Reports

High-throughput genotyping of advanced congenic lines by high resolution melting analysis for identification of *Bbaa2*, a QTL controlling Lyme arthritis

Kenneth K.C. Bramwell¹, Ying Ma¹, John H. Weis¹, Cory Teuscher² and Janis J. Weis¹

¹Department of Pathology, University of Utah, Salt Lake City, UT, USA, and ²Department of Medicine, University of Vermont, Burlington, VT, USA

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Congenic mapping is a powerful strategy to identify genomic loci regulating quantitative traits. Generating congenic lines is an iterative process of refinement that is both time and resource intensive. Here we report an alternative to traditional microsatellite marker analysis or costly high-density oligonucleotide single nucleotide polymorphism (SNP) arrays for congenic genotyping. The identification of inherited SNP variability in congenic lines using high resolution melting analysis (HRMA) represents a novel application of the method. The blocked probe HRMA approach offers a scalable, low cost, closed-tube system that benefits from rapid turnaround times, and unequivocal interpretation. The markedly higher prevalence of SNPs relative to microsatellites in the genome allows much greater flexibility for the identification of new genotyping landmarks as congenic intervals are refined. We have adopted this approach in our development of *B6.C3-Bbaa2* congenic lines for the identification of loci regulating murine Lyme arthritis severity. As a result, we have been able to fully genotype individuals prior to weaning age, and expand our number of breeding cages without increasing our colony budget. Thus far, 26 SNP markers have been successfully mapped to the *Bbaa2* locus. This has facilitated the identification of 20 novel *B6.C3-Bbaa2* congenic lines spanning the original interval.

The central dogma of molecular biology is that heritable differences in DNA lead to changes in mRNA transcripts, proteins, and ultimately to observable variations in phenotypes such as disease susceptibility (1). Inbred mouse strains provide an invaluable resource of fixed genomic variability to scientifically investigate this genetic component of disease susceptibility. The ability to generate intercross populations between susceptible and resistant inbred mouse strains, combined with powerful statistical analysis, allows regulatory loci of quantitative traits to be mapped to regions of individual chromosomes. Once identified, the breeding of congenic mouse lines allows individual regulatory loci to be isolated in the context of a controlled genetic background, and then to be narrowed to finer resolution in an

unbiased manner through additional backcrossing to parental lines. The genetic resolution is achievable through such breeding strategies is limited by the number and position of experimentally discernable genetic landmarks across the region of interest. Microsatellite markers have long been a key resource for genetic mapping due to their prevalence and ease of analysis by PCR.

The *Bbaa2* murine Lyme arthritis susceptibility locus was identified in multiple independent intercrosses between resistant and susceptible inbred strains (2–3). Roper *et al.* used composite interval mapping (CIM) on reciprocal backcross populations between C3H/HeN (C3H) and either C57BL/6 (B6) or Balb/c to identify complex regulation by up to four putative QTL within *Bbaa2Bbaa3*, with a maximum

LOD score of 10.2. Reciprocal interval specific congenic lines (ISCLs) for the *Bbaa2Bbaa3* region on chromosome 5 developed on both the B6 and C3H backgrounds each retained a significant portion of the disease susceptibility phenotype conferred by the parental line (4). However, the full *B6.C3-Bbaa2Bbaa3* congenic interval spanned 72.29 – 141.16 Mbp and included more than 870 genes and ESTs, making candidate gene selection impractical.

Efforts have been made to further refine regulatory loci within *Bbaa2* to a resolution of 1 Mb or narrower through additional ISCL backcrossing. However, given the putative complexity of the *Bbaa2* locus, the availability of microsatellite markers across the interval became a limiting factor, prompting the evaluation of alternative genotyping strat-

egies. At present, the dbSNP database contains more than 8,000 single nucleotide polymorphisms (SNPs) which

differ between B6 and C3H inbred lines across the *Bbaa2* interval (5). Although the C3H/HeN mouse strain used to

create our congenic lines is not one of the eighteen currently included in the Sanger SNP re-sequencing project (6), data for the closely related C3H/HeJ strain is available. The Sanger database contains over 19,000 high quality SNPs which differ between C3H/HeJ and C57BL/6NJ SNPs in the *Bbaa2* interval. A SNP genotyping strategy thus offered the potential to dramatically improve genetic resolution by increasing the number of discernable landmarks available. Several previously described SNP genotyping methodologies were evaluated: amplification refractory mutation system (ARMS)-PCR (7), small amplicon high resolution melting analysis (HRMA) (8), and blocked probe HRMA (9). We found that both methods of HRMA were useful for the genotyping of inherited intervals in congenic animals, with blocked probe HRMA being especially practical. Recent studies have emphasized the application of HRMA analysis for clinical diagnostics, but its application toward precise characterization of congenic mice has not previously been described.

Materials and methods

All mice used in this study were maintained in a pathogen free facility, and cared for in accordance with protocols approved by the University of Utah Institutional Animal Care and Use Committee (IACUC).

Genomic DNA was prepared from 2–3 mm tail clips from 14 to 17 day old mice by incubating in 600 μ L of 50mM NaOH at 95°C for one hour, following by neutralization with 50 μ L of 1M Tris-HCl pH 8. Samples were then centrifuged at 6000 $\times$ g for 6 min in a tabletop centrifuge and transferred to a clean tube. Two μ L of 1:10 diluted DNA was used in each PCR reaction.

ARMS-PCR is a tetra-primer SNP genotyping strategy that combines two inner SNP-specific and two outer primers in a single reaction volume (7). Reactions are designed to produce up to three amplicons of diagnostic sizes which are then evaluated by agarose gel electrophoresis, as shown in Supplemental Figure S-1A. ARMS-PCR primer sets for ten SNPs across the *Bbaa2* region were developed using the recommended design program available online. (http://cedar.genetics.soton.ac.uk/public_html/primer1.html). ARMS-PCR primer sequences are available in Supplemental Table S-1. PCR was performed in 96-well plates on a PTC-200 Thermal

Figure 1. Distribution of SNP Genotyping Assays and SNP Density Across *Bbaa2*. (A) Microsatellites from the MGD database (<http://www.informatics.jax.org/>) with assigned cM values were plotted against their basepair position to obtain a linear plot of cM versus genomic location (Black Line). Markers indicate the positions of SNP Genotyping Assays developed across the interval. (B) SNP Density across *Bbaa2* was calculated from all dissimilar B6/C3H SNPs present in the MGD database.

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Table 1. PCR-Based SNP Genotyping Assay Comparison

Estimated Reagent Cost and Throughput (per 96-well plate)				
	ARMS-PCR	Small Amplicon	Blocked Probe	RFLP
Reagents (\$)				
Taq / dNTP	100	100	100	100
Primers / Probe	2	1	2	1
Analysis	2	10	10	22
Total Cost	104	111	112	123
Time (Minutes)				
PCR Setup	30	30	30	30
Cycling	90	75	75	90
Handling	30	0	0	45
Endonuclease	0	0	0	60
Electrophoresis	60	0	0	60
Analysis	15	15	15	15
Total Time	225	120	120	300
Bbaa2 Genotyping (26 positions, 96-well plates, run back-to-back)				
	ARMS-PCR	Small Amplicon	Blocked Probe	RFLP
9 Unknowns + 3 Control Samples				
Total Cost (\$)	340	360	365	400
Total Time (Hrs)	15	8	8	20
21 Unknowns + 3 Control Samples				
Total Cost (\$)	675	720	730	800
Total Time (Hrs)	27	14	14	35
Assay Design / Utility				
	ARMS-PCR	Small Amplicon	Blocked Probe	RFLP
Success Rate	10% (1/10)	81% (9/11)	75% (12/16)	Not Tested
Interpretation	Unequivocal	Subjective*	Unequivocal	Unequivocal
* An objective genotype assignment can be made by spiking unclear samples with control DNA and repeating the HRMA				

Cycler using Platinum Taq (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. PCR products were subjected to 1% agarose gel electrophoresis in 1X TBE buffer (89 mM Tris Base, 89 mM Boric Acid, 2 mM EDTA), and bands were visualized by

ethidium bromide staining on a Gel Doc XR+ platform (Bio-Rad Laboratories, Hercules, CA, USA).

Small Amplicon HRMA employs two primers closely surrounding the SNP of interest (8). Amplicon size is kept at a minimum, between 50–70 bp.

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Table 2. Comparison of Discernable Genetic Landmarks in *Bbaa2*

<i>Bbaa2</i> Microsatellite Markers		<i>Bbaa2</i> SNP Genotyping Assays	
bp	Δ bp	bp	Δ bp
119,175,776	N.A.	119,172,092	N.A.
		119,539,274	367,182
		119,914,546	375,272
120,256,981	1,081,205	120,298,004	383,458
		120,858,582	560,578
		121,575,713	717,131
		123,489,225	1,913,512
		123,905,461	416,236
		124,668,171	762,710
		125,034,265	366,094
125,309,605	5,052,624	125,316,596	282,331
125,581,535	271,930	125,602,080	285,484
125,931,691	350,156	125,940,137	338,057
		126,607,294	667,157
127,404,260	1,472,569	127,391,403	784,109
		128,208,712	817,309
		128,954,717	746,005
129,042,314	1,638,054	129,338,863	384,146
		130,476,763	1,137,900
132,603,109	3,560,795	132,593,675	2,116,912
		133,538,654	944,979
		134,737,636	1,198,982
136,395,980	3,792,871	136,401,340	1,663,704
137,534,245	1,138,265	137,497,474	1,096,134
		139,207,488	1,710,014
141,162,992	3,628,747	141,158,294	1,950,806
Average	2,198,722	Average	879,448
Median	1,555,312	Median	746,005
StDev	1,664,367	StDev	576,607
Max	5,052,624	Max	2,116,912
Min	271,930	Min	282,331

Distribution of previously used microsatellite markers and newly mapped SNP genotyping landmarks on mouse chromosome 5.

The hydrogen bonding characteristics of the nucleotide at the SNP position alters the melting temperature of the small amplicon, which can be detected by HRMA. The melting temperature differentials of homozygous Small Amplicons are reported to vary from 0.8 – 1.2 C, while heteroduplex amplicons are reported to melt at 2.0-3.0 C lower temperatures, as shown in Supplemental Figure S-1B.

Blocked (Unlabeled) probe HRMA employs a 3'phosphorylated oligonucleotide probe that overlaps the SNP of interest but cannot be extended in the

PCR reaction, and two outer primers that produce a 65 – 150 bp amplicon (9). By including a limiting amount of primer on the same strand as the probe, asymmetric PCR produces an excess of opposite strand product to serve as a probe binding partner during HRMA. Probes form either perfect match or mismatch duplexes, with reported melting temperature differentials of 5.0 – 8.0 C, as shown in Supplemental Figure S-1C.

Small amplicon primer sets for 11 SNPs, and blocked probe primers and probes for 33 SNPs were developed using online Primer3 design software ([\[frodo.wi.mit.edu/primer3/\]\(http://frodo.wi.mit.edu/primer3/\)\). Small amplicon and blocked probe primer sets are available in Supplemental Tables S-2 and S-3, respectively. PCR and HRMA were performed in 96-well plates using Platinum Taq and 1x LCGreen Plus+ DNA intercalating dye \(Idaho Technologies, Salt Lake City, UT, USA\) on a LightCycler 480 platform \(Roche Applied Science, Indianapolis, IN, USA\). HRMA results were evaluated using LightCycler 480 Software Version 1.5.](http://</p>
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LC480 Cycling Parameters were as follows: *Initial Denaturation*: 95°C (8 min); *Amplification*: 60 cycles of 95°C denature (4 s), 65°C annealing (6 s), 72°C extension (12 s); *Melt*: 95°C denature (15 s), 50°C anneal (60 s), 50–95°C continuous ramp at 0.06°C/second, 10 measurements per degree. *Cooling*: 50°C (10 s).

Each primer set was evaluated with B6, C3H, and 1:1 mixed B6+C3H (obligate heterozygous) genomic DNA, as well as an H₂O negative control.

Due to different assay design requirements, individual SNPs were not generally compatible with all three methodologies. SNPs were selected from the Perlegen2 database (10). The Sanger Mouse Genomes Project database provides a useful additional resource to select or verify candidate SNPs for genotyping (6).

Results and discussion

The generation of congenic mouse strains is an iterative process. The ability to detect unique meiotic recombination events producing a novel isolated genomic interval in individual mice in a timely and cost effective manner is essential for efficient progress. By definition, an average of 25 out of 100 pups are expected to contain a unique crossover event within a 25 cM locus. Therefore, as targeted intervals decrease in size, the number of expected new recombinants also decreases, leading to diminishing returns and increasing colony management costs. Theoretical calculations indicate that to narrow a single 25 cM locus to 5 cM by breeding of ISCLs requires approximately 300 individuals, while further reducing each locus from 5 cM to 1 cM requires approximately 380 additional individuals per regulatory gene (11). For the complex *Bbaa2* locus, with multiple putative regulators within a 20+ cM interval, this predicts that approximately 1820 individuals, the vast majority of which will be non-informative, will

be required to achieve our goal of 1 cM resolution for each putative locus.

Congenic breeders and their young pups can be maintained in a single cage, but are generally separated into 3 or more cages at weaning age. Since so many pups are non-informative as congenic intervals become narrow, rapid genotyping that allows efficient culling prior to weaning age may therefore provide a multiplier effect on cage cost savings, depending on the unique characteristics of individual colonies and institutional protocols. This process greatly benefits from the flexibility afforded by a high-throughput genotyping assay that can be rapidly and inexpensively performed on either small or large numbers of samples and can be targeted specifically to regions of interest within a sub-interval.

We previously relied on a panel of 11 microsatellite markers, some of which were poorly spaced or tightly clustered together, to genotype *Bbaa2* recombinants. To improve upon this, several methods of SNP genotyping were evaluated.

To ensure backward compatibility with previous microsatellite-based congenic genotyping data, SNP genotyping assays

were first designed to approximate the positions of microsatellite markers in the *Bbaa2* interval. Additional assays were then designed to further improve the distribution of landmarks across the interval.

All three tested SNP genotyping methodologies produced interpretable assays. However, ARMS-PCR (7) was not adopted for routine genotyping due to concerns about assay design and optimization, throughput, and the added risk of amplicon contamination during the obligatory handling and gel electrophoresis of PCR products. Another commonly used PCR-based SNP genotyping method, restriction fragment length polymorphism (RFLP) analysis, relies on incubation of the PCR amplicon with restriction endonucleases, followed by gel electrophoresis. These steps require additional reagents and equipment, and significantly increase assay turnaround time, which dramatically reduces throughput. Only a limited sub-set of SNPs are amenable to RFLP, and if RFLP-SNP mining is limited to commonly used, cost-effective restriction endonucleases that number is reduced even further. For the 22 Mb *Bbaa2*

interval, only 538 such RFLP amenable SNPs were identified by SNP2RFLP (12) from over 19,000 high quality SNPs. Methodologically reducing the available SNPs in this way by more than 35-fold may severely impair fine mapping of single genes or SNPs within specific congenic lines, especially in low-SNP areas of the genome. Like ARMS-PCR, these manipulations also require an open-tube assay that may increase the risk of amplicon contamination of laboratory space or equipment if samples are not handled appropriately.

Small amplicon HRMA (8) was found to be a reliable and efficient methodology. Nine out of 11 small amplicon SNP assays (81%) produced interpretable results using standard reaction conditions. Heterozygous genotypes were readily apparent, due to the presence of a definitive heteroduplex melting peak. However, when scaled up to evaluate many unknown samples in parallel, inter-sample melting peak variability was observed, making it difficult to objectively assign a genotype to some individual homozygous samples. In all such cases, an objective genotype assignment could be made by repeating the HRMA analysis

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Table 3. Development of Advanced *Bbaa2* Sub-Interval Congenic Lines

Chr5 Position (Mb)																								
119.9	120.3	120.9	121.6	123.5	123.9	124.7	125.0	125.3	125.6	125.9	126.6	127.4	128.2	129.0	129.3	130.5	132.6	133.5	134.7	136.4	137.5	139.2	141.2	
B	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C
B	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	B	B	B
B	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	B	B	B	B
B	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	B	B	B	B	B
B	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	B	B	B	B	B	B	B	B
B	C	C	C	C	C	C	C	C	C	C	C	C	C	B	B	B	B	B	B	B	B	B	B	B
B	C	C	C	C	C	C	C	C	C	C	C	B	B	B	B	B	B	B	B	B	B	B	B	B
B	C	C	C	C	C	C	C	C	C	C	B	B	B	B	B	B	B	B	B	B	B	B	B	B
B	C	C	C	C	C	C	C	C	C	C	B	B	B	B	B	B	B	B	B	B	B	B	B	B
B	C	C	C	C	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
B	C	C	C	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
B	B	C	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
B	B	B	B	B	B	B	B	B	B	B	B	B	B	C	C	C	C	C	C	C	C	C	C	C
B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	C	C	C	C	C	C	C	C
B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	C	C	C	C	C	C
B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	C	C	C	C	C
B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	C	C	C	C	C
B	B	B	B	B	B	B	B	B	B	B	B	B	B	C	C	C	B	B	B	B	B	B	B	B
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B	B	B	B	B	B	B	B	B	B	B	B	B	B	C	C	C	B	B	B	B	B	B	B	B
B	B	B	B	B	B	B	B	B	B	B	B	B	B	C	C	C	B	B	B	B	B	B	B	B
	B6C3F ₁ × C3H/HeN BC1																							
	B6C3F ₁ x C57BL/6 BC1																							
	B6C3F ₂ Intercross																							
Rows - Individual <i>B6.C3-Bba2</i> congenic lines. Columns - SNP assay positions. Genotypes designated: C – C3H Homozygote, B – B6 Homozygote.																								
The bottom three rows indicate the positions of regulatory loci previously predicted for each B6C3F1 backcross and for the B6C3F2 intercross. (3)																								

after spiking the unknown sample with known B6 or C3H control DNA and looking for the presence of a heteroduplex melting peak.

Blocked probe HRMA (9) produced interpretable results under standard conditions in twelve out of sixteen assays (75%) as originally designed. Even when evaluating many unknown samples in parallel, interpretation of working blocked probe sets was objective and unequivocal. Eleven out of 17 additional blocked probe assays designed across the

region also produced interpretable assays, including three out of four designed as nearby replacements for non-working assays from the first round.

Both small amplicon and blocked probe HRMA were very rapid and efficient assays, generating SNP genotyping data in a 96-well format within 120 minutes. For very large scale applications, it is unclear whether the observed difference in assay design success rates (81% vs 75%) would strongly influence assay selection, or if other concerns would take precedence. Blocked

probe HRMA offers the additional benefit over small amplicon HRMA of theoretically greater specificity due to the addition of a third sequence-specific oligonucleotide as a probe, as well as dramatically better separation between diagnostic peaks so that spiking and HRMA repetition was not necessary. For these reasons we have adopted blocked probe HRMA as our preferred methodology for high-throughput SNP genotyping.

The estimated time, reagent cost, and utility of the ARMS-PCR, small

amplicon, blocked probe, and RFLP methods based on uniform manufacturer recommended 50 μ L PCR cycling volumes are compared in Table 1.

All *Bbaa2* HRMA genotyping assays have been successfully scaled down to 10 μ L reaction volumes in 96-well plates while retaining diagnostic integrity, further reducing reagent costs. Rapid turnaround times have enabled complete genotyping of the *Bbaa2* interval or appropriate sub-interval in all congenic offspring prior to weaning age, also reducing cage costs. This has allowed resources to be better focused on expanding breeding cages to accelerate the generation of new recombinants and on maintenance of novel sub-interval congenic lines.

The use of SNP genotyping by HRMA is becoming increasingly accessible as equipment capable of performing HRMA becomes more common in research institutions around the world. A search of the PubMed database in October 2011 identified over 240 publications using high resolution melting in the past year alone, primarily relating to human diagnostics. For this purpose, the SNPs of interest are pre-defined due to their estab-

lished linkage to or causation of human disease. While we have found that a large majority of SNPs can be genotyped in this way, not every such SNP is amenable to HRMA genotyping, depending upon the unique characteristics and complexity of surrounding DNA sequence. However, for the purposes of mapping inbred congenic mouse strains, the genomic location of a SNP rather than its potential biological function is the primary consideration, allowing flexibility for several different SNPs to be evaluated in a given area. Based on the Perlegen high-density resequencing project, SNPs differing between any two pairwise combinations of 12 classic inbred strains occur with an average frequency ranging from 1 in 440 bp to 1 in 21,000 bp in high-SNP or low-SNP regions of the mouse genome, respectively (13). Even in low frequency regions, this represents a potential resolution for congenic mapping on the order of 0.01 – 0.10 cM.

The *Bbaa2* region of mouse chromosome 5 is complex. It exhibits a nonlinear relationship between basepair position and centiMorgan distance (Figure 1A), suggesting that meiotic recombination does not occur

with uniform frequency across the interval. It also exhibits a highly variable SNP density, with some areas highly conserved between our parental B6 and C3H inbred strains of interest (Figure 1B). These characteristics impact the capacity to generate congenic animals and then to identify where recombination has occurred. Despite this complexity, diagnostic SNP genotyping assays were successfully designed at regular intervals. A total of 26 SNP genotyping assays across the *Bbaa2* region are now used for routine genotyping of backcross progeny in our congenic colony. By adopting SNP genotyping methods, all previously used microsatellite markers have been replaced with approximately equivalent high-throughput SNP assays, and 15 additional landmarks have been added. As shown in Table 2, the average and median resolutions between genotyping landmarks across the *Bbaa2* interval have improved from 2.20 Mbp and 1.56 Mbp to 0.88 Mbp and 0.75 Mbp, respectively. The ability to readily design new genotyping assays targeted to intervals containing novel breakpoints in individual mice contributes to a harmonious and efficient refinement

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process. In many cases, the prevalence of discernable SNPs will provide great enough resolution to exclude single genes, or even single SNPs within genes, from a congenic interval. This is especially important when attempting to precisely define the boundaries of similar or overlapping recombinant congenic lines. The use of HRMA based SNP genotyping may also be of special interest to anyone working with recombinant inbred (RI) lines such as those produced by the Collaborative Cross (14), or performing iterative backcrossing, such as during the transfer of a targeted gene knockout to a specific genetic background. A basic SNP genotyping panel for a broad interval surrounding the gene of interest may help speed up the backcrossing process by quickly identifying which individuals within litters inherited the narrowest surrounding interval, and would therefore best serve as breeders for successive backcrosses.

Our adoption of HRMA-based SNP genotyping has facilitated the generation, identification, and more precise discernment of 20 novel ISCLs carrying unique subintervals of the *Bbaa2* locus, as shown in Table 3. Many of these newly defined advanced congenics coincide with regions previously predicted to encode loci with positive and negative effects on Lyme arthritis severity, thereby establishing tools for the formal analysis of regulatory regions on chromosome 5. Current congenic lines allow the pairwise interrogation of intervals as small as approximately 0.6 Mbp, which will greatly facilitate efforts to identify causal genes in the region, and may be of general interest for the investigation of any phenotypic differences assigned to this region of mouse chromosome 5.

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Competing interests

The authors declare no competing interests.

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Address correspondence to Janis J. Weis, Department of Pathology, University of Utah, Salt Lake City, UT, USA. Email: janis.weis@path.utah.edu

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