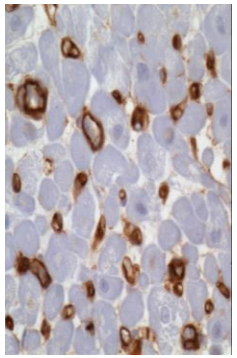
Molecular phenotype



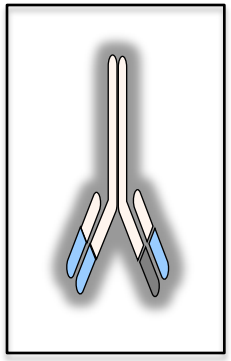
Microcirulation inflammation



Intravascular CD 68
CD 163
CD 3, CD 20



Complement activation (C4d / C3d)



Luminex SA Specificity Class / MFI

Transcripts PBT's Classifiers

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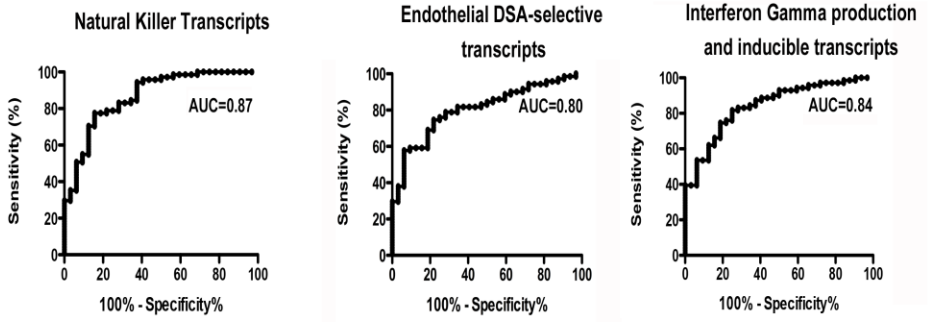
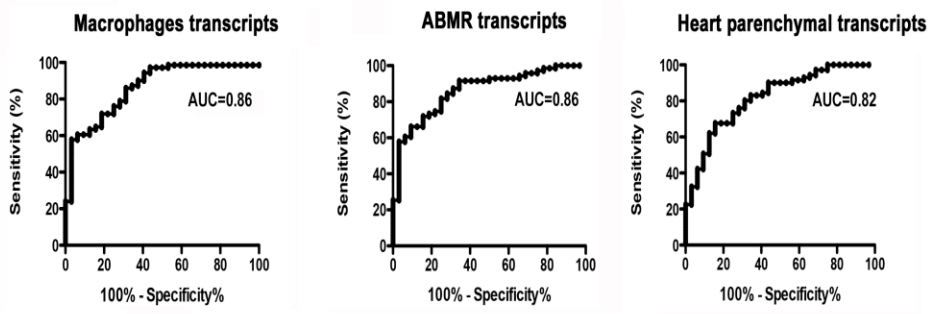
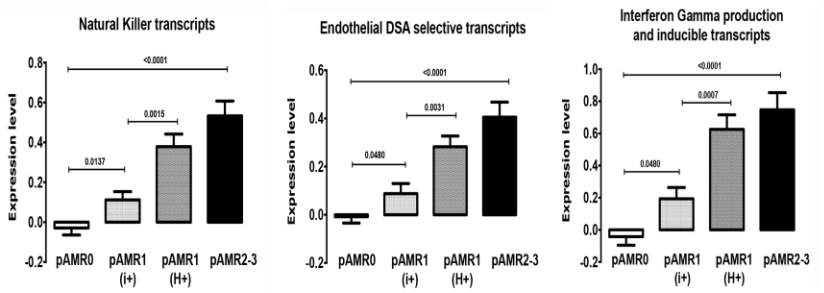
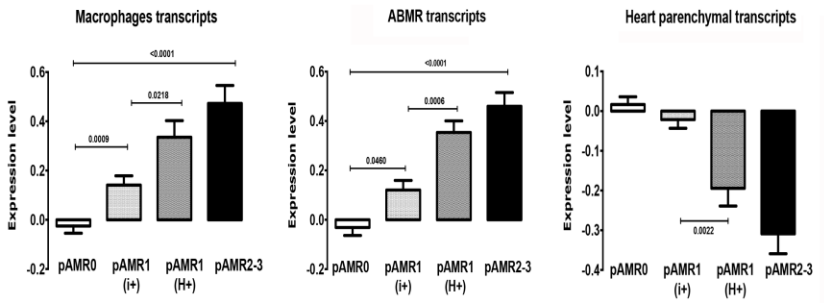
Loupy et al, Circulation 2017

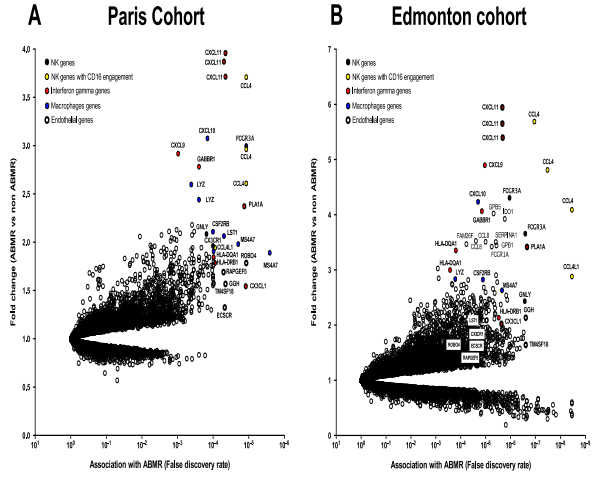
Allograft gene expression correlates with AMR diagnosis with the molecular AMR architecture being highly conserved











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Loupy et al, Circulation 2017

Edmonton FFPE studies - Methods: AMR Gene Set

CAV1	Caveolin-1	endothelial	Sis et al. Am J Transplant. 2009;9(10):2312–23
CD34	CD34 antigen	endothelial	Sis et al. Am J Transplant. 2009;9(10):2312–23
CDH13	Cadherin 13	endothelial	Hidalgo et al. Am J Transplant. 2010;10(8):1812–22
CDH5	Cadherin 5	endothelial	Sis et al. Am J Transplant. 2009;9(10):2312–23
DARC	Duffy Blood Group, Atypical Chemokine Receptor	endothelial	Hidalgo et al. Am J Transplant. 2010;10(8):1812–22
KLF4 4	Kruppel-Like Factor 4	endothelial	Sis et al. Am J Transplant. 2009;9(10):2312–23
MALL	Mal, T-Cell Differentiation Protein-Like	endothelial	Hidalgo et al. Am J Transplant. 2010;10(8):1812–22
PALMD	Palmdelphin	endothelial	Sis et al. Am J Transplant. 2009;9(10):2312–23
PECAM1	Platelet/Endothelial Cell Adhesion Molecule 1	endothelial	Sis et al. Am J Transplant. 2009;9(10):2312–23
PLA1A	Phospholipase A1 Member A	endothelial	Hidalgo et al. Am J Transplant. 2010;10(8):1812–22
PLAT	Plasminogen Activator, Tissue	endothelial	Hidalgo et al. Am J Transplant. 2010;10(8):1812–22
RHOJ	Ras homolog familiy member J	endothelial	Sis et al. Am J Transplant. 2009;9(10):2312–23
ROBO4	Roundabout, Axon Guidance Receptor, Homolog 4	endothelial	Hidalgo et al. Am J Transplant. 2010;10(8):1812–22
SELE	Selectin E	endothelial	Sis et al. Am J Transplant. 2009;9(10):2312–23
SOX7	Sex determing region Y-box 7	endothelial	Hidalgo et al. Am J Transplant. 2010;10(8):1812–22
TEK	Tyrosin-kinase, endothelial	endothelial	Sis et al. Am J Transplant. 2009;9(10):2312–23
THBD	Thrombomodulin	endothelial	Sis et al. Am J Transplant. 2009;9(10):2312–23
VWF	Von Willebrand factor	endothelial	Sis et al. Am J Transplant. 2009;9(10):2312–23
CX3CR1	CX3C chemokine receptor 1	NK cells	Hidalgo et al. Am J Transplant. 2010;10(8):1812–22
FGFBP2	Fibroblast growth factor binding protein 2	NK cells	Hidalgo et al. Am J Transplant. 2010;10(8):1812–22
KLRF1	Killer cell lectin-like receptor subfamily F, member 1	NK cells	Hidalgo et al. Am J Transplant. 2010;10(8):1812–22
MYBL1	V-Myb Avian Myeloblastosis Viral Oncogene Homolog-Like 1	NK cells	Hidalgo et al. Am J Transplant. 2010;10(8):1812–22
SH2D1B	SH2 domain containing 1B	NK cells	Hidalgo et al. Am J Transplant. 2010;10(8):1812–22
GNLY	Granulysin	NK cells	Hidalgo et al. Am J Transplant. 2010;10(8):1812–22
CXCL11	CXC chemokine ligand 11	IFNG-induced	Reeve et al. Am J Transplant. 2009;9(8):1802–10
PSMB10	Proteasome subunit, beta type, 10	IFNG-induced	Reeve et al. Am J Transplant. 2009;9(8):1802–10
RPS6	Ribosomal protein S6	mTOR pathway	Lepin EJ et al. Am J Transplant. 2006;6(7):1560–71
RPS6KB1	Ribosomal protein S6 kinase, beta 1	mTOR pathway	Lepin EJ et al. Am J Transplant. 2006;6(7):1560–71
CD74	Major Histocompatibility Complex, Class II Invariant Chain	Inflammation	Dean et al. Am J Transplant. 2012;12(6):1551–63
GATA3	GATA binding protein 3	Inflammation	Dean et al. Am J Transplant. 2012;12(6):1551–63
IFNG	Interferon, gamma	Inflammation	Dean et al. Am J Transplant. 2012;12(6):1551–63
TBX21	T-Box 21	Inflammation	Dean et al. Am J Transplant. 2012;12(6):1551–63
TNF	Tumor Necrosis Factor	Inflammation	Dean et al. Am J Transplant. 2012;12(6):1551–63
TRIB1	Tribbles Pseudokinase 1	Inflammation	Dean et al. Am J Transplant. 2012;12(6):1551–63

Material and Methods

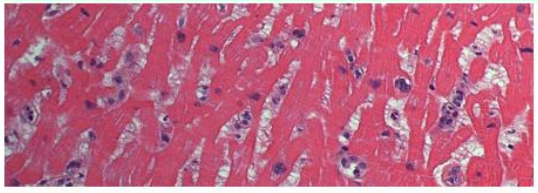
107 FFPE cardiac Bx

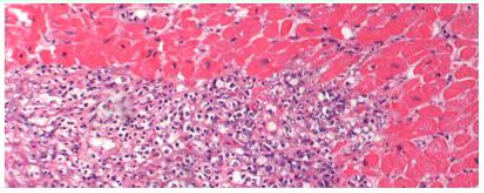
22 pAMR2	37 pAMR1 H+	11 pAMR1 I+	22 ACR	15 Normal

clinical / serologic data

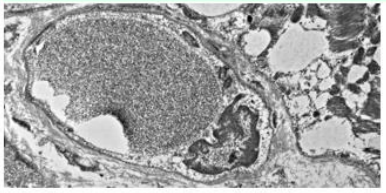
Treatment, Ejection Fraction, DSA, CAV, Graft Status, Survival

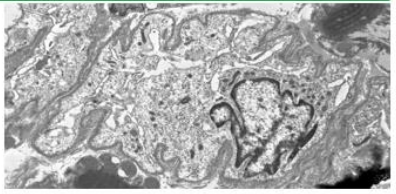
HE-slide review: identification of myocardium



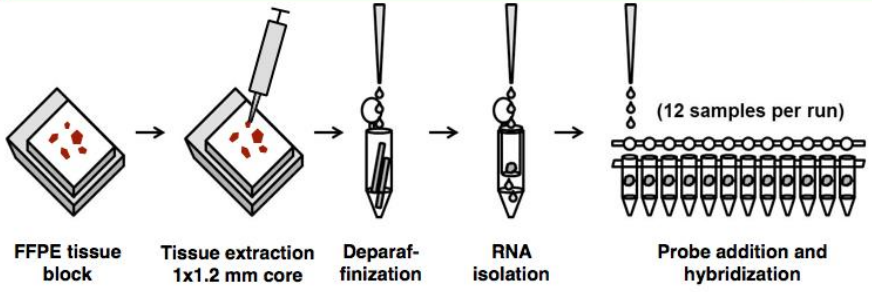


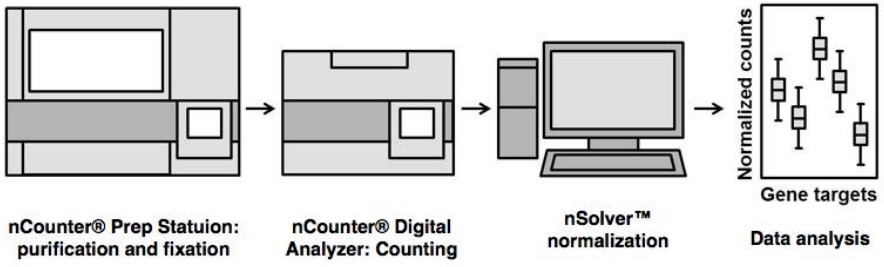
endothelial swelling measurement (TEM)





RNA isolation & multiplex gene expression quantification





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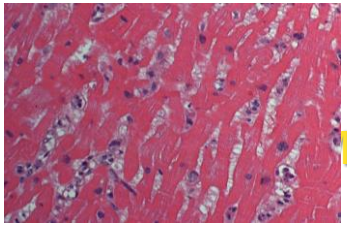
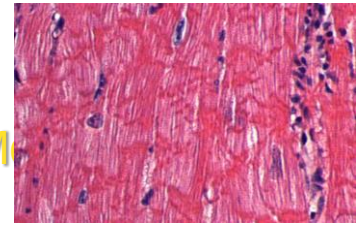
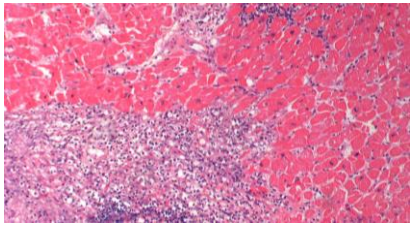
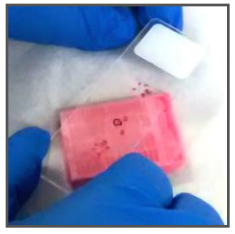
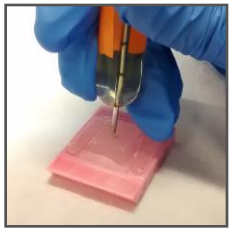
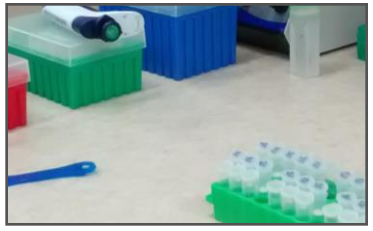
Material and Methods – Molecular Studies

107 FFPE cardiac Bx	22 ACR	22 pAMR2	37 pAMR1 H+	11 pAMR1 I+	15 Normal
clinical/serologic data	**Treatment, Ejection Fraction, DSA, CAV, Graft Status, Survival**				

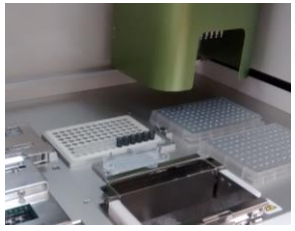
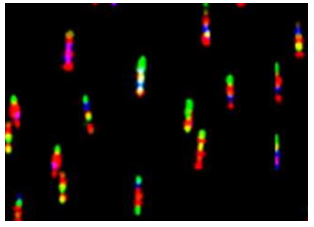
Identifying myocardium
Excluding prior Bx, thrombi, fibrosis, focal ACR sites, quilty

Micro-Punching sites Extracting RNA

NanoString nCounter™ transcript counting

	CAV1	CD34	CD74	CDH1
C	2048.72	518.6	9039.23	38.21
C	1150.81	162.9	19521.29	7.46
C	2989.02	748.47	30484.2	33.65
C	2158.1	620.3	12672.93	36.94
C	1572.4	420.4	15775.85	14.16
C	2760.51	633.81	26561.47	35.81
C	1687.41	232.16	3574.43	39.83
C	1676.49	258.61	23016.53	6.76
C	1816.13	292.51	15927.39	19.15
C	1892.68	605.28	21121.57	19.96
C	2025.92	413.04	24681.39	3.13
C	1064.25	253.82	56770.88	14.85

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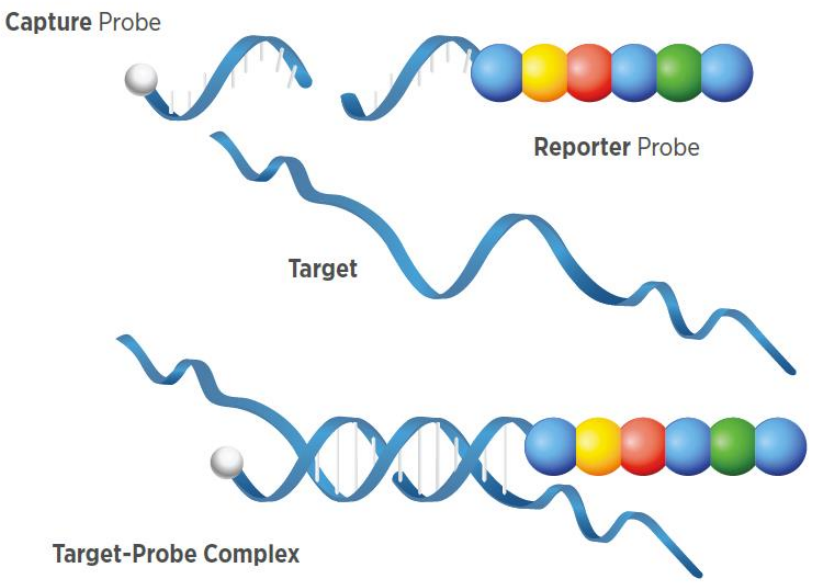
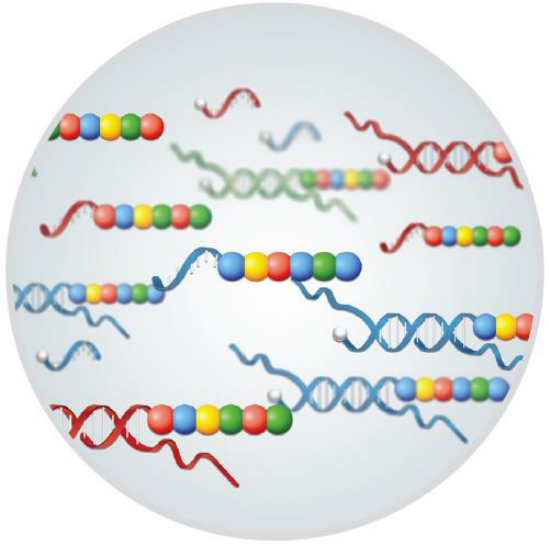
NanoString nCounter technology

1

HYBRIDIZE





solution phase hybridization

Hybridization

Barcoded probes hybridize directly to a target molecule in solution. The **Reporter Probe** carries the signal and the **Capture Probe** allows the complex to be immobilized for data collection.

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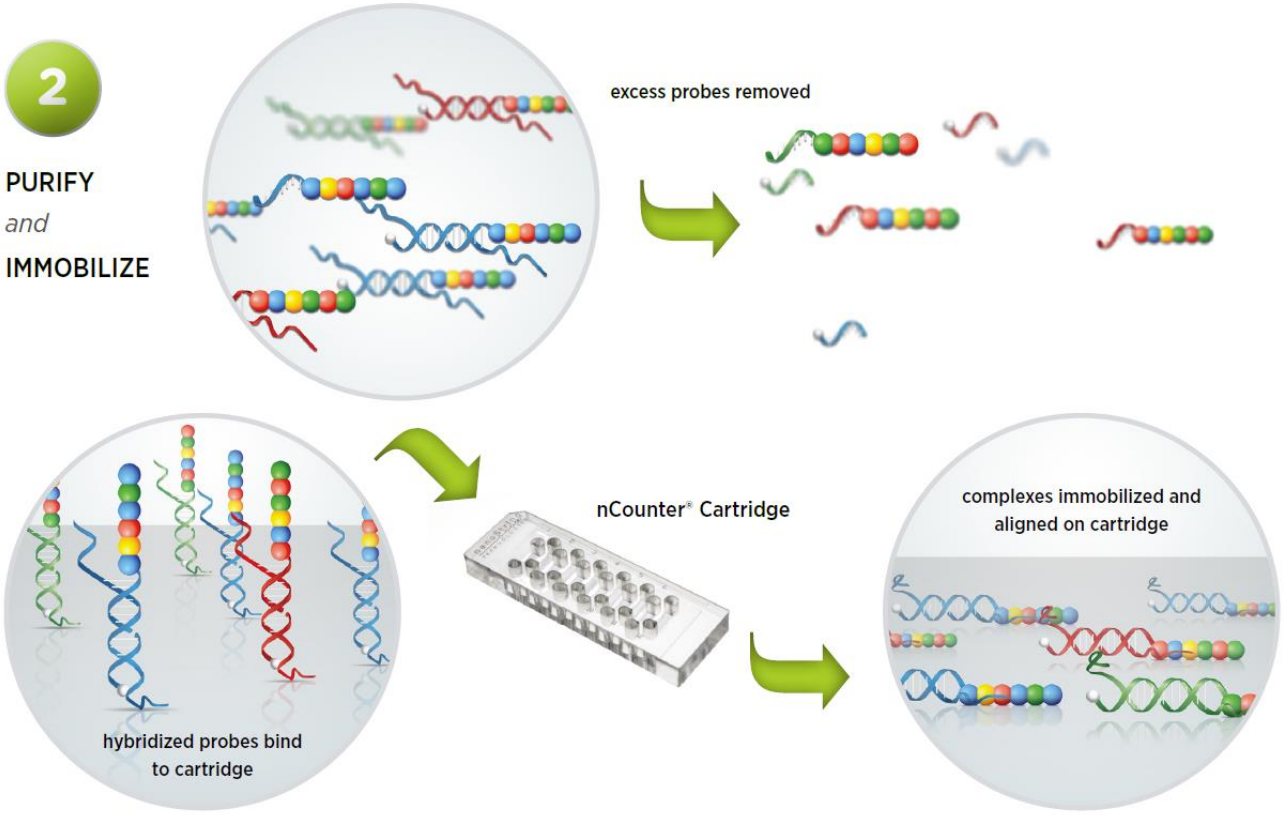
nanoString TECHNOLOGIES®

NanoString nCounter technology



Sample Processing

After hybridization, samples are transferred to the nCounter Prep Station where excess probes are removed and probe/target complexes are bound, immobilized and aligned on the nCounter Cartridge.

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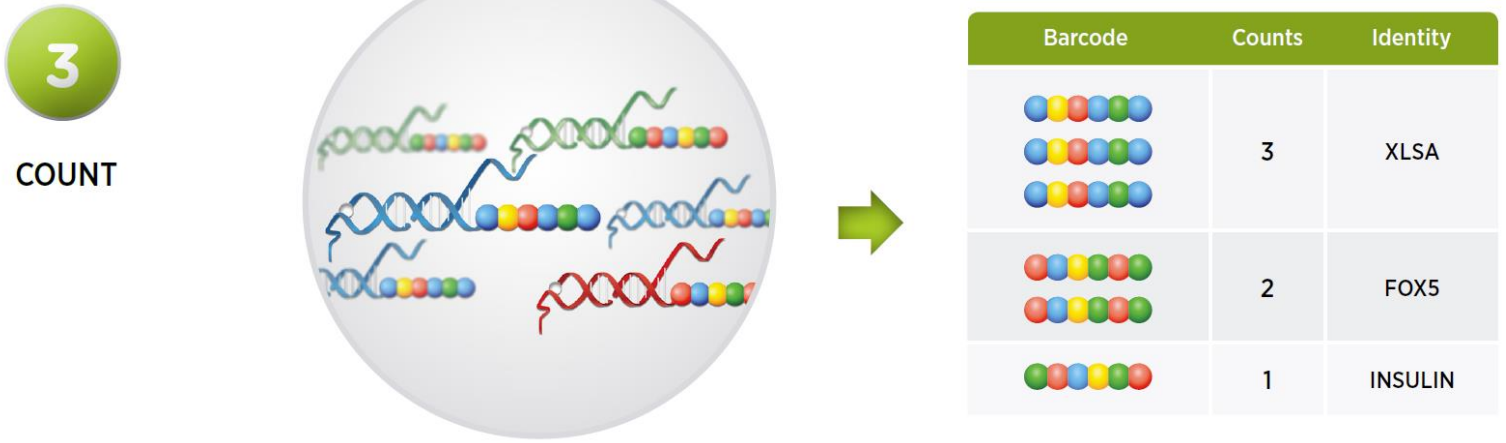

Digital Data Acquisition

Sample cartridges are placed in the nCounter Digital Analyzer for data collection. Barcodes are counted and tabulated for each target molecule.

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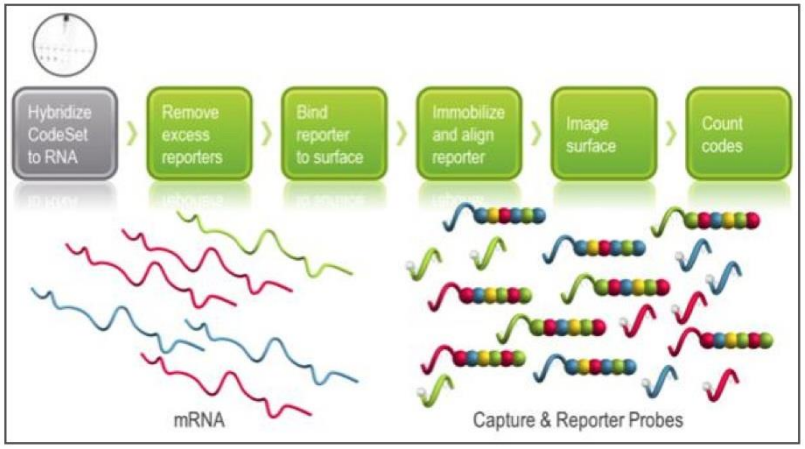

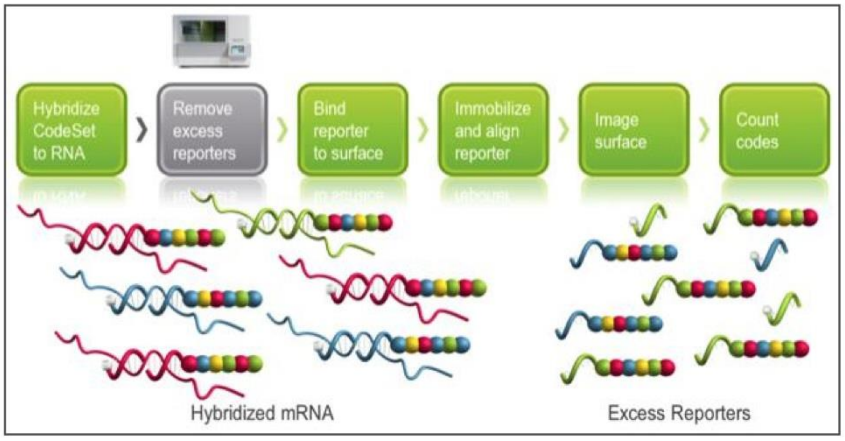

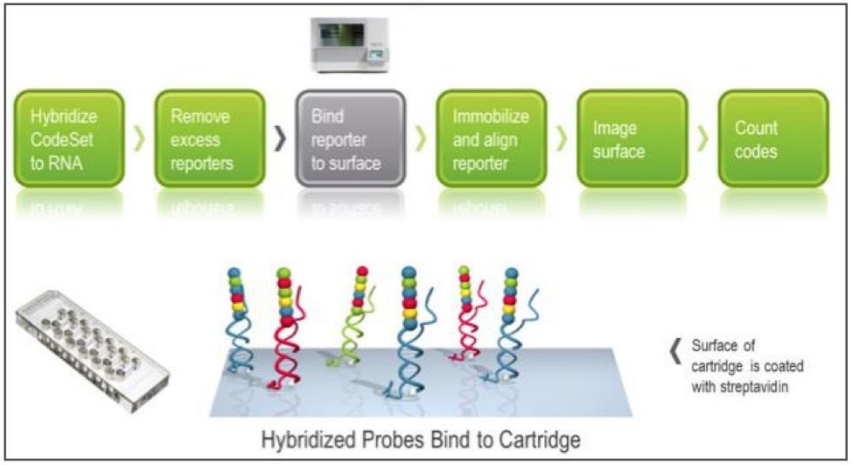

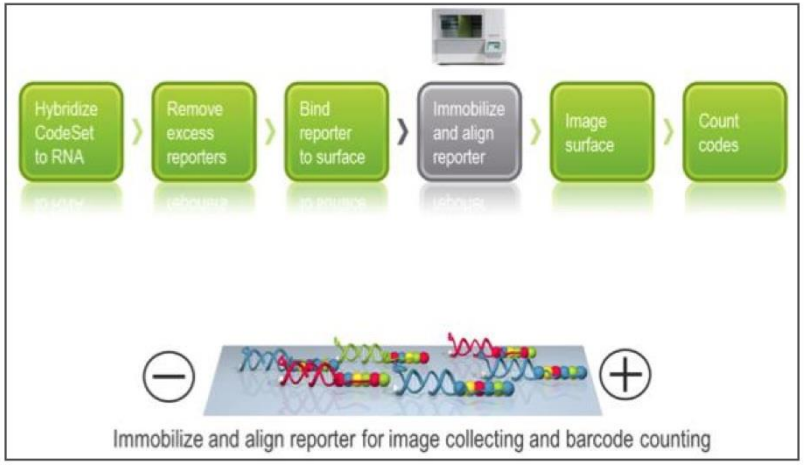

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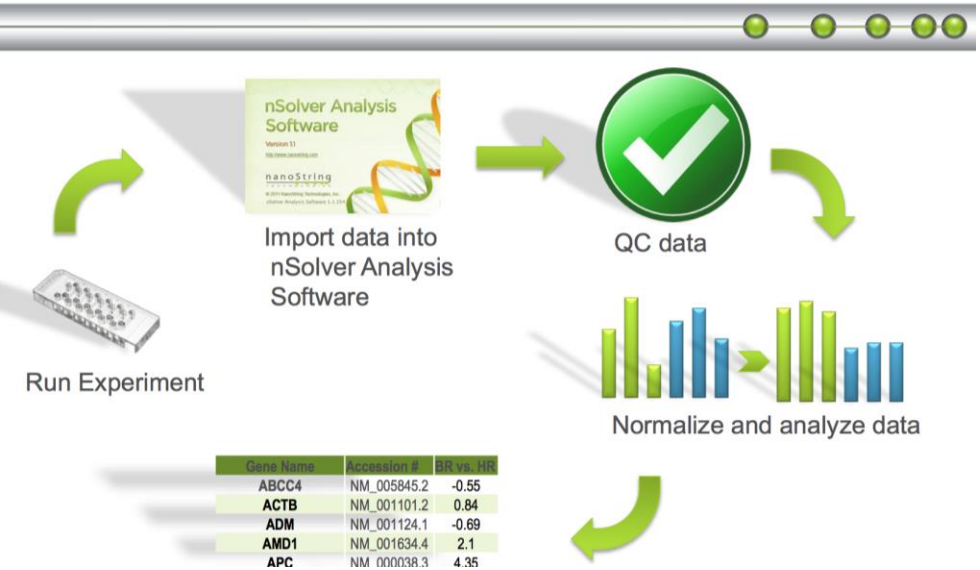
nanoString TECHNOLOGIES

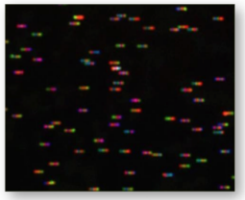
Analytics software

nSolver™ Simple results- simple analysis



Gene Name	Accession #	BR vs. HR
ABCC4	NM_005845.2	-0.55
ACTB	NM_001101.2	0.84
ADM	NM_001124.1	-0.69
AMD1	NM_001634.4	2.1
APC	NM_000038.3	4.35

Data Output: numbers of molecules detected



	Sample ID							
Target Gene	1	2	3	4	5	6	7	8
MAP2K1	1241	1283	1554	1471	1519	1604	1176	1181
MAP2K4	176	190	215	175	244	249	208	205
MAP2K6	16	12	33	19	16	14	30	31
MAP3K1	367	431	469	386	505	560	310	347
MAP3K5	27	37	33	30	40	37	65	42
MAP3K7	821	748	940	887	1027	1048	913	958
MAP3K9	82	93	132	111	127	132	117	99
MAPK1	1206	1261	1520	1376	1665	1727	1328	1379
MAPK14	678	669	843	804	902	926	798	787
MAPK3	611	595	787	698	805	901	701	682
MAPK8	326	341	466	394	428	460	386	383
MAPKAPK2	671	639	754	755	814	954	688	683

Sample Size

- 127 RNA extractions from 135 compiled blocks

  - 6 with no material on block, 1 explant sample, 1 only fibrotic tissue from prior biopsy site (3 adult, 5 pediatric biopsies)

- Selected 120 samples with highest RNA concentration (measured with NanoDrop 2000c)

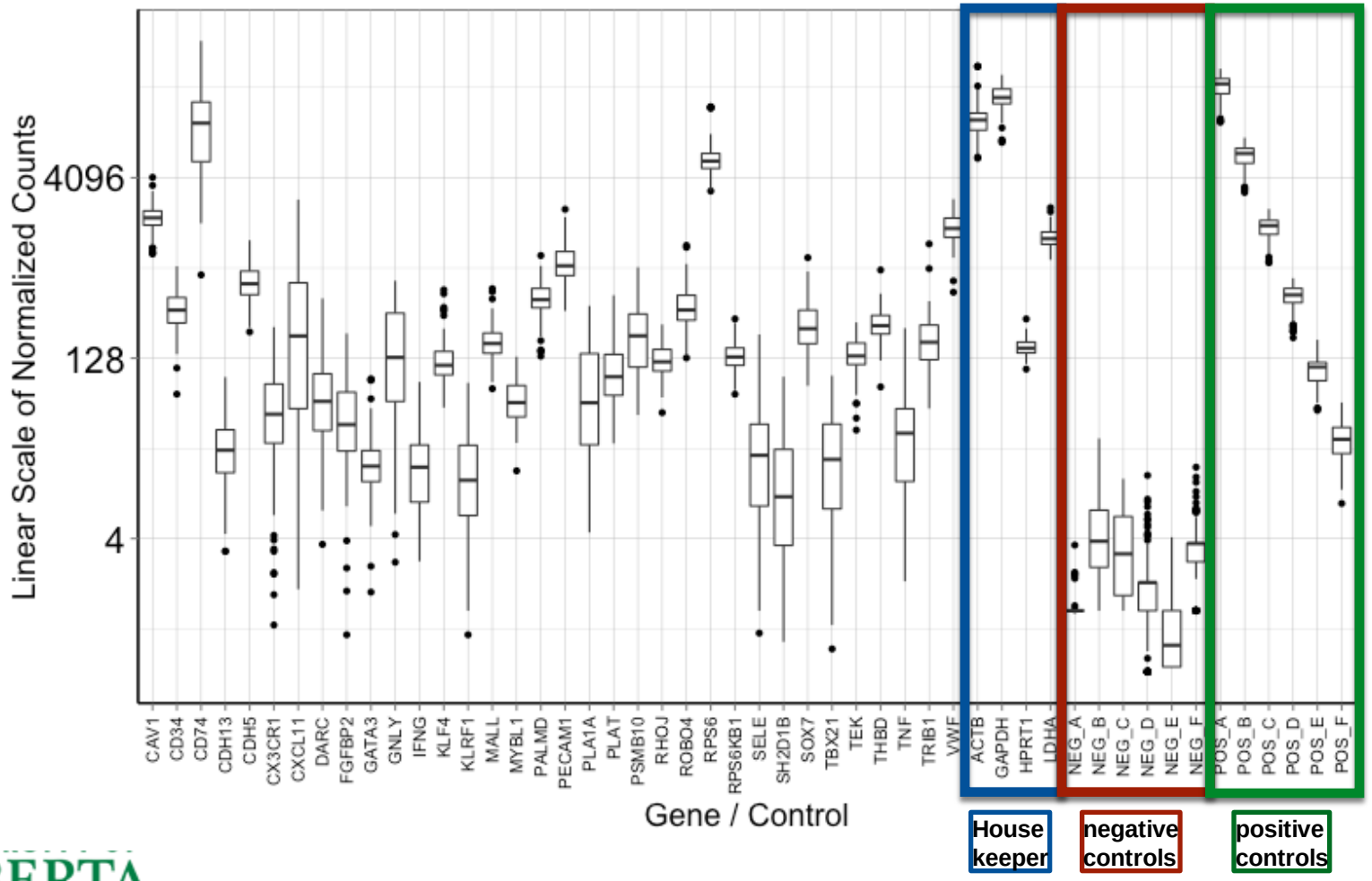
n=120	Concentration (ng/µl)	Purity A(260/280)
Minimum	3.3	1.52
Median	10.5	1.85
Mean	12.04	1.87
Max	42	3.11

- 120 total samples in 10 NanoString runs
  - 0 QC failures
  - 4 calibration repeats excluded
  - 9 pediatric cases excluded
- **107 unique samples**, **69 patients**, 3306 datapoints
- Including 56 sequential biopsies from 18 patients

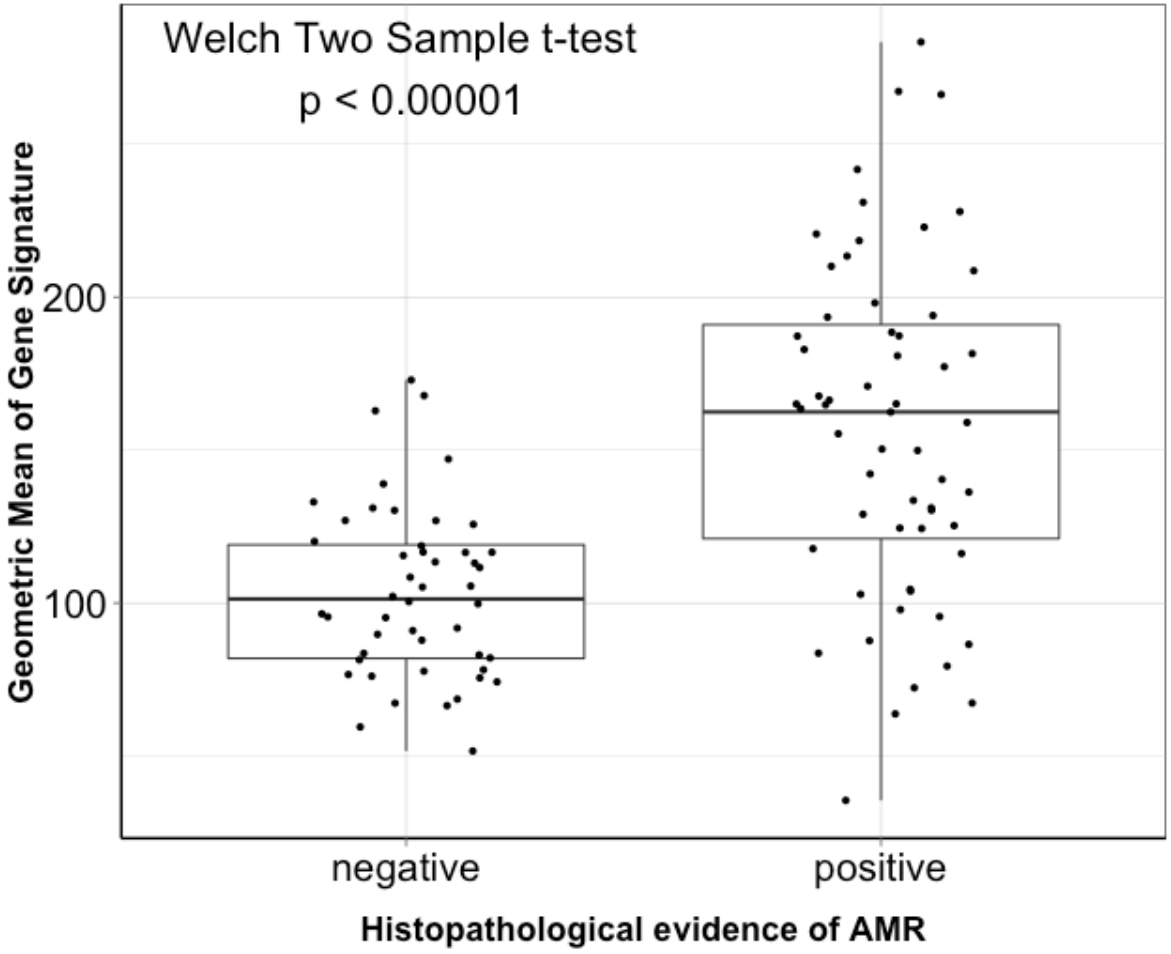
Rejection Diagnoses

n=107	AMR Histopathology	C4d+ and/or CD68+	ACR Grade 0R / > 0R	DSA pos / neg / na	n
pAMR2	pos	pos	11 / 11	17 / 5 / 0	22
pAMR1 (H+)	pos	neg	11 / 26	14 / 21 / 2	37
pAMR1 (I+)	neg	pos	5 / 6	4 / 6 / 1	11
ACR	neg	neg	0 / 22	2 / 14 / 6	22
Normal	neg	neg	15 / 0	1 / 12 / 2	15

Robust counts of positive controls, low counts of negative controls

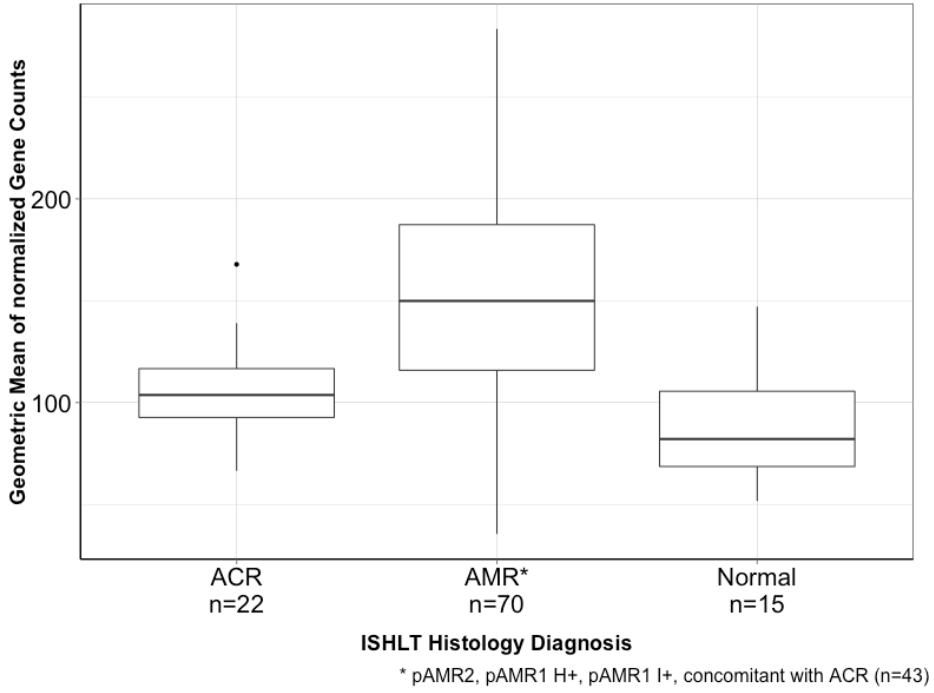


Gene Expression & AMR Histolopathology



Histologic findings of AMR are associated with higher gene set expression.

Gene Expression between AMR and ACR or Normal Controls are Significantly Different



Diagnoses	ACR	AMR
AMR	0.0009	-
Normal	0.17	<0.0001

Pairwise comparisons of Geometric Means of Gene Signature using t tests with pooled SD
P values (adjustment method: holm)

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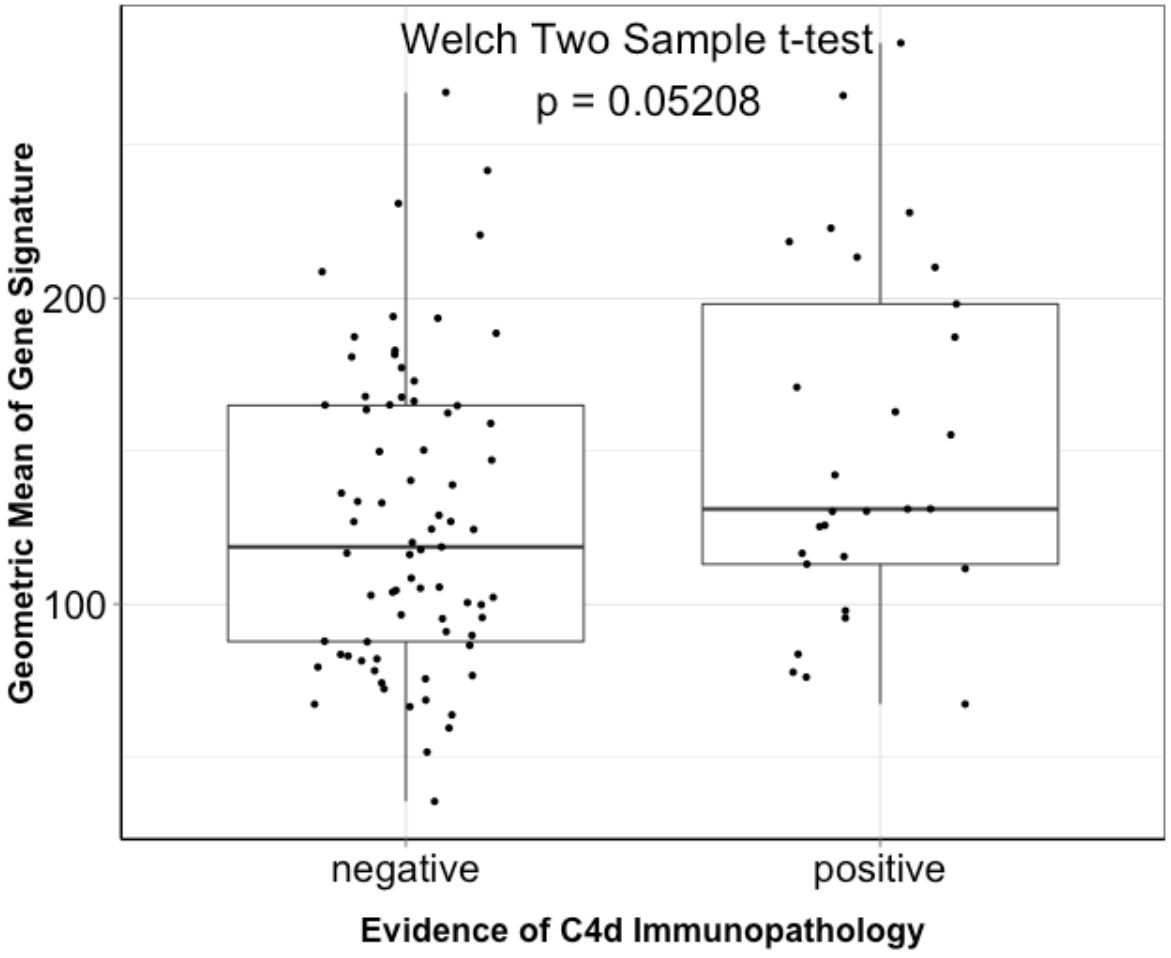
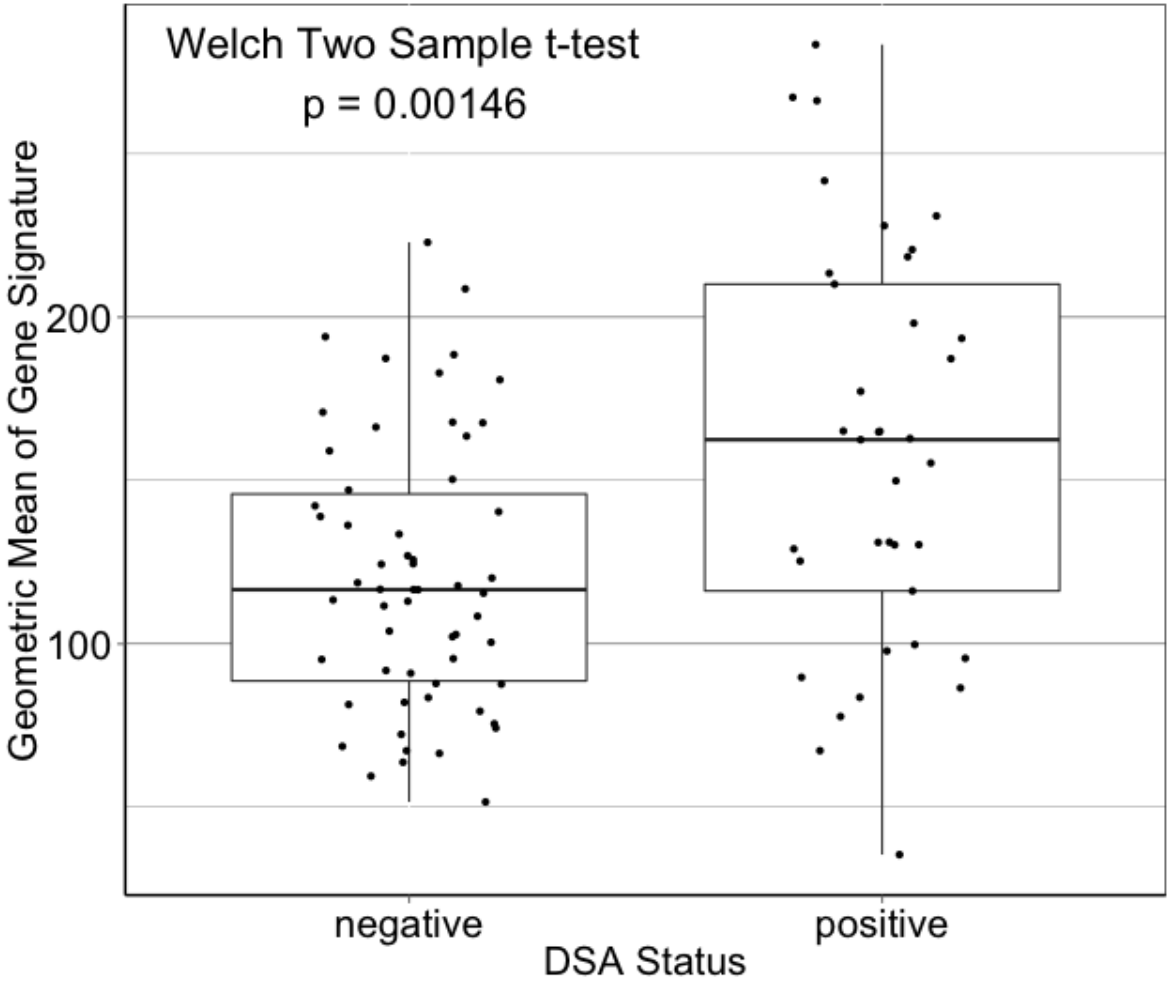
Gene Expression & AMR Immunopatholgy



Borderline association of C4d evidence and higher gene set expression.

Gene Expression & DSA Status
Higher Expression in DSA-positive Cases



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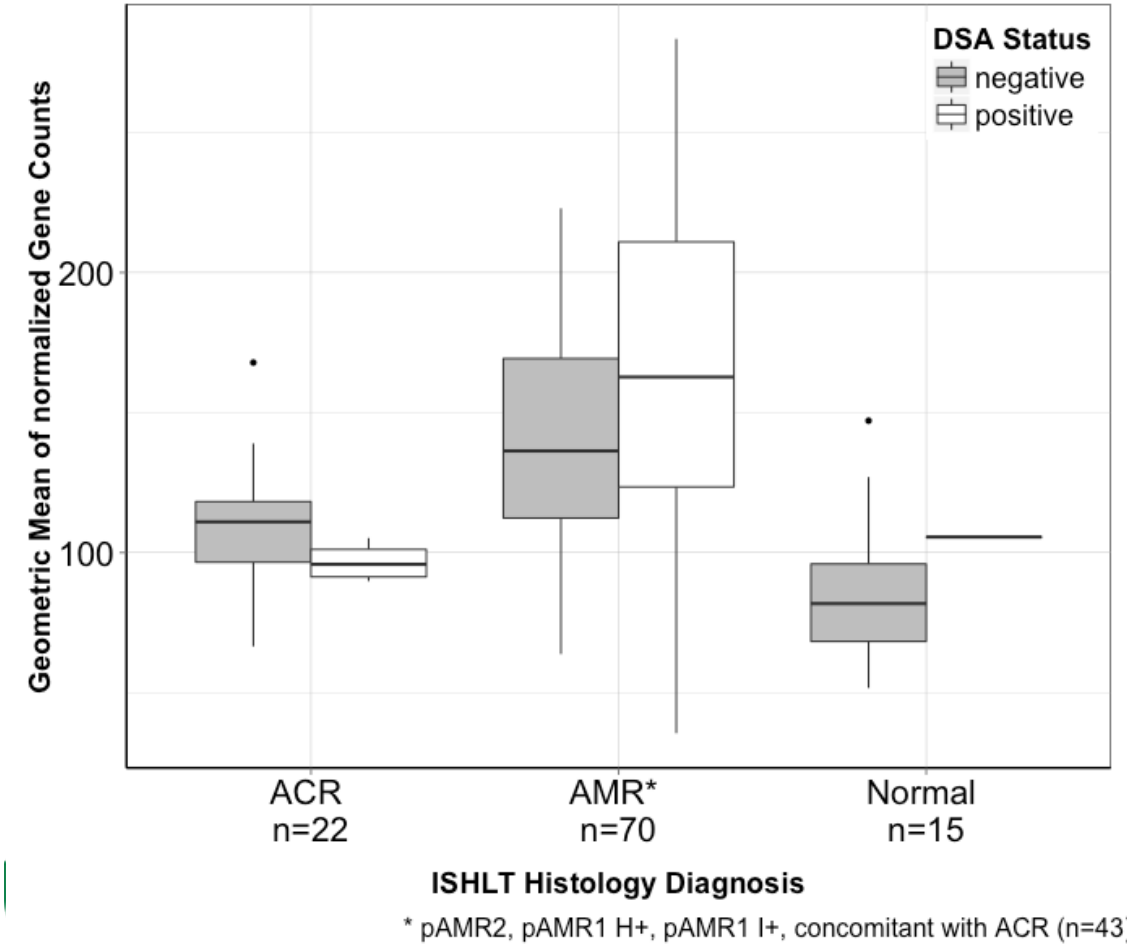
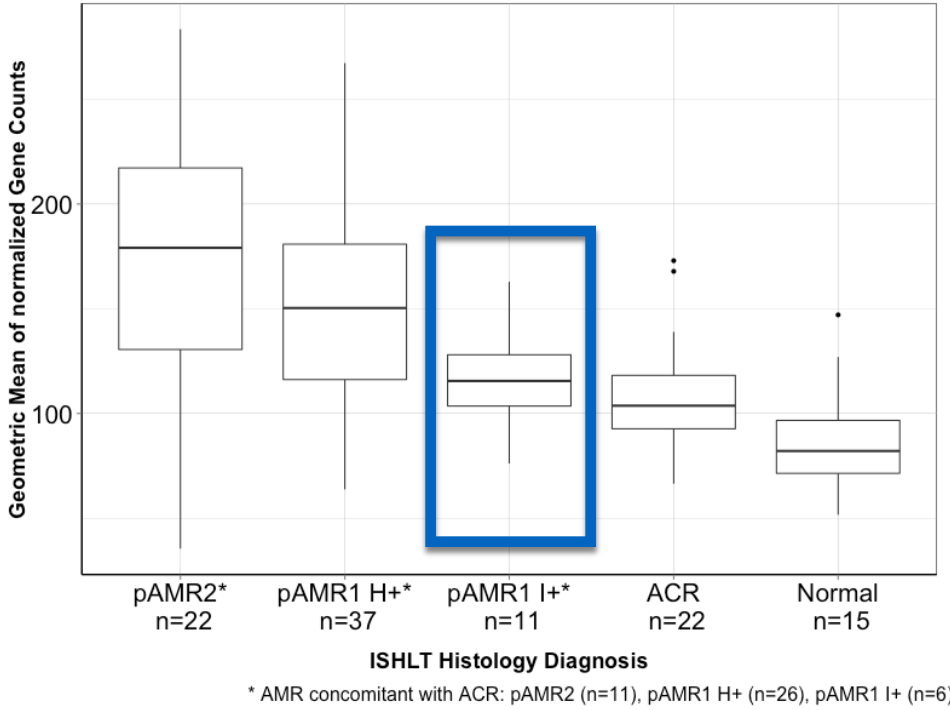
Gene Expression of ACR, AMR, Normal
No differences due to Presence of DSA



Differences between DSA-positive and DSA-negative cases within ACR (p=0.1822) or AMR (p=0.06452) group were not significant.

Significant Differences in Gene Set Expresion across 2013 ISHLT Classification Groups



Diagnoses	ACR	pAMR2	pAMR1h	pAMR1i
pAMR2	0.0001	-	-	-
pAMR1h	0.0077	0.26	-	-
pAMR1i	0.76	0.0053	0.13	-
Normal	0.41	<0.0001	0.0001	0.41

Pairwise comparisons of Geometric Means of Gene Signature using t tests with pooled SD

P values (adjustment method: holm)

pAMR1 I+ cases are neither different from pAMR1 H+ nor from ACR or normal controls.

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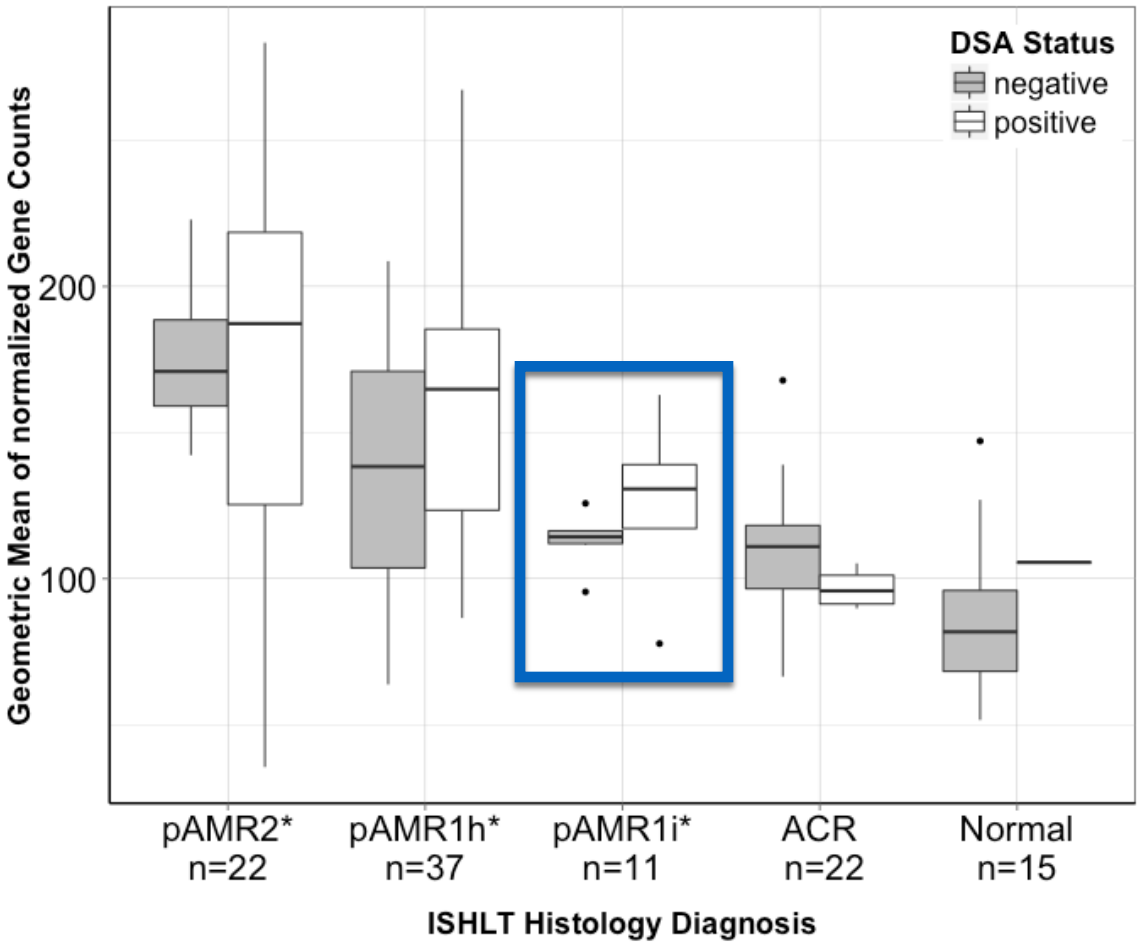
Presence of DSA Plays Minor Role in Gene Set Expression



* AMR concomitant with ACR: pAMR2 (n=11), pAMR1 H+ (n=26), pAMR1 I+ (n=6)

No differences between DSA-positive and DSA-negative cases within any specific AMR diagnosis.

pAMR1 I+ cases are neither different from pAMR1 H+ nor from ACR or normal controls.

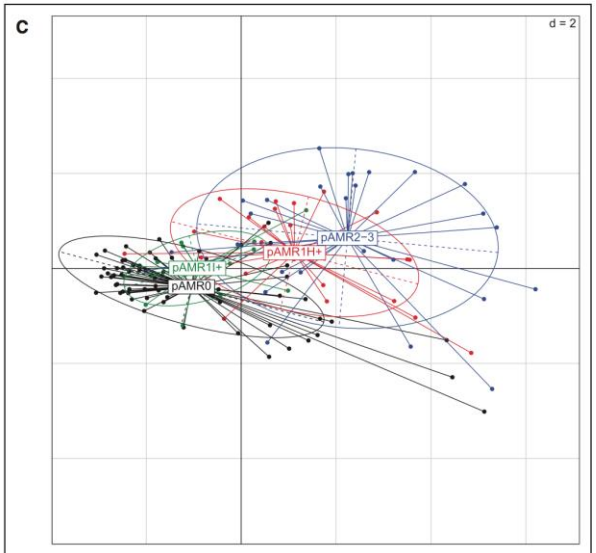
DSA-positive pAMR1 I+ appears to have higher expression (p=0.5351).

<u>More such cases need to be studied!</u>

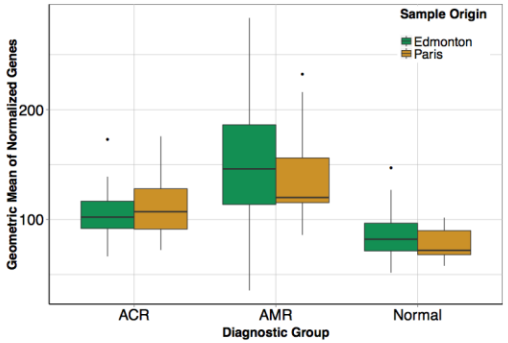
Validation set

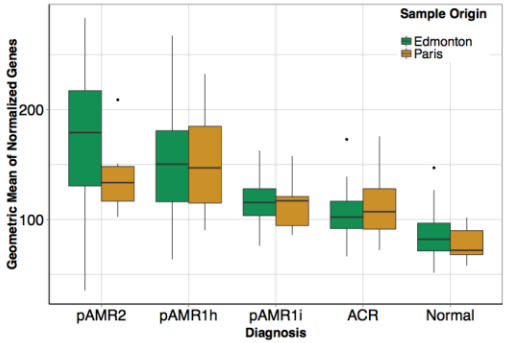
TRAINING SET VS. TEST SET

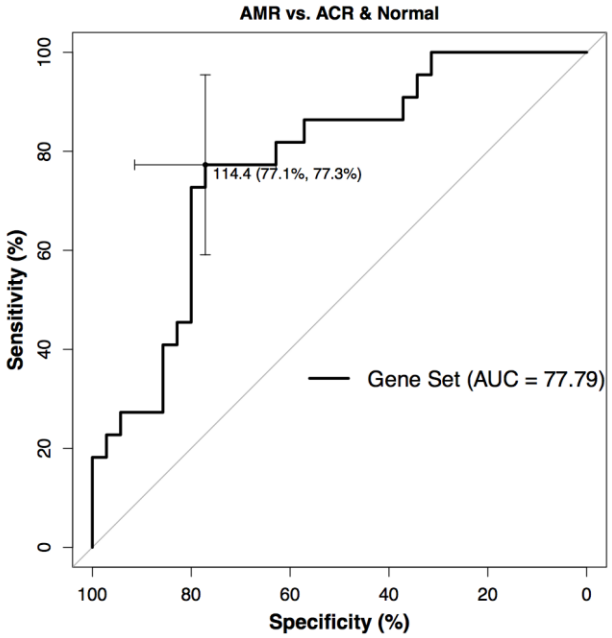
n = 163	Training Set Edmonton	Test Set Paris	p
Samples	106	57	
Patients	68	47	
AMR	70 (66%)	22 (39%)	0.00082
ACR	21 (20%)	25 (44%)	0.00233
Normal	15 (14%)	10 (18%)	0.5802
Clinical Data			
Age at Bx	48.25472	44.59649	0.09145
Donor Age	32.16832	42.34615	< 0.00001
Ischemia time	315.1771	211.9434	< 0.00001
Protocol biopsies	91 %	100 %	0.02161
DSA Status	63% pos.	52% pos.	0.1829
Days to Bx	738.6019	620.5263	0.44
Mean geomean Gene Set	132.6976	116.4008	0.02563



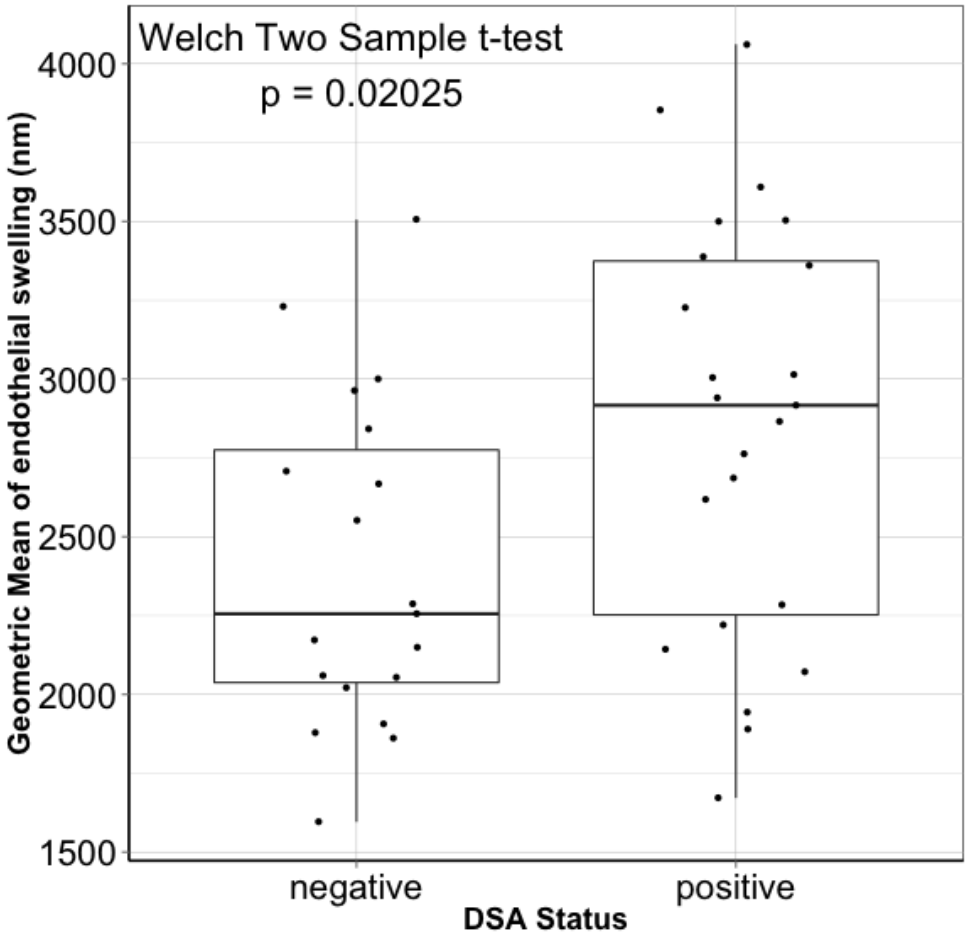
RESULTS: TEST SET CONFIRMS FINDINGS







DSA presence overall is associated with Endothelial Swelling



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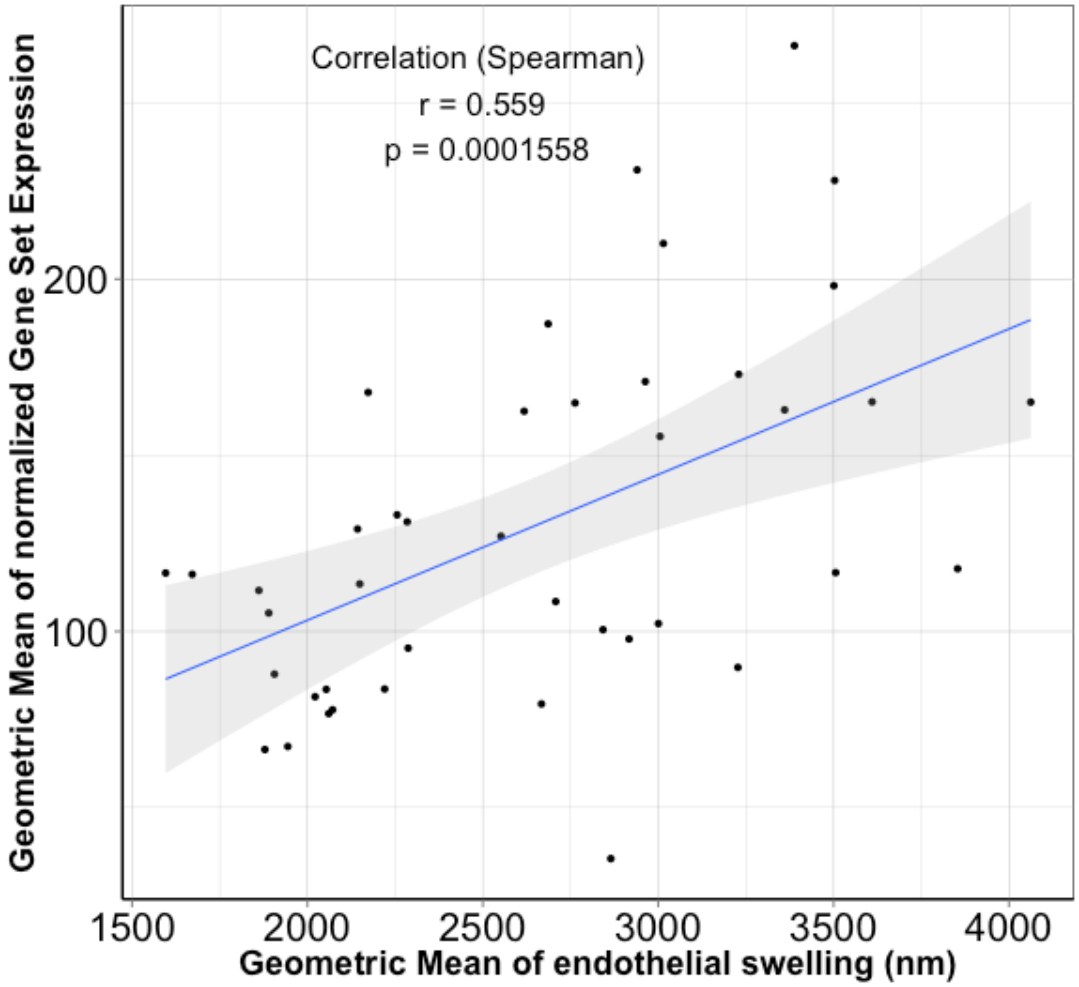
Endothelial Swelling correlates with AMR Gene Set expression



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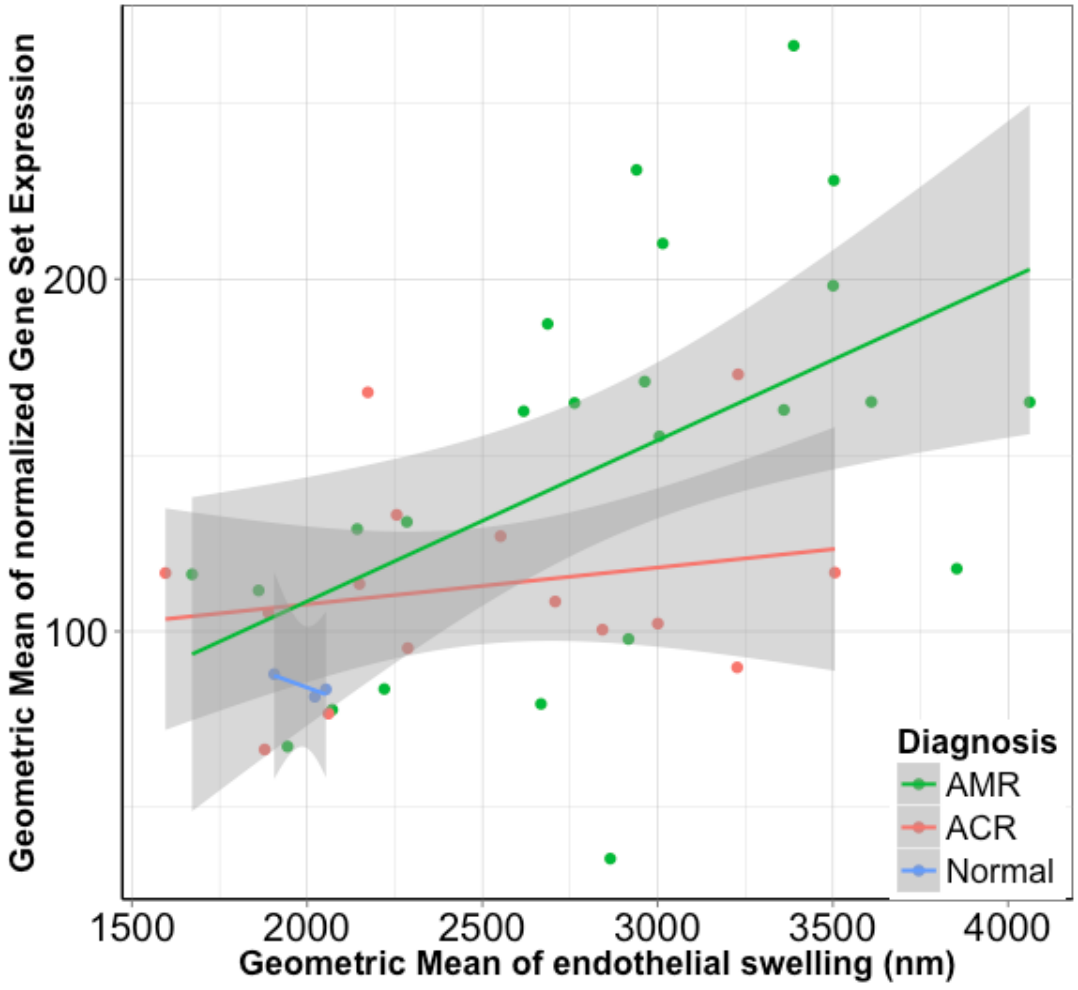
Correlation is specific to antibody-mediated rejection



AMR: rho=0.616, p<0.01

ACR: rho=0.221, p=0.43

Normal: rho=-0.5, p=1

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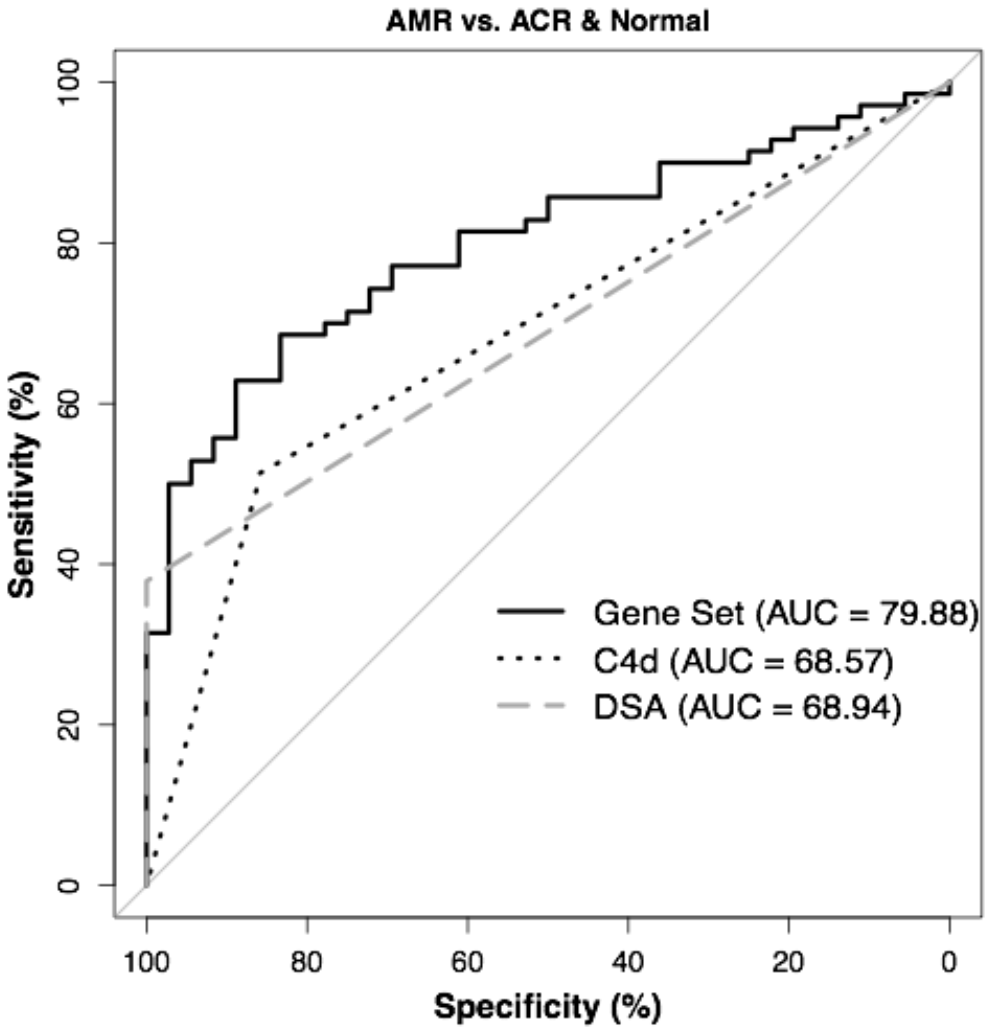
Comparison of ROC Curves Gene Set, DSA and C4d



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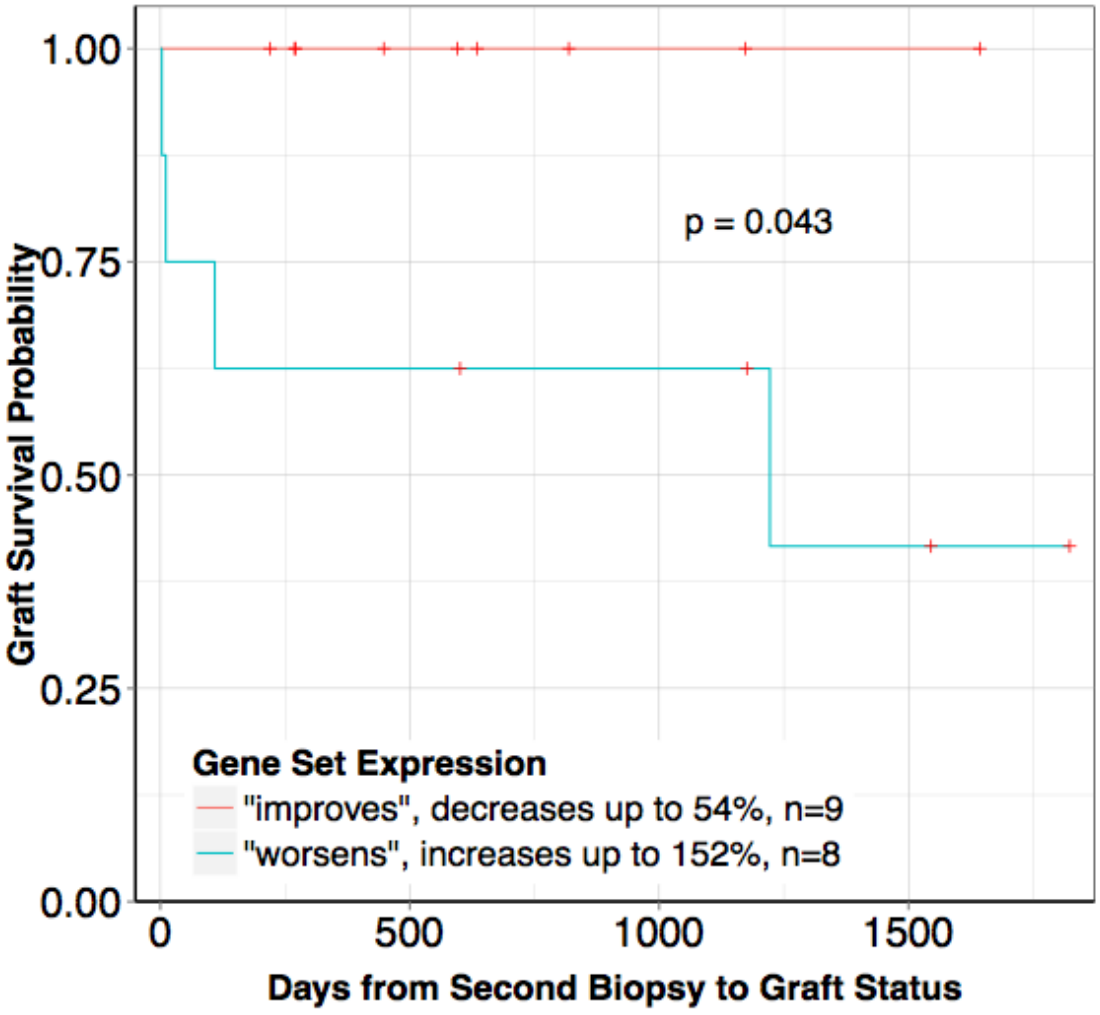
Increase of Gene Set Expression is Significantly Associated with Graft Failure



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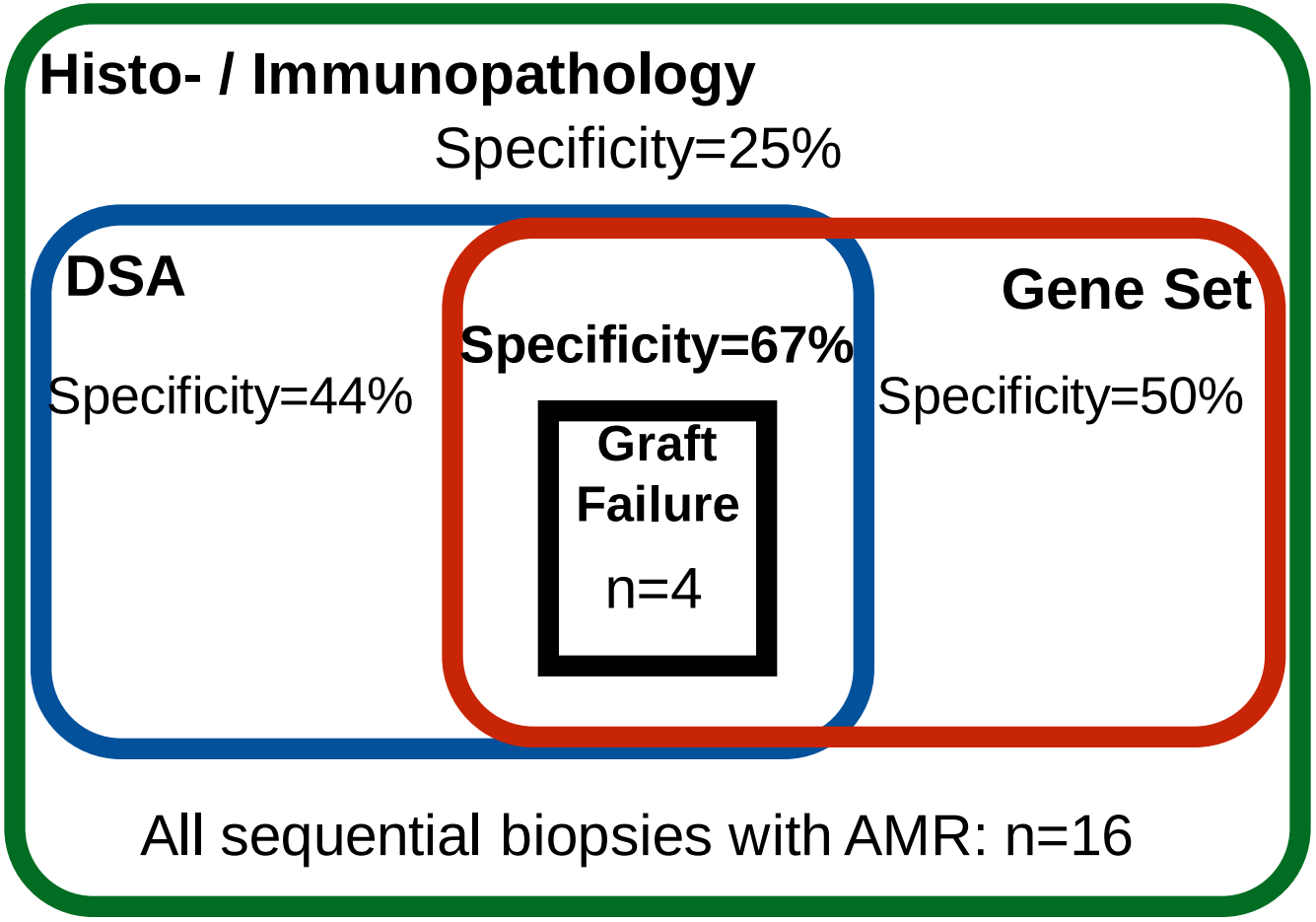
Predicting Graft Survival by Combining All Available Diagnostics Improves Specificity