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ORIGINAL RESEARCH

Abnormal Endothelial Gene Expression Associated With Early Coronary Atherosclerosis

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BACKGROUND: We examined feasibility of a unique approach towards gaining insight into heritable risk for early atherosclerosis: surveying gene expression by endothelial cells from living subjects.

METHODS AND RESULTS: Subjects aged <50 years (mean age, 37; range, 22–49) without obstructive coronary artery disease underwent coronary reactivity testing that identified them as having normal or abnormal coronary endothelial function. Cultures of Blood Outgrowth Endothelial Cells (BOEC) from 6 normal and 13 abnormal subjects passed rigorous quality control and were used for microarray assessment of gene expression. Of 9 genes differentially expressed at false discovery rate <0.1%, we here focus upon abnormal subjects having elevated expression of *HMGB1* (high mobility group box 1) which we unexpectedly found to be linked to low *LAMC1* (laminin gamma 1) expression. This linkage was corroborated by 3 of our past studies and confirmed bio-functionally. Compared with normal BOEC, abnormal BOEC released 13±3-fold more HMGB1 in response to lipopolysaccharide; and they deposited one tenth as much LAMC1 into collagen subendothelial matrix during culture. Clinical follow-up data are provided for 4 normal subjects (followed 13.4±0.1 year) and for 12 abnormal subjects (followed 9.1±4.5 years).

CONCLUSIONS: The known pathogenic effects of high-*HMGB1* and low-*LAMC1* predict that the combination would biologically converge upon the focal adhesion complex, to the detriment of endothelial shear responsiveness. This gene expression pattern may comprise a heritable risk state that promotes early coronary atherosclerosis. If so, the testing could be applied even in childhood, enabling early intervention. This approach offers a way to bridge the information gap between genetics and clinical phenotype.

Key Words: atherosclerosis ■ endothelial function ■ focal adhesion complex ■ focal adhesion kinase ■ HMGB1 ■ laminin ■ risk factor ■ shear stress

Clinical atherosclerosis emerges from complexity involving multiple promotive influences, cell types, and biologic systems. Although heritable factors are believed to account for ≥50% of disease risk,[1] only rarely does this involve a single gene exerting a large influence. Rather, the heritable component of risk most likely involves multiple genes that individually exert smaller effects. Identifying these has been a formidable challenge. We here demonstrate the feasibility of using a unique approach to bridging the information gap between genomics and clinical phenotype: assessing gene expression by endothelial cells obtained from living patients.

For this we use blood outgrowth endothelial cells (BOEC) from cultures of peripheral blood. Unlike cell types often labeled "EPC," BOEC are fully differentiated, bona fide endothelial cells that are the in vitro progeny of a circulating, marrow-derived, transplantable endothelial progenitor.[2-5] Importantly, BOEC themselves have never been exposed to

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CLINICAL PERSPECTIVE

What Is New?

- Using subjects aged <50 years shown by clinically-indicated coronary reactivity testing to have either normal or abnormal coronary endothelial function, this feasibility study surveyed gene expression by blood outgrowth endothelial cells (a stable endothelial type that can identify heritable differences in gene expression) to bridge the gap between genomics and clinical phenotype—and has thereby provisionally identified a heritable risk factor for early development of atherosclerosis.
- Compared with blood outgrowth endothelial cells from subjects having normal coronary endothelial function, blood outgrowth endothelial cells from subjects with abnormal coronary endothelial function exhibited abnormally elevated expression of *HMGB1* (high mobility group box 1) that was linked to abnormally depressed expression of *LAMC1* (laminin gamma 1).

What Are the Clinical Implications?

- This combination may be a heritable risk factor for early atherosclerosis, since known effects and functions of HMGB1 and LAMC1 predict that their abnormal expression in the observed directions would biologically converge at the focal adhesion complex and endothelial cell membrane in a manner detrimental to the vascular endothelial cell's normal responsiveness to shear stress.

Nonstandard Abbreviations and Acronyms

BOEC	blood outgrowth endothelial cells
HMGB1	high mobility group box 1
LAMC1	laminin gamma 1

inflammatory or tissue-specific influences of the in vivo environment. Even at high-fold expansion they are far more stable than other endothelial types, and they are generic endothelial reporter cells uniquely suitable for a study such as this.[3] Since they can be obtained from a known donor, BOEC enable matching of donor characteristics to endothelial features of interest, in the context of measured heritable gene expression. Indeed, we previously applied this approach to identify underlying risk for a clinical stroke phenotype affecting some children with sickle cell anemia[4] and, separately, to suggest an influence of ancestral continent-of-origin on endothelial shear stress responsiveness.[5]

The present study addresses the feasibility of using BOEC gene expression to identify risk for developing atherosclerosis at a young age. Operationally, the phenotype we focused on here is coronary endothelial dysfunction in subjects aged <50 years. Coronary endothelial dysfunction is the earliest clinically detectable form of atherosclerosis, providing gold-standard evidence for presence of atheropromotive pathobiology.[6-10] Coronary endothelial dysfunction is associated with plaque progression and increased risk of major adverse cardiovascular events.[6-9]

METHODS

This gene expression study was done in 2005 to 2007 and, hence, reflects technologies then extant. The delay in submission for publication was because the project leader paused from work for a decade because of a family medical catastrophe.

Methods described herein are sufficient to enable replication of the study. The new gene expression data underlying this report are deposited and available at Gene Expression Omnibus, series GSE132651; previously reported data extracted for use herein were previously deposited as series GSE22688 and GSE9877. Aliquots of the BOEC samples studied herein are present in our BOEC bio-bank and may be accessible by contacting the corresponding author.

Subjects

This study was approved by the Institutional Review Boards at the University of Minnesota and the Mayo Clinic. Subjects were adults, aged <50 years (all but 1 was <46 years), undergoing clinically indicated invasive angiography (at the Mayo Clinic Catheterization Laboratory) for signs and/or symptoms suggestive of angina plus risk factors. Subjects gave written informed consent.

Coronary Reactivity Testing

Patients withheld all vasoactive prescription medications for at least 24 to 48 hours, and fasted for 12 hours, before coronary angiography and coronary reactivity testing.[8,10] Following diagnostic angiography and exclusion of obstructive coronary artery disease, we positioned a Doppler guidewire (Flowire, Volcano Therapeutics Inc, Rancho Cordova, CA) within a coronary-infusion catheter into the mid-left anterior descending (LAD) coronary artery.[11,12] We gave escalating intracoronary doses of acetylcholine (10^{-6}, 10^{-5}, and 10^{-4} mol/L for 3 minutes at each concentration), infused selectively into the mid-LAD. We measured coronary artery diameter offline (by an independent investigator) in the segment 5 mm distal to the tip of the Doppler wire, using a quantitative

coronary angiography program (Medis Corp, Leiden, the Netherlands) as previously described.[11] We calculated coronary blood flow in the LAD from the Doppler derived time velocity integral and vessel diameter, where coronary blood flow =π×(coronary artery diameter/2)^2×(average peak velocity/2). Coronary endothelial dysfunction was defined as >20% decrease in mid-LAD diameter and/or <50% increase in coronary blood flow in response to acetylcholine infusion.[13-15] Subjects were thus identified as having normal or abnormal coronary endothelial dysfunction. These data for individual subjects are summarized in Table S1.

BOEC Culture

At time of angiography, before heparin administration, we drew 50- to 100-mL venous blood into citrate anti-coagulant and sent it to the University of Minnesota. Within 4 hours of venipuncture, we established BOEC cultures using an updated version of the long-term culture method we originally developed.[2] Using Histopaque-1077 and blood diluted with Ca++/Mg++ free Hanks buffered salt solution, we obtained blood mononuclear cells. These were washed twice with BOEC culture medium (EBM2 basal medium plus EGM-2 SingleQuot and 10% fetal bovine serum) and resuspended in 4 mL of the same. All 4 mL of cell suspension was added to a single well of a 6-well culture plate previously coated with collagen I. Cultures were incubated at 37°C in a humidified environment having 5% CO_2. After 16 hours, a gentle wash with culture medium removed debris and unattached cells. Thereafter, culture medium was changed daily for 7 days and thereafter on every other day. Subcultures were established on collagen I whenever cells reached 60% to 70% confluence. An important aspect of the method is fastidious application and meticulous performance of each of the extraordinary precautions we originally adopted (Data S1).

We harvested cells at a nominal 10^6-fold expansion to collect ≈3×10^7 BOEC. This degree of expansion falls well within a broad safe expansion window wherein (deliberately induced) acquired effects have washed out, yet long before onset of gene expression instability.[2] For the resulting 28 unique-patient BOEC cultures, we used fresh cells for quality control and RNA preparation, and we cryopreserved aliquots for later experimental use.

Nineteen BOEC cultures passed our multi-parameter quality control testing requiring: cobblestone morphology; staining positive for VE-cadherin, von Willebrand Factor, and P1H12 (CD146); staining negative for CD45 and CD14; a single population of cells at the sensitivity of light microscopy; and normal cytogenetics. Our previous studies documented that cultures meeting all these criteria additionally: are negative for CD133 and positive for multiple additional endothelial antigens; are 100% endothelial by FACS; display typical endothelial features such as VCAM-1 upregulation in response to tumor necrosis factor/interleukin-1, uptake of acetylated low-density lipoprotein, and tube formation in Matrigel; exhibit presence of Weibel Palade bodies; and display endothelial lineage fidelity by gene expression.[2,3] Each of the 9 quality control failures was because of cytogenetics analysis returned as being abnormal for culture acquired abnormalities. This left us with BOEC from the 6 normal and 13 abnormal subjects reported herein (Table 1).

Gene Expression

From each culture we isolated total RNA that was then reverse transcribed, quality verified, labeled, fragmented, and applied to Affymetrix U133A microarrays (assay for 14 500 well characterized genes and 18 400 transcripts/variants). To minimize possible batch effects, all samples for gene expression were profiled in a single batch at the University of Minnesota Microarray Core facility. As previously described,[4,5] we used the robust multi-array average method to background-adjust, quantile-normalize, and summarize expression using median polish algorithm, as implemented in the software Genedata Expressionist Pro3.1PP (Basel, Switzerland). Our analysis applied the R function "t test" for the Welch t test (we report uncorrected *P* values) and the R package "samr" for Significance Analysis of Microarrays that reports false discovery rate (FDR) with 500 permutations and a delta value of 0.719[16,17]; the code

Table 1. Subjects at Time of Enrollment

	Coronary Endothelial Function	
	Normal (n=6)	Abnormal (n=13)
Age, y	36.8±10.4 (24–49)	37.4±6.1 (22–45)
BMI, kg/m^2	23.5±4.1 (20.1–31.0)	27.7±3.9 (22.8–35.2)
C-reactive protein, nmol/L	4±1 (3–6)	18±23 (1–76)
C-reactive protein, mg/L	0.4±0.1 (0.3–0.6)	1.8±2.3 (0.1–7.6)
Men	2/6	7/13
White	6/6	13/13
Hypertension	0/6	2/13
Diabetes mellitus	0/6	0/13
Hyperlipidemia	0/6	10/13
Smoker, active	2/6	3/13
Smoker, never	3/6	7/13
Family history positive	3/6	11/13
History chest pain	6/6	13/13

BMI indicates body mass index.

used is provided in Data S1. We thus used the permutation-based Significance Analysis of Microarrays method and its associated FDR q-values as the primary criteria for statistical inference, while using the parametric, yet robust, Welch test[18] as secondary evidence of statistical significance.

Hierarchical Clustering

We conducted 2 clustering analyses,1 using only the universe of 43 transcripts exhibiting differential expression at $P<0.001$, and 1 using the universe of 9 transcripts exhibiting FDR <0.1%. We used R function "hclust" for unsupervised hierarchical clustering, using normalized gene expression, complete linkage, and 1 minus Pearson correlation as the distance measure. Expression level per probe was centered and normalized to have variance 1 before clustering. To assess relative discriminatory importance amongst these 43 transcripts, we constructed a random forest using normal/abnormal expression ratio as the response and each of the 43 transcripts as predictors. We determined variable importance by the mean decrease in the Gini coefficient in R package "randomForest."

Informatics

To identify biological inter-relationships we searched using databases: Enrichr, Ensembl, DIANA-TarBase v.8, miRbase, PathwayCommons, genomatix, Ingenuity Pathway Analysis, and the cardiovascular literature. BOEC gene expression data for the present 19 study subjects have been deposited in the NCBI Gene Expression Omnibus repository with accession number GSE132651 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132651). Other data sets used herein were deposited in the past: GSE22688 and GSE9877.

Bio-Functional Validation Testing

From cryopreserved aliquots, we re-established BOEC cultures from normal and abnormal subjects to seek bio-functional confirmatory data.

To assay HMGB1 (high mobility group box 1) content of culture medium, we used 1.9×10^5 BOEC at 85% confluence, switched to serum-free medium, and incubated ±100 ng/mL lipopolysaccharide. After 24 hours an ELISA measured HMGB1 released into medium.

To assay LAMC1 (laminin gamma 1) deposition into subendothelial matrix, we plated BOEC onto collagen I coated wells in sufficient number to reach confluence in 2 days. We then maintained them for 14 days, changing culture medium 3 times a week. Then, after rinsing with PBS, we treated culture wells with 0.5% Triton for 10 minutes to eliminate cell bodies but leave behind extracellular matrix on the well.[19] An in situ ELISA measured LAMC1 in matrix.

RESULTS

Characteristics of the 6 normal and 13 abnormal study subjects at time of coronary and gene studies reveal that, on average, the abnormal subjects had somewhat higher body mass index, C-reactive protein, and risk factor burden (Table 1). All but one subject was aged <46 years.

The reporter cells used here, BOEC, have themselves never been exposed to in vivo signaling effects.[2-5] Rather, they are the in vitro outgrowth progeny of a circulating, marrow-derived progenitor cell. Further, the necessary degree of their expansion in culture is sufficient for (deliberately) induced inflammatory responses to fully wash out yet is still many logs below the expansion degree at which phenotypic or gene expression drift is seen. Unlike other endothelial cell types, BOECs are very stable.

Single Gene Expression

At the significance threshold of FDR <10%, 29 transcripts exhibited differential expression for abnormal versus normal subjects (Table 2). Among the 9 transcripts exhibiting differential expression at the highly stringent threshold of FDR <0.1%, there was little overlap in the expression value ranges for normal versus abnormal groups (Figure 1).

The 43 transcripts exhibiting differential expression at the threshold of Welch $P<0.001$ are provided in Table S2.

Focus on HMGB1

Of the identified genes, we herein focus on the increased expression of *HMGB1* by abnormals (1.4-fold, FDR <0.1%, $P=5.7\times10^{-5}$) because of this protein's known and prominent role in arterial disease pathogenesis, specifically including the biology of atherosclerosis, per Discussion.[20] Arguing that high-*HMGB1* expression amongst abnormal subjects is not reflective of any proinflammatory in vivo milieu, *HMGB1* elevation was not accompanied by differential expression by any of 40 other inflammatory genes (Table S3).

Inverse Expression Linkage

Inspection of individual subject expression values suggested a sub-cluster of 7 abnormal with highest *HMGB1* expression and a sub-cluster of 7 abnormal with lowest *LAMC1* expression (Figure 1). These 2 subclusters were composed, nearly perfectly, of the same subjects, and there was a strong inverse correlation

Table 2. Differentially Expressed Transcripts at Threshold of False Discovery Rate <10%, Listed in Order of False Discovery Rate and Then by *P* Value

		False Discovery Rate		FOLD	
Probe Set	Gene	(%)	*P* Value	(Abnormal/ Normal)	NAME
209041_s_at	*UBE2G2*	≤0.1	2.9×10^{-6}	1.28	Ubiquitin conjugating enzyme E2 G2
209181_s_at	*RABGGTB*	≤0.1	8.5×10^{-6}	1.27	Rab geranylgeranyltransferase subunit beta
203622_s_at	*PNO1*	≤0.1	1.3×10^{-5}	1.42	Partner of NOB1 homolog
202855_s_at	*SLC16A3*	≤0.1	2.0×10^{-5}	1.84	Solute carrier family 16 member 3 (MCT4)
208996_s_at	*POLR2C*	≤0.1	4.3×10^{-5}	1.34	RNA polymerase II, subunit C
214938_x_at	*HMGB1*	≤0.1	5.7×10^{-5}	1.40	High mobility group box 1
212714_at	*LARP4*	≤0.1	1.1×10^{-4}	1.20	La ribonucleoprotein 4
213825_at	*OLIG2*	≤0.1	1.3×10^{-4}	1.11	Oligodendrocyte transcription factor 2
219082_at	*AMDHD2*	≤0.1	1.3×10^{-4}	0.82	Amidohydrolase domain containing 2
218447_at	*CMC2*	6.75	3.2×10^{-5}	1.35	C-X9-C containing motif containing 2
220890_s_at	*DDX47*	6.75	4.1×10^{-5}	1.22	DEAD box helicase 47
216149_at	*LRRC37BP1*	6.75	5.7×10^{-5}	1.13	Leucine rich repeat containing 37B pseudogene 1
220016_at	*AHNAK*	6.75	2.3×10^{-4}	1.15	AHNAK nucleoprotein
211999_at	*H3F3B*	6.75	3.7×10^{-4}	1.24	H3 histone family member 3B
208672_s_at	*SFRS3*	6.75	5.7×10^{-4}	1.30	Serine and arginine rich splicing factor 3
212394_at	*EMC1*	6.75	6.1×10^{-4}	1.13	ER membrane protein complex subunit 1
202856_s_at	*MCT4*	6.75	7.9×10^{-4}	1.72	Solute carrier family 16 member 3
200700_s_at	*KDELR2*	6.75	8.1×10^{-4}	1.17	KDEL endoplasmic reticulum protein retention receptor 2
201574_at	*ETF1*	6.75	8.2×10^{-4}	1.22	Eukaryotic translation termination factor 1
201862_s_at	*LRRFIP1*	6.75	9.7×10^{-4}	1.49	LRR binding FLII interacting protein 1
207094_at	*IL8RA*	6.75	1.3×10^{-3}	1.11	C-X-C motif chemokine receptor 2
214058_at	*MYCL1*	6.75	1.6×10^{-3}	1.11	MYCL proto-oncogene, bHLH transcription factor
201862_s_at	*LRRFIP1*	6.75	1.8×10^{-3}	1.49	LRR binding FLII interacting protein 1
218948_at	*QRSL1*	6.75	3.3×10^{-3}	1.26	Glutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1
216302_at	*HNRPC*	6.75	3.9×10^{-3}	1.11	Heterogeneous nuclear ribonucleoprotein C (C1/C2)
202564_x_at	*SNX15*	8.64	1.1×10^{-4}	0.86	Sorting nexin 15
200770_s_at	*LAMC1*	8.64	1.5×10^{-4}	0.71	Laminin subunit gamma 1
214150_x_at	*ATP6V0E*	8.64	4.2×10^{-4}	0.80	ATPase H+ transporting V0 subunit e1
221097_s_at	*KCNMB2*	8.64	5.8×10^{-3}	1.08	K^+ Ca-activated channel subfamily regulatory beta subunit

between *HMGB1* and *LAMC1* expression for the 19 study subjects (Figure 2A)

Providing indirect corroboration of this unexpected observation, we uncovered the same high-*HMGB1*/low-*LAMC1* relationship in our past studies of BOEC gene expression for separate groups of: 38 healthy 20- to 29-year-olds (Figure 2B),[5] 27 random normal subjects (Figure 2C),[4] and 20 children with sickle cell anemia (Figure 2D).[4] There was no apparent effect of sex on this high-*HMGB1*/low-*LAMC1* relationship.

Hierarchical Clustering

Hierarchical clustering analysis using the universe of 43 transcripts exhibiting differential expression at $P<0.001$ generated 2 primary clusters, one containing all normals and the other containing all abnormals (Figure S1A). The latter cluster had 2 secondary sub-clusters that separate the abnormals having highest versus lowest *HMGB1* expression. This suggests that *HMGB1* expression is an important—but not sole—discriminator.

Indeed, a random forest analysis of the same transcript universe estimated the strongest discriminators to also include *MCT4* (*SLC16A3*), *RABGGTB*, *LAMC1*, *UBE2G2*, *POLR2C*, *PNO1*, and *HMGCS1* (Figure S2). That these help discriminate between lowest- versus highest-*HMGB1* expressers (regardless of subject group) was supported by another hierarchical clustering analysis using only the 9 transcripts having FDR <0.1% (Figure S1B). This accurately generated 2 primary clusters: 1 composed of those abnormal subjects having the highest *HMGB1* expression, and the other containing those abnormals having the lowest *HMGB1* expression plus all normals.

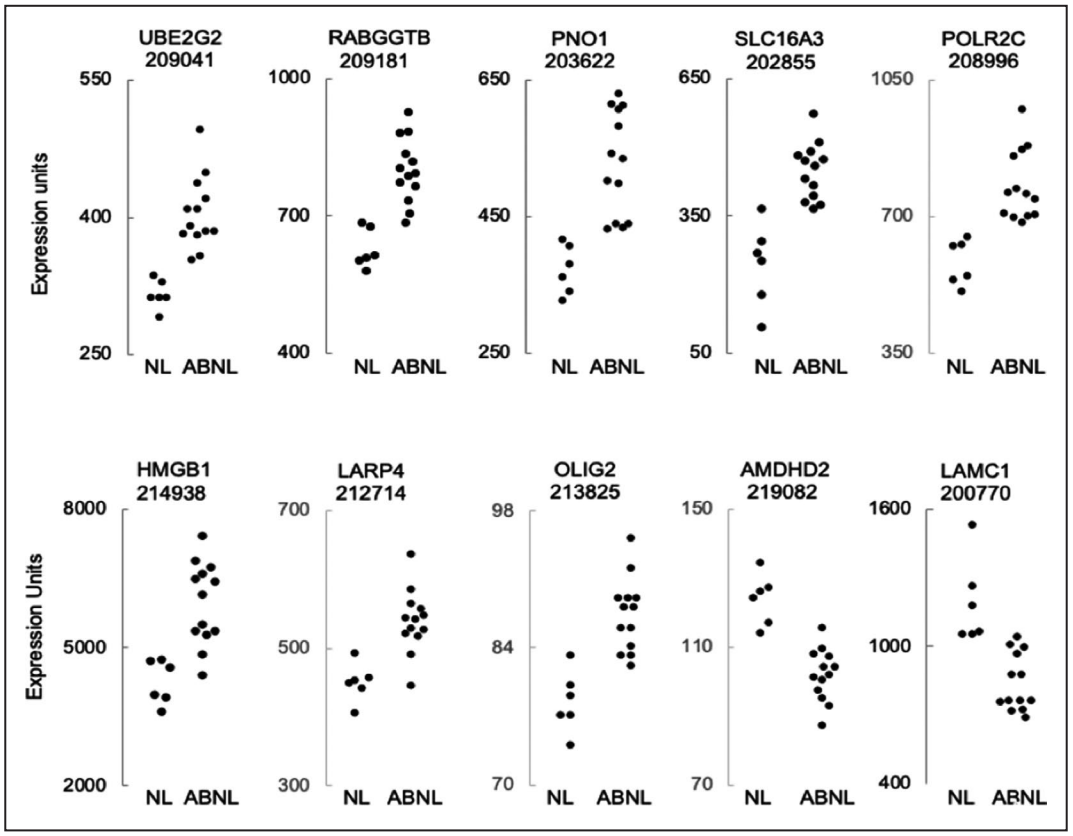

Figure 1. For individual normal and abnormal study subjects, expression values are plotted for the 9 transcripts (identified by gene name and Affymetrix probe set number) exhibiting differential expression at false discovery rate <0.1%, and also for LAMC1 (laminin gamma 1).
For the abnormal group, note the suggestion of an outlying cluster of 7 subjects with highest-*HMGB1* (high mobility group box 1) and an outlying cluster of 7 subjects with lowest-*LAMC1*. Gene names are expanded in Table 2.

Bio-Functional Validation

To bio-functionally test for elevated HMGB1 protein, we measured its release from BOEC incubated± lipopolysaccharide. Consistent with their abnormally high *HMGB1* gene expression, BOEC from abnormal released 13 ±3-fold greater HMGB1 than normal BOEC released; $P=8.5\times10^{-5}$ (Figure 3, left). This provides bio-functional confirmation that higher *HMGB1* gene expression by abnormal subjects is real.

To bio-functionally test for lowered LAMC1 protein, we incubated BOEC on plates originally coated with collagen and measured LAMC1 deposition into the subendothelial matrix after 14 days. Consistent with their abnormally depressed *LAMC1* expression, BOEC from abnormals deposited, on average, one tenth as much LAMC1 as identically-incubated normal BOEC (Figure 3, right). Despite the discernable difference, the result from this test was not statistically significant, likely because of small sample size.

Clinical Follow-Up

Since this clinical study was performed in 2005 to 2007, follow-up data are available but incomplete (institutional review board rules prohibit contacting non-Mayo subjects from this closed study) (Table 3). For 4 normals followed for 13.4±0.1 years, 1 experienced a major adverse cardiovascular event. For 12 abnormals followed for 9.1±4.5 years, 4 of 12 developed major adverse cardiovascular events. Interestingly, of these 4 abnormals who developed major adverse cardiovascular events, 3 were among the top-4 highest *HMGB1* expressors.

DISCUSSION

In addressing risk for early coronary endothelial dysfunction, the earliest clinical form of atherosclerosis,[8-10] we surveyed gene expression by Blood Outgrowth Endothelial Cells as an approach to bridging the troublesome gap between genetics and clinical phenotype. Our operational definition of "early" was subject age <50 years, and coronary reactivity testing identified subjects as having normal or abnormal coronary endothelial function. Our results indicate that the approach is, indeed, feasible and possibly can shed light upon underlying risk. Comparing abnormals versus normals we identified differential expression of 29 transcripts at FDR <10%,

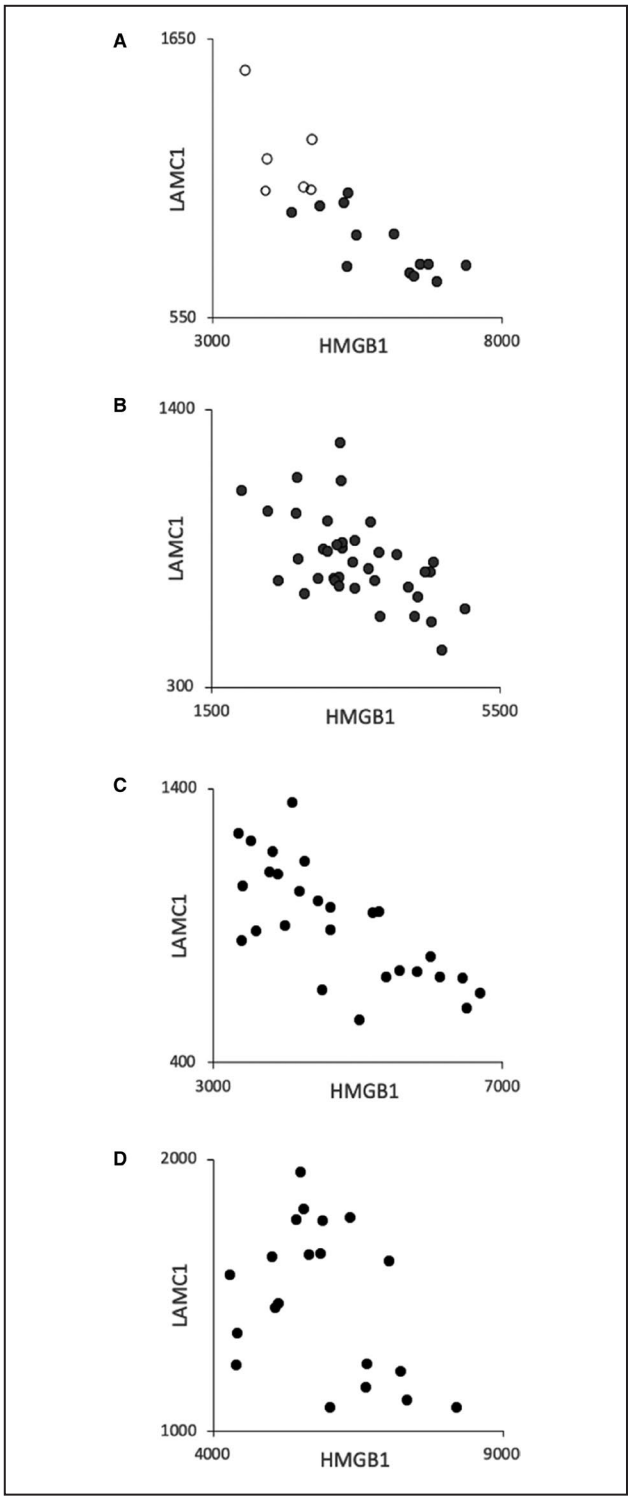

Figure 2. Inverse correlation between high-HMGB1 (high mobility group box 1) and low-LAMC1 (laminin gamma 1) expression. Scales display expression units which differ amongst panels because each study was done in a different time frame.
A, The present study in which normal are in open symbols and abnormal in closed symbols, r=−0.844. **B**, Healthy 20- to 29-year-olds, r=−0.569.[5] **C**, Random 23- to 69-year-olds, r=−0.728.[4] **D**, Children with sickle cell anemia, r=−0.383.[4] Gene expression data are deposited at Gene Expression Omnibus, series GSE13 2651, GSE22688, GSE9877, GSE9877, respectively.

of which 9 were significant at the stringent threshold of FDR <0.1%.

Of the latter group, we here focus on the abnormals having elevated expression of *HMGB1* in apparent linkage with lowered expression of *LAMC1*. We first review the most relevant aspects of HMGB1 and LAMC1 proteins, as each has unambiguous implications for atherogenesis. Then we focus on their expected gene-gene interactions that predict a remarkable, pathobiological convergence that would jeopardize endothelial cell responsiveness to shear stress.

HMGB1

HMGB1 expression was greater for abnormal subjects (1.4-fold, FDR <0.1%, P=5.7×10^{-5}), and the ranges for normal versus abnormal subjects barely overlapped. HMGB1, an "alarmin", is one of the damage-associated molecular pattern molecules. Its roles are diverse, remarkable, and highly relevant to atherosclerosis.[20-22]

Nucleus

HMGB1 is the most abundant non-histone nuclear protein, and it regulates multiple nuclear functions, among them gene expression. For example, HMGB1 enhances binding of nuclear factor-κB p50/p50 and p65/p50 to DNA, and it is reportedly required for p50 to be functional.[23] Although it traffics bidirectionally across the nuclear membrane, HMGB1 is normally highly skewed towards the nucleus. In monocytes, however, the skew is heavily towards cytoplasm.[24]

Cytoplasm

In cytoplasm, HMGB1 is part of many regulatory protein complexes, and it promotes translocation of nuclear factor-κB1, RelA and SP1 into the nucleus. Notably, HMGB1 associates with cytoplasmic Src, exerting an inhibitory influence that is discussed in the Gene-Gene Interactome section below.

Export

During necrosis or in response to injury or stimuli, HMGB1 is passively or actively exported from various cell types.[20-22] For example, endothelial cells release it in response to tumor necrosis factor,[25] abnormal shear stress,[26] or hypoxia.[20] Smooth muscle cells release it in response to cholesterol.[27] Neutrophils disgorge it into neutrophil extracellular traps,[28] and platelets contribute it to thrombi.[29] Monocytes/macrophages release HMGB1 in response to inflammatory stimuli and when manifesting their inflammatory reprogramming.

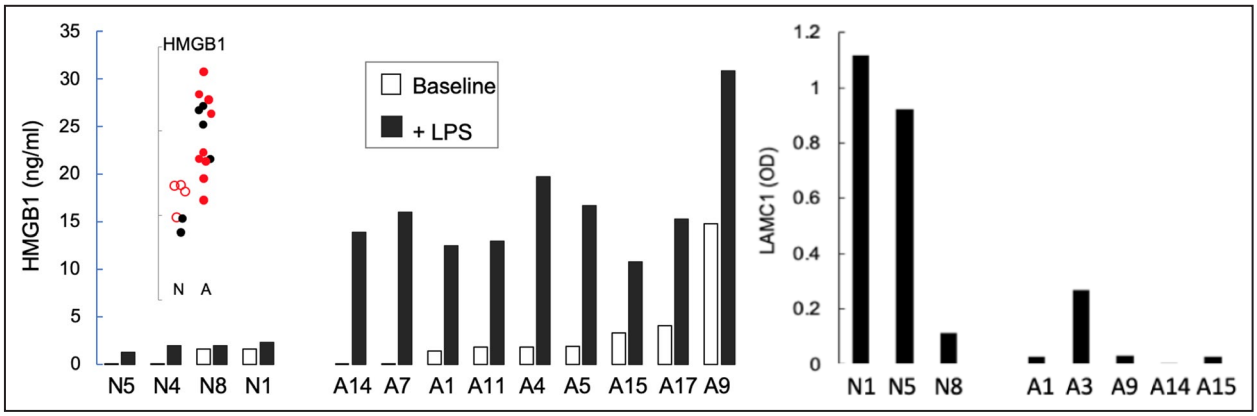

Figure 3. Bio-functional testing corroborates high-*HMGB1* (high mobility group box 1) and low-*LAMC1* (laminin gamma chain 1) expression in abnormal subjects (A) vs normal subjects (N). Left, HMGB1 content of culture medium over blood outgrowth endothelial cells measured ± lipopolysaccharide stimulation for 24 hours. Baseline release (white bars) tended to be higher for abnormal vs normal subjects (P=0.158). In response to lipopolysaccharide (black bars), abnormal blood outgrowth endothelial cells released 13.3-fold greater HMGB1 (P=8.5×10^{-5}). The specific subjects studied are indicated in red in the embedded *HMGB1* expression plot. Right, LAMC1 deposition into collagen subendothelial matrix by abnormal blood outgrowth endothelial cells over 14 days incubation was, on average, one tenth that by normal blood outgrowth endothelial cells. LPS indicates lipopolysaccharide.

Extracellular

Upon release, HMGB1 can act as a cytokine or chemokine, able to induce and amplify inflammation by engaging multiple receptors, especially toll-like receptor 4 and the receptor for advanced glycation end-products. Indeed, HMGB1 is a dominant driver of sterile inflammation.[20-22] It activates monocytes/macrophages and platelets, as well as endothelial and smooth muscle cells that then adopt a proliferative phenotype. It causes endothelial barrier hyperpermeability, facilitating egress of WBC and atherogenic lipid, and it activates endothelial NADPH oxidase.[22]

Atherogenesis

Each of the above HMGB1 effects is atheropromotive in nature and would plausibly be exaggerated in those with inherently high *HMGB1* expression. Indeed, HMGB1 already is believed to promote all stages of atherosclerosis from inception to plaque rupture, and it is specifically implicated in coronary, peripheral and cerebral arterial disease.[20] For example, antibody-mediated neutralization of HMGB1 attenuated atherosclerosis by >50% in apolipoprotein E deficient mice,[30] and genetic deletion or neutralization of HMGB1 prevented intimal hyperplasia in response to carotid wire injury.[31]

Table 3. Available Subject Data at Subsequent Clinical Follow-Up

	Coronary Endothelial Function	
	Normal	Abnormal
Number available	4/6	12/13
Age at coronary study, y	38.0±10.4 (24–49)	40.8±1.6 (38–42)
Follow-up duration, y	13.4±0.1 (13.3–13.5)	9.1±4.5 (1.2–14.0)
Experienced MACE, n	1/4	4/12
Age at MACE, y	55	50±6 (39–54)
Study-to-MACE interval, y	13	1, 12, 12, 12

For the normal subject this was a myocardial infarction. For the abnormal subjects it was: 1 new diagnosis of congestive heart failure; 1 stroke; 1 myocardial infarction; 1 peripheral artery disease and carotid endarterectomy needed. MACE indicates major adverse cardiovascular events.

SNP Insight

We did not conduct SNP testing here, but extant data link increased HMGB1 expression to arterial disease. The *HMGB1* 3814 polymorphism (rs2249825) is predicted to create a strong enhancer effect on *HMGB1* expression,[32] and leukocytes from such individuals actually do exhibit exaggerated HMGB1 release when challenged with lipopolysaccharide[33]—just as we saw here for BOEC from abnormals. Studied in Chinese, this SNP is associated with hypertension,[34] increased cerebral ischemia size,[35] and exaggerated inflammation in sepsis.[33]

LAMC1

LAMC1 expression was lower for abnormal subjects (0.71-fold, FDR=8.64%, P=4.2×10^{-4}), and ranges for normal versus abnormal subjects barely overlapped. Laminins are important to all vessel wall cells and engage them with functional reciprocity.[36]

Endothelial cells produce the 2 laminin heterotrimers (α4β1γ1 and α5β1γ1) of their basement membrane contact layer, and assembly of these heterotrimers specifically requires involvement of LAMC1,

the laminin γ1 chain.[36] Indeed, experimental knockout of *LAMC1* resulted in embryonic lethality with failure to make basement membranes.[36,37] Remarkably, laminin α5β1γ1 was experimentally found to be necessary for endothelial response to shear stress.[38]

Thus, even if LAMC1 production is just lowered, harmful consequences may be predictable. One is enhanced endothelial barrier hyperpermeability.[39] Another consequence is that in endothelial basement membranes if laminin is missing it is maladaptively replaced with fibronectin, replacing laminin-enforced endothelial quiescence with fibronectin-mediated inflammatory signaling.[40] Particularly notable is that endothelial cells on laminin are shear responsive, but those on fibronectin are not.[41] Thus, depressed LAMC1 production could replicate several key features of atherogenesis.[42]

Atherogenesis

We find nothing in the literature that directly links LAMC1 to atherosclerosis, but there are some suggestive data. First, a study of quantitative trait loci identified the *Ath44* region of chromosome 1 as being associated with aortic root lesion size in murine atherosclerosis; *LAMC1* is a gene in this region.[43] Second, an analysis of bio-functional pathways enriched in advanced versus early coronary atherosclerosis identified focal adhesion (critical in shear responsiveness) to be an implicated functional module, within which *LAMC1* was one of the abnormally downregulated genes associated with arteriopathy severity.[44]

Gene-Gene Interactome Impacting Endothelial Function

For all these reasons, we predict that the combination of high-*HMGB1* plus low-*LAMC1* expression in endothelium would converge biologically and detrimentally at the focal adhesion complex that is required for endothelial cell mechanosensing of shear stress.[36,41,45] Specifically, focal adhesion complex function would be impaired by HMGB1 because it inhibits the reciprocal phosphorylations[46] between Src and FAK (focal adhesion kinase) that enable focal adhesion complex to participate in normal shear sensing. As for LAMC1, it is necessary for the proper basement membrane engagement with endothelial abluminal integrins[36-38,41] that is required for their clustering, an on-switch for focal adhesion complex function. That could be jeopardized if LAMC1 production is low.

We suggest that these known effects of high-HMGB1 and of low-LAMC1 would synergistically undermine the endothelial cell's ability to respond adaptively and optimally to shear stress. Thus, we hypothesize that impairment of shear responsiveness in this manner would maladaptively foster endothelial dysfunction and, thereby, earlier development of detectable atherogenesis.

Concordance of High-*HMGB1* and Low-*LAMC1* Expression

The unexpected linkage between high-*HMGB1* and low-*LAMC1* expression revealed by our data is corroborated via archived data sets from our 3 previous studies of BOEC gene expression (Figure 2). This suggests an underlying regulatory relationship, although our data cannot inform as to mechanism. The primary aberrancy could be high-*HMGB1,* or low-*LAMC1,* or something else affecting both. So, to illustrate relevance but simplify discussion, we here arbitrarily assume that elevated *HMGB1* expression is the primary aberrancy that drives lower LAMC1 expression.

Suppression of *LAMC1* is possible via microRNA (miR) species already implicated in atherosclerosis.[47,48] Perhaps most intriguing is miR-21 that is induced by both HMGB1 and oscillatory flow regimes,[49,50] and miR-21 is known to target *LAMC1*.[48,49] Interestingly, miR-21 is the most abundant miR in normal BOEC (Hebbel and Steer, unpublished observation, 2012), and it is upregulated in human atherosclerotic tissue.[47,48] Other *LAMC1*-targeting miRNAs are identified, as well as some that target *LAMC1* transcription factors. One of these is *ESR1* (estrogen receptor alpha) that not only is hypermethylated in atherosclerosis but also is a target of miR-206, another miR that can be induced by HMGB1.[51]

Caveats and Limitations

This study probes the early onset of atherosclerosis, but we recognize that "early" here can mean truly earlier onset and/or accelerated progression and/or earlier symptom awareness. The gene expression changes we have highlighted are perhaps consistent with each. Regardless, we surmise that such changes would not only nudge an individual's homeostatic balance in a maladaptive direction but also enhance susceptibility to risk factors. For example, elevated constitutive *HMGB1* expression could result in its exaggerated release from endothelium residing in anatomically atheroprone areas.[26]

We emphasize that all of our subjects were studied by coronary catheterization because they had an episode of chest pain. Thus, our normal subjects were not truly normal individuals. Rather, our labels " normal" and "abnormal" refer specifically to their coronary endothelial function status. Consistent with this, our abnormal subjects did exhibit somewhat greater

frequency of some risk factors, most notably hyperlipidemia and elevated C-reactive protein. Experimentally, HMGB1 can induce C-reactive protein,[52] but hyperlipidemia can stimulate HMGB1 release of HMGB1.[27] We find no evidence that C-reactive protein or lipid increase HMGB1 expression.

Cultured cells always raise concerns about laboratory-induced variations. However, our prior standardization, reproducibility, and validation studies,[2-5] plus our extensive experience with BOECs, justify confidence that our data identify true differences between endothelial cells from abnormal versus normal. We emphasize that a critical factor in achieving this is the fastidious application of all our extraordinary culture precautions described in Data S1.

Further, measured BOEC gene expression is not influenced by in vivo signaling exposures[2-5] as the BOEC themselves have never been exposed to inflammation or tissue-specific signaling. Their 10^6-fold expansion is logs beyond what is needed for inflammatory signaling effects to wash out, and it is logs before any phenotypic or genotypic drift appears.[3,4] BOEC are far more stable in culture than other endothelial types. Thus, we believe that here, as in our previous studies of this nature,[4,5] results likely reflect heritable differences between abnormal and normal subjects.

Of course, it is impossible to absolutely exclude the possibility that, in the abnormal subjects, an atherogenic environment created durable epigenetic changes within the circulating endothelial progenitors, changes then passed to their outgrowth progeny, the BOEC. Somewhat mitigating this concern, elevated *HMGB1* expression by the BOEC from abnormal subjects was not accompanied by differential expression of any of 40 other inflammation-responsive genes.

Finally, we do not know if these expression changes are actually endothelial specific. If not, perhaps they could be identified in a more easily accessible cell type. For other cell types, however, there would be magnified concern about acquired influences or artifacts. And, of course, if interest lies in the functional biology of endothelial cells, BOEC uniquely offer the opportunity to examine this.

CONCLUSIONS

Our data reveal an association between high-*HMGB1* plus low-*LAMC1* expression with coronary endothelial dysfunction at age <50 years. This pathobiologically-relevant, probably-heritable combination could create risk via a detrimental biological convergence that maladaptively impairs endothelial mechanosensing. Our results may have practical clinical implications since the approach can be applied even in children,[4] perhaps enabling identification of those who would most benefit from earlier, more aggressive medical intervention.

We recognize, of course, that this feasibility study is too limited to be definitive. Nonetheless, it does support the notion that BOEC can be used to bridge the information gap between genomics and clinical phenotype in understanding atherosclerosis risk. Indeed, BOEC comprise a unique platform that enables matching donor characteristics with endothelial functional assessment with various "omics." In that regard, sufficient BOEC can easily be produced to enable studying all "omics" simultaneously for each endothelial cell culture. We suggest that this would be uniquely useful for achieving the truly integrative endothelial "omics" that may be key in understanding the heritable component of atherosclerosis risk.

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Disclosures

None.

Supplementary Materials

Data S1
Tables S1–S3
Figures S1–S2

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SUPPLEMENTAL MATERIAL

Data S1.

SUPPLEMENTAL METHODS

BOEC culture

The present study was enabled by our prior developmental, standardization, and validation studies performed in 2000-2003 and described elsewhere (text references 2-5). These involved BOEC cultures from >150 unique individuals, with gene expression surveyed on >80. At our period of peak activity, we successfully established BOEC cultures 2-3 times per week, with a success rate of ~90%. As developed by the Hebbel Lab (Univ of Minnesota) our method includes taking each of the following extraordinary precautions to avoid culture variation effects and to maximize reproducibility.

[a] Necessary supplies, reagents, microarray chips, culture medium ingredients are acquired in sufficient quantity prior to study onset so there is no risk that lot number of anything could change in mid-study.

[b] At venipuncture, the first few ml of peripheral venous blood is discarded.

[c] Blood is maintained at room temperature between venipuncture and culture by using special shipping boxes [8] that provide this protection.

[d] Although in the past we have successfully established BOEC cultures up to 8 hours after venipuncture, we make every effort to minimize the venipuncture-to-culture delay. In the present study the interval between blood attainment and starting culture set-up was <4 hours.

[e] Any single step of the overall process (i.e., culture set-up/maintenance, quality control assessments, RNA preparation, cell biology experiments) is always performed by the same trained and highly-experienced technician known to consistently achieve highly reproducible results. For example, for the present study the single BOEC culture technician was available around-the-clock daily for ~2 years.

[f] All cultures are set up in the same culture room, using the same culture hood, using the same temperature/humidity/gas-controlled incubator. For our studies, a culture room is dedicated to BOEC specifically, and only the designated BOEC culture technician has access.

[g] All cultures are passed to the same extent, a nominal million-fold expansion providing ~$3x10^7$ BOEC. They are always harvested 4 hours after the last change of culture medium and when at 85-90% confluence. This degree of expansion is solidly within what we previously found to be a “safe window” of expansion: deliberately acquired gene expression changes (from IL-1/TNF) have completely washed out; and the cells are several logs of expansion shy of developing instability of phenotype or gene expression.

[h] Quality Control: All cultures are subjected to rigorous quality control measures, with success indicated by: cobblestone morphology; positive for VE-cadherin and vWF and P1H12(CD146); negative for CD45 and CD14; single population of cells at level of light microscopy. In addition, all cultures are submitted for cytogenetics analysis (to enable later exclusion of data from any exhibiting culture-acquired cytogenetic abnormalities).

Our prior standardization and validation studies revealed that BOEC cultures passing this multi-parameter screen additionally: are negative for CD133; 100%

endothelial at level of FACS; positive for multiple endothelial antigens (flk1, PECAM-1, VCAM-1, ICAM-1, CD34, CD51, thrombomodulin); exhibit typical endothelial behaviors such as “in vitro angiogenesis”, acLDL uptake, VCAM-1 upregulation in response to TNF/IL-1. Also, gene expression profiling confirms endothelial lineage identity, and EM reveals Weibel Palade bodies.

Antibodies Used

Antibody	Source	catalog #	Working dilution or concentration
anti-vWF	Sigma	F3520	1 μg/ml
anti-VE-cadherin	Santa Cruz	sc-6458	1 μg/ml
anti-CD146	Hebbel lab	P1H12	5 μg/ml
anti-CD45	Santa-Cruz	sc-25590	1 μg/ml
anti-CD14	Santa Cruz	sc-9150	1 μg/ml
anti-HMGB1	ABCAM	ab190377	1:500 dilution
anti-LAMC1	Sigma	sab 4051727	1 μg/ml

Code used for samir:

```
require(samr)
x = as.matrix(expressionDat)
y = c(rep(2,13),rep(1,6))
data=list(x=x,y=y, geneid=probe.id, genenames= gene.id, logged2=F)
samr.obj<-samr(data, resp.type="Two class unpaired", nperms=500)
delta.table <- samr.compute.delta.table(samr.obj)
delta=0.719
samr.plot(samr.obj,delta)
siggenes.table<-samr.compute.siggenes.table(samr.obj,delta, data, delta.table)
```

Table S1. **Coronary reactivity assessment summary.**

Definition(s) of abnormal coronary endothelial function:
less than 50% increase in coronary blood flow (CBF) in response to highest dose acetylcholine
and/or
more than 20% reduction in coronary artery diameter (CAD) in response to highest-dose acetylcholine

subject	% change CBF in response to highest-dose acetylcholine	% CAD change in response to highest-dose acetylcholine	subject group assignment
A	52	-15	NL
B	99	-9	NL
C	133	0	NL
D	129	0	NL
E	555	49	NL
F	57	-20	NL
G	63	-30	ABNL
H	20	-42	ABNL
I	-24	-28	ABNL
J	56	-30	ABNL
K	48	7	ABNL
L	7	-4	ABNL
M	31	-21	ABNL
N	46	-10	ABNL
O	-10	-26	ABNL
P	-24	-36	ABNL
Q	22	-33	ABNL
R	-57	-35	ABNL
S	-100	-100	ABNL

Table S2. Differentially expressed transcripts at threshold of Welch P<0.001,listed in order of P value.

PROBE SET	GENE	FDR (%)	P	FOLD (ABNL/NL)	EXPANDED NAME
209041_s_at	UBE2G2	≤0.1	$2.9x10^{-6}$	1.28	ubiquitin conjugating enzyme E2G2
209181_s_at	RABGGTB	≤0.1	$8.5x10^{-6}$	1.27	Rab geranylgeranyltransferase subunit beta
203622_s_at	PNO1	≤0.1	$1.3x10^{-5}$	1.42	partner of NOB1 homolog
202855_s_at	SLC16A3	≤0.1	$2.0x10^{-5}$	1.84	solute carrier family 16 member 3
218447_at	CMC2	6.75	$3.2x10^{-5}$	1.35	C-X9-C containing motif containing 2
220890_s_at	DDX47	6.75	$4.1x10^{-5}$	1.22	DEAD box helicase 47
208996_s_at	POLR2C	≤0.1	$4.3x10^{-5}$	1.34	RNA polymerase II, subunit C
214938_x_at	HMGB1	≤0.1	$5.7x10^{-5}$	1.40	high mobility group box 1
216149_at	LRRC37BP1	6.75	$5.7x10^{-5}$	1.13	leucine rich repeat containing 37B pseudogene 1
212714_at	LARP4	≤0.1	$1.1x10^{-4}$	1.20	La ribonucleoprotein domain family member 4
202564_x_at	SNX15	8.64	$1.1x10^{-4}$	0.86	sorting nexin 15
213825_at	OLIG2	≤0.1	$1.3x10^{-4}$	1.11	oligodendrocyte transcription factor 2
219082_at	AMDHD2	≤0.1	$1.3x10^{-4}$	0.82	N-acetylglucosamine-6-phosphate deacetylase
200770_s_at	LAMC1	8.64	$1.5x10^{-4}$	0.71	laminin subunit gamma 1
212601_at	ZZEF1	>10	$1.5x10^{-4}$	0.88	zinc finger, ZZ type with EF hand domain
213826_s_at	H3F3B	>10	$1.8x10^{-4}$	0.84	H3 histone, family 3B
44120_at	ADCK2	>10	$2.1x10^{-4}$	0.91	aarF domain containing kinase 2
220016_at	AHNAK	6.75	$2.3x10^{-4}$	1.15	AHNAK nucleoprotein
203202_at	KRR1	>10	$2.8x10^{-4}$	1.29	KRR1, small subunit processome component homolog
208765_s_at	HNRNPR	>10	$3.5x10^{-4}$	1.18	heterogeneous nuclear ribonucleoprotein R
207734_at	LAX1	>10	$3.7x10^{-4}$	1.08	lymphocyte transmembrane adaptor 1
211999_at	H3F3B	6.75	$3.7x10^{-4}$	1.24	H3 histone family member 3B
205822_s_at	HMGCS1	>10	$4.0x10^{-4}$	1.48	3-hydroxy-3methylglutary-Coenzyme A synthase 1
217370_x_at	FUS	>10	$4.2x10^{-4}$	1.29	FUS RNA binding protein
214150_x_at	ATP6V0E	8.64	$4.2x10^{-4}$	0.80	ATPase H+ transporting V0 subunit e1

221255_s_at	EMC6	>10	$4.4x10^{-4}$	1.21	ER membrane protein complex subunit 6
217370_x_at	FUS	>10	$4.2x10^{-4}$	1.29	RNA binding protein FUS
222382_x_at	NUP205	>10	$5.1x10^{-4}$	1.16	nuclear pore complex protein Nup205
201965_s_at	SETX	>10	$5.7x10^{-4}$	0.85	senataxin
208672_s_at	SFRS3	6.75	$5.7x10^{-4}$	1.30	serine and arginine rich splicing factor 3
212394_at	EMC1	6.75	$6.1x10^{-4}$	1.13	ER membrane protein complex subunit 1
219836_at	ZBED2	>10	$6.2x10^{-4}$	1.14	zinc finger, BED-type containing 2
202722_at	HMGCL	>10	$6.7x10^{-4}$	0.86	hydroxymethylglutaryl-CoA lyase mitochondrial
202856_s_at	SLC16A3	6.75	$7.0x10^{-4}$	1.72	solute carrier family 16 member 3
208990_s_at	HNRPH3	>10	$7.1x10^{-4}$	1.31	heterogeneous nuclear ribonucleoprotein H3 (2H9)
211933_s_at	HNRNPA3	>10	$7.2x10^{-4}$	1.21	heterogeneous nuclear ribonucleoprotein A3
214409_at	RFPL3S	>10	$7.3x10^{-4}$	1.19	RFPL3 antisense [ncRNA]
215558_at	C6orf133	>10	$8.1x10^{-4}$	1.11	chromosome 6 open reading frame 13315
204647_at	HOMER3	>10	$8.2x10^{-4}$	0.74	homer scaffold protein 3
201574_at	ETF1	6.75	$8.2x10^{-4}$	1.22	eukaryotic translation termination factor 1
214882_s_at	SFRS2	>10	$8.7x10^{-4}$	1.27	splicing factor, arginine-serine rich 2
200700_s_at	KDELR2	6.75	$8.7x10^{-4}$	1.17	KDEL endoplasmic reticulum protein retention R2
201862_s_at	LRRFIP1	6.75	$9.7x10^{-4}$	1.49	LRR binding FLII interacting protein 1
210269_s_at		>10	$9.9x10^{-4}$	0.85	DNA segment on X & Y 155 expr. sequence

Table S3. ABNLs vs NLs did not significantly differ in expression of 40 inflammation-response genes (for each gene, all transcripts are listed).

Gene `	Probest	Fold Difference ABNL/NL)	P
AGER	210081_at	1.00	0.987
	217046_s_at	0.87	0.076
CAT	215573_at	0.98	0.616
C3	217767_at	0.90	0.077
	211922_s_at	0.81	0.110
	201432_at	0.83	0.044
C4	214428_x_at	1.02	0.554
	208451_s_at.	1.00	0.985
C5	205500_at	1.04	0.600
CCL2	216598_s_at	0.90	0.827
CCL3	205114_s_at	1.00	0.974
CRP	205753_at	0.99	0.885
	37020_a	0.94	0.215
F3	204363_at	1.00	0.971
HMOX1	203665_at	1.01	0.978
ICAM1	215485_s_at	0.87	0.305
	202638_s_at	0.73	0.294
	202637_s_at	0.84	0.371
IFNB1	208173_at	0.97	0.631
IL1β	205067_at	1.00	0.936
	39402_at	0.98	0.515
IL2	207849_at	0.98	0.645
IL6	205207_at	1.20	0.213
IL8	202859_x_at	0.90	0.678
	211506_s_at	0.68	0.412
JUN	213281_at	1.00	0.981
	201466_s_at	0.94	0.607
	201465_s_at	0.92	0.281
	201464_x_at	0.91	0.313
MYC	202431_s_at	1.09	0.272
NFKB1	209239_at	1.02	0.785
NFKB2	209636_at	0.88	0.089
	207535_s_at	0.84	0.309
	211524_at	1.04	0.384
NFKBIA	201502_s_at	0.86	0.267
NOS1	207309_at	1.06	0.202
	207310_s_at	0.98	0.576
NOS3	205581_s_at	1.08	0.781
PPARG	208510_s_at	0.74	0.154
PTK2	207821_s_at	0.95	0.514
	208820_at	0.92	0.241

REL	206036_s_at	0.99	0.862
RELA	201783_s_at	1.01	0.717
	209878_s_at	1.01	0.901
RELB	205205_at	0.88.	0.236
SELE	206211_at	0.65	0.357
SEL	206049_at	0.91	0.617
SP1	214732_at	1.04	0.434
TGFβ	203084_at	0.94	0.230
	203085_s_at	0.96	0.750
TLR2	204924_at	0.79	0.216
TLR4	221060_s_at	0.81	0.410
TP53	211300_s_at	1.06	0.660
	201746_at	0.98	0.857
TNF	207113_s_at	1.01	0.770
SOD1	200642_at	1.02	0.757
SRC	221281_at	0.95	0.370
	213324_at	0.97	0.668
	221284_s_at	0.92	0.225
VCAM1	203868_s_at	1.14	0.799
VEGF	212171_x_at	0.95	0.368
	210513_s_at	0.91	0.402
	211527_x_at	0.87	0.244
	210512_s_at	0.84	0.407
	211527_x_at	0.87	0.244
	212171_x_at	0.95	0.368

Figure S1. Hierarchical clustering analyses. A. Clustering using the universe of all 43 transcripts significant at P<0.001 suggests *HMGB1* is important but not the sole discriminator between NLs vs ABNLs. The low-*HMGB1* subjects in the ABNL group are outlined in the inset. **B**. Clustering using only the 9 transcripts significant at FDR<0.1% reveals substructure discriminating all lowest *HMGB1* expressers (those encircled in the inset) from the highest *HMGB1* expressers, regardless of subject group.

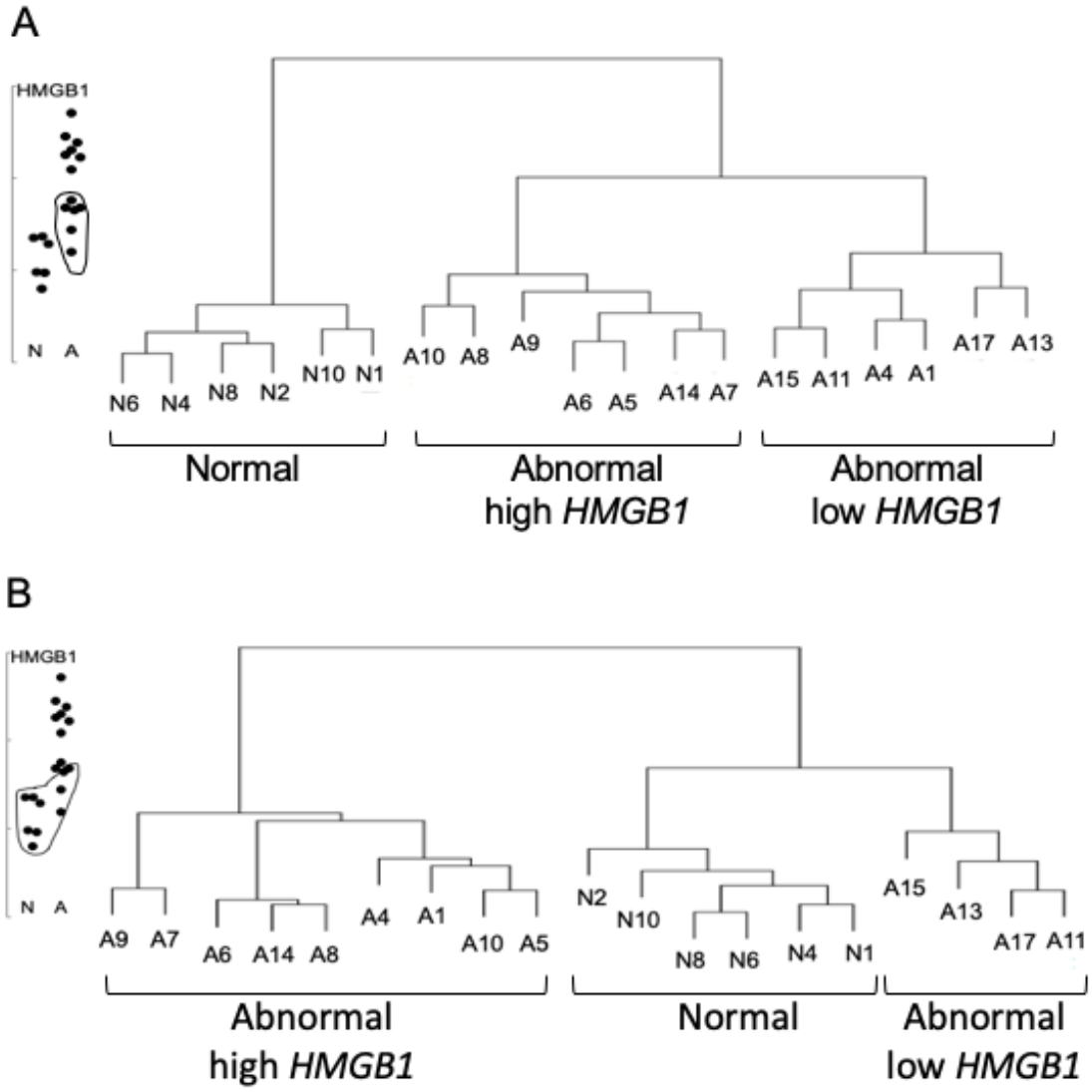
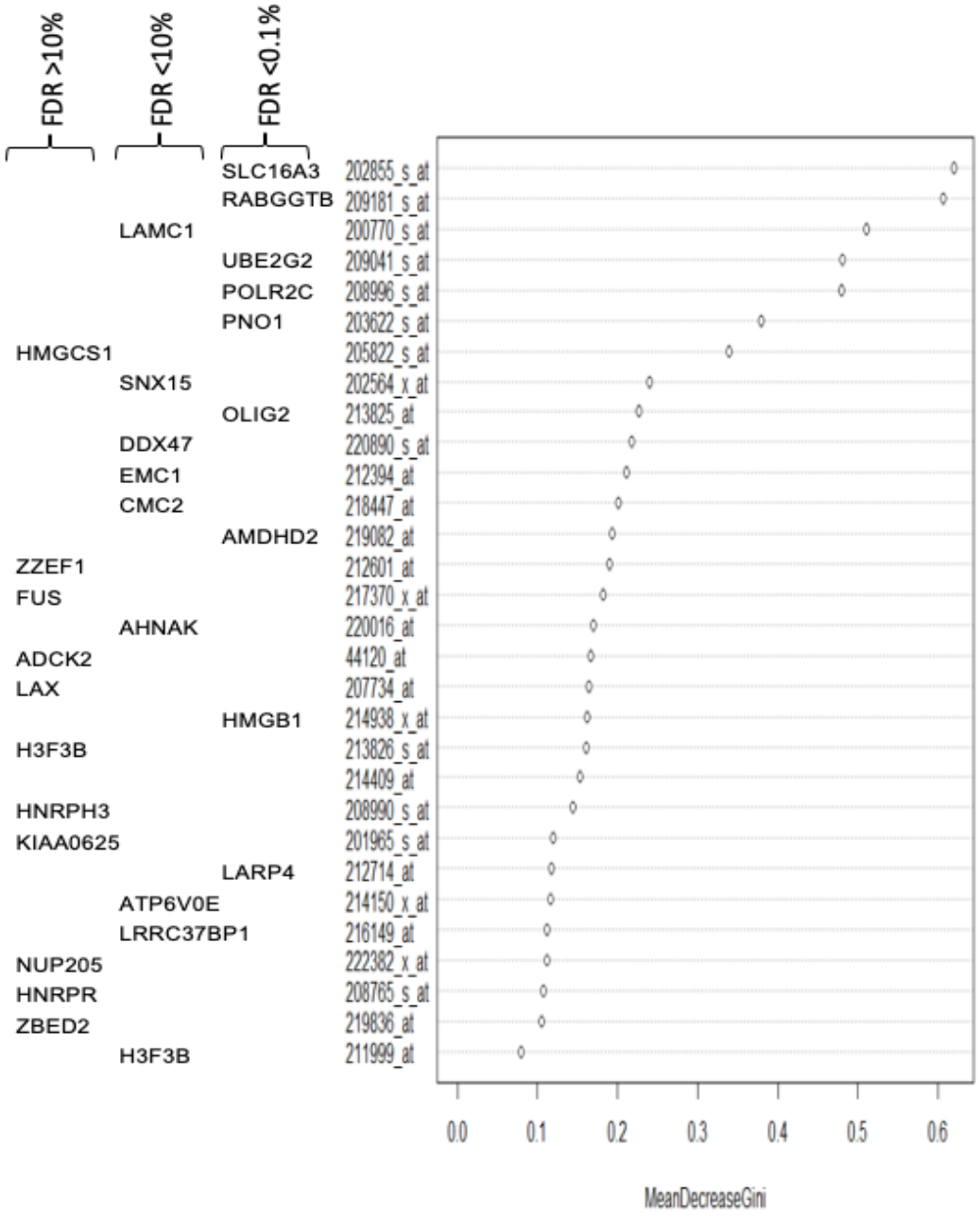


Figure S2. Random forest analysis.



We applied a random forest approach (using the universe of 43 transcripts significant at P<0.001 that yielded the clustering pattern shown in Supplemental Figure 1A) to estimate relative degree of contribution made by individual transcripts/genes in correctly separating ABNLs from NLs. Stronger contribution is rightwards on the horizontal axis. At the left, transcripts are listed by gene names in one of three columns indicating their significance level: FDR>10% (far left), FDR<10% but >0.1% (middle), FDR <0.1% (right).