Genome-wide association study of café-au-lait macule number in neurofibromatosis type 1

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Abstract
Background: Neurofibromatosis type 1 (NF1) is a tumor-predisposition disorder that arises due to pathogenic variants in tumor suppressor NF1. NF1 has variable expressivity that may be due, at least in part, from heritable elements such as modifier genes; however, few genetic modifiers have been identified to date.

Methods: In this study, we performed a genome-wide association analysis of the number of café-au-lait macules (CALM) that are considered a tumor-like trait as a clinical phenotype modifying NF1.

Results: A borderline genome-wide significant association was identified in the discovery cohort (CALM1, N = 112) between CALM number and rs12190451 (and rs3799603, $r^2 = 1.0; p = 7.4 \times 10^{-8}$) in the intronic region of RPS6KA2. Although, this association was not replicated in the second cohort (CALM2, N = 59) and a meta-analysis did not show significantly associated variants in this region, a significant corroboration score (0.72) was obtained for the RPS6KA2 signal in the discovery cohort (CALM1) using Complementary Pairs Stability Selection for Genome-Wide Association Studies (ComPaSS-GWAS) analysis, suggesting that the lack of replication may be due to heterogeneity of the cohorts rather than type I error.
1 | INTRODUCTION

Neurofibromatosis type 1 (NF1) is a common, monogenic tumor-predisposition disorder that arises due to germline pathogenic variation in NF1 (OMIM 613113). The protein product of NF1, neurofibromin, regulates the conversion of the active form of RAS (RAS-GTP) to its inactive form (RAS-GDP; Welti, D’Angelo, & Scheffzek, 2008). Individuals haplo-insufficient for NF1 have increased levels of RAS-GTP, dysregulation of cell growth, and thus have an increased risk of a variety of benign and malignant tumors, predominantly of the central and peripheral nervous system (e.g., neurofibromas, pilocytic astrocytomas, optic pathway gliomas; Huson, 2008). Soft-tissue sarcomas (malignant peripheral nerve sheath tumors, rhabdomyosarcomas), neuroendocrine tumors (somatostatinomas, pheochromocytomas), and leukemia (juvenile myelomonocytic leukemia) are also associated with NF1 (Brems, Beert, de Ravel, & Legius, 2009).

Although the first, modern medical description of NF1 was published in 1882 by von Recklinghausen, novel tumor associations continue to be discovered, including gastrointestinal stromal tumors (Stewart et al., 2007), glomus tumors (Brems, Park, et al., 2009), and breast cancer (Sharif et al., 2007; Uusitalo et al., 2016).

NF1 has variable expressivity, even among members of the same family. This phenotypic complexity probably derives from multiple etiologies (e.g., epigenetic phenomena and stochastic events) as well as from heritable elements, such as modifier genes (Carey & Viskochil, 1999). There is clinical evidence that modifier genes are a major component of phenotypic variation in NF1. Easton, Ponder, Huson, and Ponder (1993) first examined intra-familial correlation in phenotypic features (Easton et al., 1993). In that study, among six pairs of monozygotic twins, the highest correlations were for the number of café-au-lait spots ($r = 0.85, p < .05$) and cutaneous neurofibromas ($r = 1.00, p < .01$). Correlation decreased for first- and second-degree relatives. Four of the five binary traits studied in that paper also showed significant familial clustering (Easton et al., 1993). Szudek, Joe, and Friedman (2002) observed similar patterns of familial correlation that also suggested a role for genetic factors (Szudek et al., 2002; Szudek et al., 2002). A statistical analysis of NF1 phenotypes and severity in a French cohort provided additional evidence for the existence of genetic modifiers in NF1 (Sabbagh et al., 2009). The heritability of café-au-lait macules, a hallmark feature of NF1, was the highest (0.62 ± 0.08) among the traits considered.

In people with NF1, CALM number is a phenotype that has a number of advantages: compared to other NF1 clinical features it is relatively stable during patients’ life (although some “fading” is observed), is readily quantifiable and can be non-invasively measured. Moreover, CALMs are “tumor-like” in that they arise from a somatic inactivation (a second hit) of the remaining normal copy of NF1 in skin melanocytes, which, like the Schwann cells, also originate from the common neural crest cell progenitor (Maertens et al., 2007). In previous work using a gene expression-based, multi-platform approach, variants were identified in the intergenic region of DPH2 (OMIM 603456) and ATP6V0B (OMIM 603717) and in MSH6 (OMIM 600678) that influence CALM number in NF1 (Pemov et al., 2014). In the current work, a genome-wide association study (GWAS) was employed for the first time to identify additional variants associated with CALM number in NF1. Furthermore, the potential functional effects of these variants were evaluated in both normal skin and blood tissues using publicly available databases.

2 | METHODS

2.1 | NF1 patients and CALM number in CALM1 and CALM2 cohorts

CALM1 and CALM2 studies were approved by the Institutional Review Boards (IRB) and the NCI Special Studies IRB. All participants provided written informed consent prior to joining the studies. The NCI Special Studies IRB also approved the overall GWAS. The study was carried out according to the principles of the Declaration of Helsinki.

Quantitative trait café-au-lait macule count data were collected from two cohorts, CALM1 (CALM number counted by DRS) and CALM2 (CALM number counted by AB), as described previously (Pemov et al., 2014). Most of the patients in the CALM1 cohort were adults, while most patients in the CALM2 cohort were children (Table 1). All patients in the CALM2 cohort underwent whole-body MRI and had at least one plexiform neurofibroma. In both cohorts, all CALMs ≥ 5 mm in longest diameter were counted.
A Wood’s lamp, which makes CALMs more visible, was used for CALM counting in CALM1, but not in CALM2. The log-transformed CALM number, log10 (CALM + 10), was analyzed adjusting for age, sex and body surface area (m^2) = \sqrt[(60)]{weight(kg) \cdot height(cm)} defined as in Mosteller, 1987. Differences in age, sex, body surface, and CALM number were compared by two-sided t-test assuming unequal variance between cohorts.

### 2.2 | Genotypes for CALM1 samples for GWAS

Genotyping for the CALM1 samples was performed on Illumina Infinium HumanOmni-1Quad bead-chips. SNPs from 22 autosomes were included if the SNP GenTrain score was >0.3 and minor allele frequency (MAF) was >0 (non-monomorphic). Samples with genotyping rate \geq 99% and calling rate \geq 99% were retained. Cryptic relatedness and population stratification were checked with multidimensional scaling (MDS) analysis estimating pairwise identity by descent (IBD) for all pairs of founders using independent SNPs in PLINK (Purcell et al., 2007). SNPs with MAF \geq 0.2 in founders were pruned for independence, first with pairwise linkage disequilibrium (LD) correlation measure r^2 < 0.2 within 50 SNPs, then again within 200 SNPs. Lastly, SNPs with MAF < 0.1 in European–Americans were excluded. The quality control protocol yielded 608,561 SNPs in 112 European–Americans with non-missing CALM number. The CALM1 cohort included 57 unrelated individuals, 16 families with 2 members each, 5 families with 3 members each, and 2 families with 4 members each.

### 2.3 | Genotypes for CALM2 samples for GWAS

Genotyping for the replication cohort CALM2 samples was performed on Illumina Infinium Omni2.5 bead-chips. Quality control measures were performed as described above although the retention rates for genotyping and calling rates were both lowered to \geq 98% due to the smaller sample size. Samples with genotyping rate <95% from remaining European–Americans and SNPs with MAF < 0.2 were excluded—yielding 623,562 SNPs in 59 European–Americans with non-missing CALM number, including two sibling pairs. To ensure compatibility between the genotyping data between the two cohorts, LitfOver (Hinrichs et al., 2006) was used to convert genome positions in the CALM1 cohort (NCBI build 36, UCSC hg18) to corresponding positions in the CALM2 cohort (NCBI build 37 (UCSC hg19).

### 2.4 | Targeted sequencing of candidate loci

Targeted sequencing of candidate loci was carried out in 171 European–American samples using the AmpliSeq/IonTorrent platform, as per the manufacturer’s instructions. A genotype was considered missing if its genotype quality score (GQ) was <10, its read depth (DP) score was <10 or the ratio of the genotype quality score (GQ) divided by genotype read depth (DP), GQ/DP was <0.5. Monomorphic SNPs and SNPs with a calling rate <90% and samples with a genotyping rate <90% were excluded leaving 176 SNPs in 157 samples. Quality control for targeted gene sequencing data was done using SNP & Variation Suite v8.3.4 (http://goldenhelix.com/products/SNP_Variation/index.html) and PLINK (Purcell et al., 2007).
2.5 Tests of association

In each cohort, tests of association between each SNP and log-transformed CALM number, adjusted for age, sex, and body surface area, were performed with EMMAX (Kang et al., 2010) with an IBS kinship matrix considering possible spurious association due to genetic relatedness. Simple linear regression (SLR) ignoring genetic relatedness was also performed; these results are not shown because the correlations of the p-values for EMMAX and SLR for the CALM1 and CALM2 cohorts were 0.95 and 0.99, respectively. In the targeted gene sequencing samples, tests of association were performed by SLR between each SNP in RPS6KA2 (OMIM 601685; GenBank NM_021135.6) and the log-transformed CALM number, adjusted for age, sex, and body surface area, for the CALM1 and CALM2 cohorts, both separately and combined. An additional covariate, cohort (CALM1, CALM2), as well as age, sex, and body surface area were adjusted in the mega analysis for combined-targeted gene sequencing data. Candidate loci were defined as those with suggestive genome-wide significance ($p < 5 \times 10^{-8}$). ComPaSS-GWAS score significance level ($\alpha$ = 0.6) is comparable to a traditional genome-wide association studies (GWAS) significance level ($\alpha$ = 5 x 10^{-8}). Simulations have shown (Sabourin et al., 2018) that a ComPaSS-GWAS score greater than 0.6 is comparable to a traditional genome-wide association significance level ($p < 5 \times 10^{-8}$). ComPaSS-GWAS score greater than 0.4 and less than 0.6 are considered to be suggestively significant findings.

2.6 Meta-analysis

Meta-analyses between the CALM1 and CALM2 cohort studies were performed with METAL (Willer, Li, & Abecasis, 2010) with weights proportional to the square-root of the sample size for each study (Stouffer, Suchman, Devinney, Star, & Williams, 1949), with genomic control correction of input statistics enabled. Heterogeneity analyses including I$^2$ statistics, the percentage of variation between cohorts due to heterogeneity rather than chance, were also performed in meta-analyses.

2.7 ComPaSS-GWAS analysis

The second approach for corroboration of SNP associations used ComPaSS-GWAS (Sabourin et al., 2018), a method based on complementary pairs stability selection (Shah & Samworth, 2013) with a regression based GWAS analysis. ComPaSS-GWAS approximates replication by randomly splitting a sample in half (into a pseudo-discovery and pseudo-replication set) multiple times and looking for corroboration of results between each random split. ComPaSS-GWAS returns a corroboration score between 0 and 1, indicating the proportion of random splits where the SNPs were corroborated for the specified within-split significance parameter. The primary focus of ComPaSS-GWAS is for situations where appropriate independent replication data are either not available or when the replication cohort is too small to sufficiently bolster the primary cohort’s findings in a meta-analysis. ComPaSS-GWAS may also be useful when heterogeneity between the cohorts affects the ability of a meta-analysis to produce confident results. A high ComPaSS-GWAS score in a single cohort could also indicate that the lack of replication may be a result of the differences in cohorts rather than the individual cohort analysis result being a false positive. In this study, ComPaSS-GWAS scores were obtained using a within-split significance parameter of 10^{-3} based on 100 random splits. Simulations have shown (Sabourin et al., 2018) that a ComPaSS-GWAS score of 0.6 is comparable to a traditional genome-wide association significance level ($p < 5 \times 10^{-8}$). ComPaSS-GWAS score greater than 0.4 and less than 0.6 are considered to be suggestively significant findings.

2.8 LD structures

Haploview 4.2 (Barrett, Fry, Maller, & Daly, 2005) was used to generate LD plots across the genome based on D' and r$^2$ values to evaluate LD structures among significant SNPs variants in Table 2 for each cohort.

2.9 Functional annotation and quantitative-trait loci analysis of SNPs

Custom tracks on the UCSC Genome browser (http://genome.ucsc.edu) were used to screen NIH Roadmap Epigenomics and ENCODE experimental data containing the SNP regions of interest for evidence for regulatory relevance in normal skin cells (melanocytes, fibroblasts and keratinocytes). The online tools HaploReg (http://www.broadinstitute.org/mammals/haploreg/haploreg.php) and RegulomeDB (http://regulome.stanford.edu) were used as complementary analyses and to confirm the location of each SNP in relation to protein-coding and/or non-coding RNA genes. While many regulatory regions can act in a tissue-specific manner, it is unknown whether variants in such regions also have tissue-specific effects, and complex phenotypes are often caused by dysfunction of multiple tissues or cell type. This suggests the potential for the involvement of skin and other tissues/cells such as blood (immunity) and fibroblasts (regulatory role in pigmentation) in CALM phenotype. To evaluate if a SNP could influence gene expression in-cis or act as an expression quantitative trait loci (eQTL) in blood and skin tissues, all coding genes were identified that mapped within ±1 Mb of the index SNPs (associated SNPs of interest). eQTL results for all CALM-associated SNPs and collated genes based on 749 normal skin tissues (414 sun-exposed and 335 non-sun-exposed), 369 normal blood samples and 300 transformed fibroblast cell lines were then extracted from GTEx (V7,
| SNP ID   | Chr | Position (hg19) | Nearest neighbor gene | Type          | MAF<sup>a</sup> | CALM1 EMMAX beta | CALM1 p-value | CALM1 score | CALM2 EMMAX beta | CALM2 p-value | CALM2 score | CALM2 Meta p-value | Meta I² (%) | Meta p-value | Heterogeneity |
|----------|-----|----------------|-----------------------|---------------|-----------------|-----------------|----------------|-------------|-----------------|----------------|-------------|----------------------|-------------|-------------|--------------|
| rs4513317 | 2   | 101280697      | PDCL3, NPAS2          | Intergenic    | 0.357           | 0.018           | 3.43 × 10⁻¹    | 0           | 0.134           | 6.61 × 10⁻⁶ | 0.50        | 6.80 × 10⁻⁴           | 89.6        | 1.94 × 10⁻³ |
| rs7615501 | 3   | 53175557       | RFT1, PRKCD           | Intergenic    | 0.429           | −0.030          | 1.27 × 10⁻¹    | 0           | 0.124           | 7.53 × 10⁻⁷ | 0.57        | 9.55 × 10⁻²           | 95.8        | 9.88 × 10⁻⁷ |
| rs4856490 | 8   | 84147884       | LINC02008, LINC00971  | Intergenic    | 0.143           | 0.116           | 1.13 × 10⁻⁶    | 0.54         | ND              | ND            | ND          | ND                    | ND          | ND          | ND          |
| rs6872422 | 5   | 7422768        | ADCY2                 | Intronic      | 0.370           | −0.026          | 1.51 × 10⁻¹    | 0           | 0.107           | 3.10 × 10⁻⁶ | 0.43        | 1.16 × 10⁻¹           | 95.3        | 3.97 × 10⁻⁶ |
| rs11742602| 6   | 7440607        |                        | Intergenic    | 0.415           | ND              | ND            | ND          | 0.125           | 1.19 × 10⁻⁷ | 0.75        | ND                    | ND          | ND          | ND          |
| rs1025291 | 6   | 7445289        |                        | Intergenic    | 0.305           | −0.033          | 6.91 × 10⁻²    | 0           | 0.124           | 4.27 × 10⁻⁷ | 0.67        | 1.35 × 10⁻¹           | 96.2        | 2.55 × 10⁻⁷ |
| rs4710070 | 6   | 166995232      | RPS6KA2               | Intronic      | 0.292           | 0.096           | 1.08 × 10⁻⁶    | 0.57         | ND              | ND            | ND          | ND                    | ND          | ND          | ND          |
| rs3799603 | 6   | 167001286      |                        | Intergenic    | 0.312           | 0.100           | 7.40 × 10⁻⁸    | 0.72         | −0.006          | 8.40 × 10⁻¹ | 0           | 2.27 × 10⁻⁵           | 91.0        | 8.87 × 10⁻⁴ |
| rs3778385 | 6   | 167003381      |                        | Intergenic    | 0.299           | 0.096           | 6.15 × 10⁻⁷    | 0.63         | −0.0004         | 9.89 × 10⁻¹ | 0           | 5.63 × 10⁻⁵           | 88.4        | 3.28 × 10⁻³ |
| rs12190451| 6    | 167005259      |                        | Intergenic    | 0.312           | 0.100           | 7.40 × 10⁻⁸    | 0.72         | ND              | ND            | ND          | ND                    | ND          | ND          | ND          |
| rs799585  | 6   | 167016947      |                        | Intergenic    | 0.390           | −0.086          | 3.64 × 10⁻⁶    | 0.40         | −0.034          | 2.48 × 10⁻¹ | 0           | 9.31 × 10⁻⁶           | 68.0        | 7.70 × 10⁻² |
| rs10946177| 6    | 167018721      |                        | Intergenic    | 0.305           | 0.099           | 4.34 × 10⁻⁷    | 0.68         | 0.027           | 3.71 × 10⁻¹ | 0           | 3.94 × 10⁻⁶           | 80.2        | 2.48 × 10⁻² |
| rs10215358| 7    | 131363167      | PODXL, LOC101928782   | Intergenic    | 0.448           | 0.085           | 5.84 × 10⁻⁸    | 0.46         | −0.029          | 2.62 × 10⁻¹ | 0           | 2.62 × 10⁻³           | 92.2        | 3.58 × 10⁻⁴ |
| rs12765442| 10   | 25905421       | GPR158, LINC00836     | Intergenic    | 0.110           | −0.124          | 6.09 × 10⁻⁶    | 0.51         | ND              | ND            | ND          | ND                    | ND          | ND          | ND          |
| rs868692  | 11   | 25905810       |                        | Intergenic    | 0.110           | −0.124          | 6.09 × 10⁻⁶    | 0.51         | ND              | ND            | ND          | ND                    | ND          | ND          | ND          |
| rs868691  | 11   | 25905876       |                        | Intergenic    | 0.110           | −0.124          | 6.09 × 10⁻⁶    | 0.51         | ND              | ND            | ND          | ND                    | ND          | ND          | ND          |
| rs1951646 | 14   | 38146748       | TTC6                  | Intronic      | 0.442           | 0.073           | 4.37 × 10⁻²    | 0.28         | 0.061           | 4.67 × 10⁻² | 0.01        | 7.67 × 10⁻⁶           | 0           | 4.28 × 10⁻¹ |
| rs34047645| 15   | 84611367       | ADAMTSL3              | Exonic        | 0.112           | −0.116          | 3.91 × 10⁻⁶    | 0.53         | ND              | ND            | ND          | ND                    | ND          | ND          | ND          |
| rs8093709 | 18   | 44134749       | LoxHDI                | Intronic      | 0.297           | ND              | ND            | ND          | 0.144           | 6.49 × 10⁻⁶ | 0.32        | ND                    | ND          | ND          | ND          |
| rs732108  | 18   | 44135027       |                        | Intergenic    | 0.297           | ND              | ND            | ND          | 0.144           | 6.49 × 10⁻⁶ | 0.32        | ND                    | ND          | ND          | ND          |

Note: ND, no SNP data analyzed; p-values for EMMAX and Meta-analysis < 1 × 10⁻⁵ and ComPaSS-GWAS score > 0.4 are shown in bold and italic fonts, respectively. GenBank IDs for genes listed in the table, from top to bottom: NM_020465.5; NM_002518.4; NM_052859.4; NM_0006254.4; NR_147146.1; NR_033860.1; NM_020546.3; NM_021135.6; NM_001018111.3; NR_110836.1; NM_020752.3; NR_108067.1; NM_001310135.2; NM_207517.3; NM_144612.6. If multiple transcripts exist for a gene, only an ID for transcript variant 1 is shown.

<sup>a</sup>Minor allele frequency in CALM1 samples or CALM2 samples if not in CALM1.
dbGaP Accession phs000424.v7.p2), (12 lead index SNPs and 1 surrogate variant in perfect LD). In addition, the influence of a SNP on metabolite levels in blood was evaluated by extraction of association results for each SNP and metabolites in blood from the Metabolomics GWAS (http://metabolomics.helmholtz-muenchen.de/gwas/).

3 | RESULTS

3.1 | GWAS for CALM1 and CALM2 cohorts

Descriptive information for the CALM1 and CALM2 cohorts and p-values comparing difference between cohorts by t-test analyses are presented in Table 1. The cohorts were significantly different with respect to age (p = 2.2 × 10^{-16}) and body surface area (p = 6.6 × 10^{-12}). Figure 1 presents the -log_{10}(p-value) of tests of association using EMMAX on 608,561 SNPs from 112 samples in the CALM1 cohort (top panel) and 623,562 SNPs from 59 samples from the CALM2 cohort (middle panel). The bottom panel presents -log_{10}(p-value) from the meta-analysis of the 322,455 SNPs that were shared in common between the two cohorts. Table 2 presents SNPs that were suggestively genome-wide significant (p < 1.0 × 10^{-5}) using EMMAX for either the CALM1 or CALM2 GWAS or the meta-analysis. p-values < 1 × 10^{-5} are shown in bold and italics.

In the CALM1 cohort GWAS, two SNPs in LD (r^2 = 1, D’ = 1) on chromosome 6, rs12190451 and rs3799603, in the intronic region of RPS6KA2, had a borderline genome-wide significant association (p = 7.4 × 10^{-8}) with CALM number (Figure S1a,b). Four additional significant SNP associations in the CALM1 cohort in this intronic region were significant at the suggestive genome-wide level (p < 1.0 × 10^{-5}). Five of the six SNPs in RPS6KA2 appeared to be in LD, but rs3799585 was not in high LD (r^2 < 0.28, D’=1) with the other SNPs (Figure S1a,b, Block 2). Figure 2 presents a regional visualization of EMMAX results for RPS6KA2 in CALM1 samples (LocusZoom [Pruim et al., 2010]). In addition to the six intronic SNPs in RPS6KA2, other SNPs that were suggestively significant at a genome-wide association level included: an intergenic SNP on chromosome 3 (rs4856490, near LINC00971), an intergenic SNP on chromosome 7

**FIGURE 1** Manhattan plots of p-values obtained from the genome-wide EMMAX analyses. (a) CALM1 cohort; (b) CALM2 cohort; (c) Meta-analysis of CALM1 and CALM2. The red horizontal line corresponds to the genome-wide significance level of p < 5 × 10^{-8} and the blue line corresponds to suggestive genome-wide significance level of p < 1.0 × 10^{-5}.
(rs10215358) residing between *PODXL* and *LOC101928782*, three intergenic SNPs in LD ($r^2 = 1$, D’=1) between *GPR158* and *LINC00836* on chromosome 10 (rs12765442, rs868692, and rs868691) and an exonic SNP in *ADAMTSL3* on chromosome 15 (rs34047645).

Similarly, SNPs in the CALM2 cohort SNPs that were suggestively significant at the genome-wide level ($p < 1.0 \times 10^{-5}$) included one intergenic SNP on chromosome 2 (rs4513317 between *PDCL3* and *NPA52*); one intergenic SNPs on chromosome 3 (rs7615501 between *RFT1* and *PRKCD*); three intronic SNPs in *ADCY2* on chromosome 5 (rs6872422, rs11742602, rs10252921); and two SNPs in LD ($r^2 = 1$, D’=1) in an intronic region of *LOXHD1* on chromosome 18 (rs8093709, rs732108). The SNPs in *ADCY2* and *LOXHD1* were in high LD within each gene (Figure S1c,d, Blocks 1 ($r^2 > 0.7$, D’=1) and 3 ($r^2 = 1$, D’=1)).

Of the ten SNPs in common between the two cohorts, eight SNPs had an $I^2$ statistic, the proportion of the variance between the cohort due to heterogeneity rather than chance, greater than 80%, and three had an $I^2$ statistic greater than 95% ($p < 1 \times 10^{-5}$). And, in six of these eight SNPs, the regression coefficients (betas) were opposite in the direction between the two cohorts, although the differences were quite small.

### 3.3 Cohort heterogeneity

In addition to the significant differences between the cohorts for age and body surface area (Table 1), there were striking differences between the cohorts in terms of the distribution of CALM number and age (Figure 3). CALM number decreased as age increased in the CALM1 cohort, but CALM number increased with age up to about 18 years of age in the CALM2 cohort. Linear regression modeling for CALM number versus age for two groups was performed (>18 years old versus ≤18 years old). The model $p$-value for the group of 124 NF1 patients older than 18 years of age (right side of the vertical dashed line) was $1.04 \times 10^{-8}$ and the $p$-value for the group of 47 NF1 patients less than 18 years of age (left side of the vertical dashed line) was 0.04, confirming the opposite relationship between CALM number versus age by 18 years of age. Taking into consideration that most of the patients in the CALM1 cohort were older than 18 years and most of the patients in the CALM2 cohort were younger than 18 years.
(Table 1), the opposite age distribution for CALM number could contribute to the opposing SNP effects observed in each independent GWAS. These findings suggest that the CALM2 GWAS was not an appropriate replication cohort for the CALM1 GWAS. To address this issue ComPaSS-GWAS was used on each cohort separately.

3.4 | ComPaSS-GWAS for CALM1 and CALM2

ComPaSS-GWAS corroboration scores using EMMAX were obtained for all SNPs, presented in Table 2. In the CALM1 cohort, the two most significant SNPs identified by EMMAX in RPS6KA2 intron on chromosome 6 (rs3799603 and rs12190451) also had the highest ComPaSS-GWAS corroboration score (0.72). These SNPs were in LD (r^2 = 1; D'=1). Similarly, the two most significant SNPs identified by EMMAX in ADCY2 on chromosome 5 in the CALM2 cohort (rs11742602 and rs1025291) also had the highest ComPaSS-GWAS corroboration scores of 0.75 and 0.67. The ComPaSS-GWAS analyses confirmed the findings by EMMAX within each cohort of CALM1 and CALM2 and suggest that these findings may be replicated if larger more homogeneous samples can be obtained.

3.5 | Targeted sequencing of RPS6KA2

Because of the marginally/borderline significant associations in the intronic region of RPS6KA2 in the CALM1 cohort GWAS, ~24 Kb (chr6:166,995,059–167,018,915) of intron 1 of RPS6KA2 was sequenced (transcript variant 1, NM_021135.6) in order to investigate other possible variants. There were 15 RPS6KA2 SNPs with suggestive genome-wide significance (p < 1.0 x 10^-5) using simple linear regression (SLR) in the CALM1 cohort. However, no SNP was significant in the CALM2 cohort, or in meta- or mega-analyses of combined cohorts (Table S1).

3.6 | Functional annotation of CALM-associated SNPs

Using Roadmap and ENCODE experimental data from normal penile foreskin cells (including melanocytes, fibroblasts, and keratinocytes) and peripheral blood mononuclear cells, each of the CALM-associated SNPs at p-value < 1.0 x 10^-5 presented in Table 2 were annotated and mapped to potential DNA regulatory regions (e.g., transcriptional start site [TSS], enhancers, and DNaseI/open chromatin sites; Table S2). RPS6KA2 rs12190451, rs3778385, and rs4710070 mapped to either an active enhancer or region flanking an active enhancer in penile skin fibroblasts and/or melanocytes. Variation at rs4710070 also altered a CpG dinucleotide. ADCY2 rs11742602 and RPS6KA2 rs1025291 were located in heterochromatin regions in fibroblasts, keratinocytes and melanocytes. The SNP rs7615501 on chromosome 3 was shown to map to an intergenic promoter region in fibroblasts. SNPs rs4513317 (chromosome 2), rs4856490 (chromosome 3), rs10215358 (chromosome 7), and rs12765442 (chromosome 10) were located in heterochromatin regions in fibroblasts, keratinocytes and melanocytes. The SNP rs34047645 in exon 18 of ADAMTSL3 and rs3799603 in intron 1 of RPS6KA2

![FIGURE 3](https://www.example.com/image.png)  
Comparison of café-au-lait macule number distribution by patients' age in CALM1 and CALM2 cohorts. Samples in CALM1 are depicted with open circles and samples in CALM2 are depicted with asterisks. Vertical dashed line is drawn at age of 18 years. Age on the X axis is shown in years.
(Figure 4) did not locate to regulatory regions in skin or peripheral blood cells. SNP-containing DNA regions shown to interact with specific transcription factor proteins or alter protein binding motifs are also indicated in Table S2.

3.7 | QTL analysis of CALM-associated SNPs

The effect of each of the CALM-associated SNPs in Table 2 were further evaluated for effects on gene expression in-cis using publicly available data from the National Institutes of Health Genotype-Tissue Expression GTEx data (V7) from normal whole blood, and skin (sun-exposed [lower leg] and not sun exposed [suprapubic]) tissues as well as transformed fibroblast cell lines (Table S3). Neither of the two genome-wide significantly-associated SNPs (rs3799603 and rs12190451) in CALM1 were identified as potential eQTLs for RPS6KA2 or other coding genes in-cis. Likewise, none of the variants identified by targeted gene sequencing of intron 1 of RPS6KA2 in Table S1 were eQTLs for RPS6KA2 or other
genes **in-cis** (data not shown). rs11742602 and rs1025291 which showed the strongest association with CALM number in CALM2 were not eQTLs for ADCY2 or other genes **in-cis** in the blood or skin tissues examined. In contrast, the C allele (Gly713Arg) of rs34047645 in exon 18 of ADAMTSL3, which was associated with CALM number in CALM1 results in significantly lower expression of GOLGA6L4 (beta = -0.41, p = 4.2 x 10^-5) in sun-exposed skin and higher expression of CSGP4P11 (beta = 0.29, p = 7.1 x 10^-5) in not sun-exposed skin. Also, the G allele of the intergenic SNP rs7615501 on chromosome 3 which correlated with CALM number in CALM2 resulted in significantly higher expression of RFT1 (beta 0.15, p = 2.4 x 10^-5) in transformed fibroblasts and sun-exposed skin, and significantly lower expression of GLT8G1 and PHF7 in sun-exposed skin (Table S3). After correction for multiple comparisons, none of the remaining SNPs were eQTLs for any coding genes **in-cis** in the GTEx tissues examined (Table S3).

Using published association results (beta and p-values) from the genome-wide association studies (GWAS) of the human blood metabolome (http://metabolomics.helmholtz-muenchen.de/gwas/; Shin et al., 2014; Suhre et al., 2011), we further evaluated if any CALM-associated SNPs in Table 2 could act as metabolite QTLs (mQTLs) in blood (Table S4). After correcting for multiple comparisons (alpha of 0.05/20 tests = p-value ≤ 2.5 x 10^-3), **RPS6KA2** SNPs rs4710070 and rs10946177 (r^2 = 0.83) were significant mQTLs for glutamine (beta = 0.01, p-value = 9.8 x 10^-5) and prolyl-4-hydroxyproline (beta = 0.02, p-value = 6.1 x 10^-4), respectively. In addition, rs12765442, rs868692, and rs868691 on chromosome 10, which were in LD (r^2 = 1, D'=1) were mQTLs for uridine (beta =−0.022, p-value = 1.9 x 10^-4) and hypoxanthine (beta = −0.032, p-value = 3.7 x 10^-4) in blood.

4 DISCUSSION

To identify common variants that influence the number of café-au-lait macules in individuals with NF1, a GWAS was performed in two cohorts with CALM number being treated as a quantitative trait. In the CALM1 cohort, multiple, significant SNPs were identified in the intronic regions of **RPS6KA2** (but not in CALM2) and in CALM2 cohort SNPs were identified in **ADCY2** (OMIM103071; but not in CALM1). Meta-analysis of the two cohorts identified no genome-wide level significant SNPs, although three SNPs (two in **RPS6KA2** (rs3799585 and rs10946177)) and one SNP in **TTC6** (rs1951646) were significant at a suggestive genome-wide association level (p-value < 1 x 10^-5). Post-genotyping analysis of the cohorts showed significant differences in age and body surface area, differences between the distribution of CALM number and age, and a large number of the variants shared in common between the two cohorts suggested that differences may likely be due to heterogeneity rather than type I error.

To address this heterogeneity issue, Complimentary Pairs Stability Selection—Genome-Wide Association Study or ComPaSS-GWAS (Sabourin et al., 2018), a method that approximates replication by randomly splitting the data into pseudo-discovery and pseudo-replication sets, was used to identify SNPs in each cohort separately. ComPaSS-GWAS identified four SNPs with EMMAX in the CALM1 cohort (in **RPS6KA2**) and two SNPs in the CALM2 cohort (in **ADCY2**) with ComPaSS-GWAS corroboration score >0.6. Based on previous simulation studies, this suggests that these findings could be replicated in larger and/or more homogeneous samples.

The only SNPs that had a significant ComPaSS-GWAS corroboration score (>0.6), were located in the introns of **RPS6KA2** and **ADCY2**. These two genes belong to RAS-MAPK and cyclic AMP pathways, respectively. These two pathways interact in the cell via common members and converge on the family of transcription factors that control gene expression by binding to cAMP responsive element (CREBs), found in the promoter regions of a number of genes. Both MAPK and cAMP pathways are known to play important roles in proliferation, differentiation, apoptosis and DNA repair among other cellular functions (Anjum & Blenis, 2008; Houslay, 2006; Sassone-Corsi, 2012).

RPS6KA2 (ribosomal protein S6 kinase A2, legacy name **RSK3**) encodes a 90 kDa protein that belongs to a family of closely related (75%–80% amino-acid identity) and evolutionarily conserved serine-threonine kinases that are phosphorylated and activated by RAS-MAPK pathway kinases, encoded by **MAPK3** (OMIM017975) and **MAPK1** (OMIM 176948; Anjum & Blenis, 2008). RPS6KA2 has been identified as a modifier of epidermal growth factor receptor activity in pancreatic cancer (Milosevic et al., 2013). Although no known human phenotypes are associated with **RPS6KA2**, **RPS6KA3** (OMIM 300075) underlies Coffin-Lowry syndrome (CLS; Trivier et al., 1996), a rare X-linked disorder of cognitive delay and skeletal malformations. However, no pigmentary abnormalities are reported in CLS.

The cAMP pathway has been shown to be involved in NF1 pathogenesis. **ADCY2** (adenylate cyclase 2) is a member of a family of genes that encode enzymes catalyzing synthesis of cyclic adenosine 3’,5’-monophosphate (cAMP) from adenosine triphosphate (ATP; Sassone-Corsi, 2012). In mammalian brain, NF1/RAS may affect cellular cAMP through protein kinase C zeta and cell-type specific G-protein coupled receptors (Anastasaki & Gutmann, 2014). Additionally, a study of 243 NF1 patients that focused on association of 2,761 unique SNPs in 22 key regulators of intracellular cAMP levels with optic pathway glioma (OPG), identified an adenylyl cyclase
gene, ADCY8 (OMIM 103070), to be significantly associated with OPG risk (Warrington et al., 2015).

Functional annotation of CALM-associated SNPs in this study revealed that the majority of RPS6KA2 SNPs including the significantly associated rs12190451 in CALM1 mapped to potential enhancers or enhancer-like regions and/or resided in strong open chromatin regions in primary skin cells. The ADCY2 SNPs located to heterochromatin and/or polycomb-repressed regions in fibroblasts and keratinocytes, but not melanocytes. However, none of the RPS6KA2 or ADCY2 associated SNP (and with ComPaSS-GWAS scores >0.6) were identified as eQTLs for the respective genes or any genes in cis in sun-exposed, non-exposed skin tissues, whole blood, and transformed cultured fibroblasts from GTEx (Table S3). The possibility remains, however, that these SNPs may influence mRNA expression of these genes in melanocytes as both RSK2 and ADCY2 proteins are expressed in melanocytes (https://www.proteinatlas.org/).

RPS6KA2 rs3799585 which was associated with CALM number in CALM1 (p-value = 4.34 × 10^{-7}), but not CALM2, has been reported to be a trans-eQTL for SOCS5 (OMIM 607094) on chromosome 2 in monocytes (p-value = 3.15 × 10^{-8}; Fairfax et al., 2014) suggesting that the other haplotype SNPs may also influence monocyte levels of SOCS5 mRNA. We show here using RoadMap and Encode data that rs3799585 together with the significant signal rs3799603 (p-value = 7.4 × 10^{-8}) locate to a promoter region in monocytes, but not in skin cells, and unlike rs3799585, rs3799603 can influence the binding of several transcription factors including YY1 (Choudhury & Ramsey, 2016). SOCS5 is a member of the suppressor of cytokine signaling (SOCS) family of proteins and plays an important role in several immune response pathways including IL4 and JAK-STAT signaling. IL4 (OMIM 147780) can directly inhibit melanogenesis in normal healthy melanocytes via STAT3 signaling. IL4 (OMIM 147780) can directly inhibit melanogenesis in normal healthy melanocytes via STAT3 signaling. Therefore, IL4-induced JAK2-STAT6 signaling in melanocytes could be considered a potential mechanism for influencing pigmentation in skin.

We also observed that RPS6KA2 rs4710070 and rs10946177 (r^2 = 0.83) can influence the level of glutamine and prolyl-4-hydroxyproline metabolites, respectively, in blood (Table S4). Both SNPs were associated with increased CALM number in CALM1 only. Proliferating cells display an intense appetite for glutamine, reflecting its versatility as a nutrient and mediator of intermediary metabolism (DeBerardinis & Cheng, 2010). A role for glutamine in extracellular signal-regulated protein kinase (ERK) signaling/pathways has also been identified in intestinal epithelial cells (DeBerardinis & Cheng, 2010). Prolyn-hydroxyproline (Pro-Hyp) has also been shown to stimulate the growth of skinfibroblasts in rats and consequently increases fibroblasts migrating from the skin (Shigemura et al., 2009).

The lack of replication in this study may likely be due to heterogeneity between discovery (CALM1) and replication (CALM2) cohorts. Several factors may be responsible. The data from this study show an increase in the CALM number with age up to ~18 years and a decrease in CALM number after 18 years (Figure 3). CALM number is known to increase in early childhood (Boyd et al., 2010; Shah, 2010) and then decrease with age (Duong et al., 2011; Huson, Harper, & Compston, 1988). Clinically different features of the patients of the two cohorts due to ascertainment bias of recruitment could explain other parts of the heterogeneity. For the CALM1 cohort, all NF1 patients were eligible, regardless of specific phenotypic features. For the CALM2 cohort, only NF1 patients with plexiform neurofibromas were recruited. Since plexiform neurofibromas can be associated with overlying hyper-pigmentation (Shah, 2010), the high prevalence (essentially 100%) of these tumors in CALM2 that may have inflated the CALM number. Furthermore, plexiform neurofibromas tend to grow during childhood and especially puberty (Dombi et al., 2007; Nguyen et al., 2012); the associated dyspigmentation may also follow this trend. This may partially explain the increase in CALM number we observed in patients <18 years of age; the mini-peak in CALM number around 11–12 years of age is particularly notable (Figure 3).

Difference in phenotyping of NF1 traits is another possible cause of heterogeneity between cohorts. The Wood's lamp method (UV light in a dark room) method was used to find CALM for CALM1; this is helpful to find fainter CALM in older people. However, counting of CALM by Wood's lamp was not used for CALM2. Accurate and robust quantitative phenotyping of clinical traits in NF1 is technically challenging, highly time- and labor-consuming and remains one of the major obstacles in identification of genetic modifiers in NF1. Another possible interpretation of the lack of replication could be that multiple genetic modifiers are involved in NF1, each with a limited effect on the phenotype, but few of them could be identified in a modestly sized GWAS and none in a small one. However, as ComPaSS-GWAS results suggest, present findings within each cohort might be biologically plausible given larger or more homogeneous cohorts.

In summary, we performed the first GWAS for CALM number in neurofibromatosis type 1. We used café-au-lait macule number as a tumor-like and easily quantifiable clinical phenotype to identify genome-wide variation that is associated with this trait. We identified SNPs in intronic region of RPS6KA2, a member of RAS-MAPK signaling pathway, that were associated with CALM number at a suggestively significant genome-wide level. RPS6KA2 is biologically plausible as a genetic modifier of NF1 and warrants further investigation and functional studies.
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CONFLICT OF INTERESTS
DRS performs contract clinical telegenetics services for Genome Medical, Inc, in accordance with relevant NCI ethics policies.

AUTHOR CONTRIBUTIONS
HS and PLH analyzed data, interpreted results, wrote and prepared manuscript for publication. AP managed clinical samples, wrote and prepared manuscript for publication, and submitted manuscript for publication. JAS analyzed data. AMB collected clinical samples and data. SB oversaw high-throughput genotyping and sequencing of DNA. KT and WL analyzed high-throughput genotyping and sequencing data. FNLCR (collectively) processed, SNP-array genotyped and sequenced DNA samples. BCW oversaw clinical portion of the study. DRS originated the study, collected and analyzed clinical data, contributed to experimental design, and wrote the manuscript. AFW contributed to experimental design, oversaw data analyses, and wrote the manuscript.

DATA AVAILABILITY STATEMENT
Data available on request from the authors.

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SUPPORTING INFORMATION

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