The efficacy of abatacept in patients with early diffuse systemic sclerosis (dcSSc) was analyzed to test the hypothesis that patients in the inflammatory intrinsic gene expression subset would show the most significant clinical improvement. 84 participants with dcSSc were randomized to receive abatacept or placebo for 12 months. RNA-seq was performed on 233 skin paired biopsies at baseline, 3- and 6-months. Improvement was defined as a 5 point or >20% change in modified Rodnan skin score (mRSS) between baseline and 12 months. Samples were assigned to intrinsic gene expression subset (inflammatory, fibroproliferative, or normal-like). In the abatacept arm, change in mRSS was most pronounced for the inflammatory (p<0.001) and normal-like (p=0.03) subsets relative to placebo. Participants on placebo remained in their molecular subset while inflammatory participants treated with abatacept moved toward normal-like. The CD28 costimulation pathway decreased in patients that improved on abatacept (FDR=5.88x10^-4) and was specific to the inflammatory subset (FDR=0%). Patients in the inflammatory subset had elevation of the CD28 costimulation pathway at baseline relative to fibroproliferative (p = 0.0026) and normal-like (p=0.0001) participants. There was a correlation between improved ΔmRSS and baseline expression of the CD28 costimulation pathway (R=-0.62, p=0.02). This study provides an example of precision medicine in SSc clinical trials.
Machine-learning Classification Identifies Early Systemic Sclerosis Patients that Improve with Abatacept treatment by Modulating CD28-Pathways

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Abstract
The efficacy of abatacept in patients with early diffuse systemic sclerosis (dcSSc) was analyzed to test the hypothesis that patients in the inflammatory intrinsic gene expression subset would show the most significant clinical improvement. 84 participants with dcSSc were randomized to receive abatacept or placebo for 12 months. RNA-seq was performed on 233 skin paired biopsies at baseline, 3- and 6-months. Improvement was defined as a 5 point or >20% change in modified Rodnan skin score (mRSS) between baseline and 12 months. Samples were assigned to intrinsic gene expression subset (inflammatory, fibroproliferative, or normal-like). In the abatacept arm, change in mRSS was most pronounced for the inflammatory (p<0.001) and normal-like (p=0.03) subsets relative to placebo. Participants on placebo remained in their molecular subset while inflammatory participants treated with abatacept moved toward normal-like. The CD28 costimulation pathway decreased in patients that improved on abatacept (FDR=5.88x10^{-4}) and was specific to the inflammatory subset (FDR=0%). Patients in the inflammatory subset had elevation of the CD28 costimulation pathway at baseline relative to fibroproliferative (p = 0.0026) and normal-like (p=0.0001) participants. There was a correlation between improved ΔmRSS and baseline expression of the CD28 costimulation pathway (R=-0.62, p=0.02). This study provides an example of precision medicine in SSc clinical trials.

Brief Summary (25 words)
Machine-learning Classification in the Abatacept in Systemic SclErosis Trial (ASSET) identified a Subset of Patients that Improve on Therapy via Modulation of a CD28-Related Pathway
Introduction

Systemic Sclerosis (SSc) is a rare, progressive autoimmune disease of unknown etiology that is characterized by extracellular matrix (ECM) deposition, as well as vascular and immunologic abnormalities (1, 2). Patient heterogeneity is an inherent feature of SSc, as manifested through variable skin fibrosis, autoantibodies, organ involvement, and progression. There are two reported clinical subtypes defined by the extent of skin involvement: diffuse cutaneous systemic sclerosis (dcSSc) and limited cutaneous systemic sclerosis (lcSSc). This study focused on dcSSc patients.

Four intrinsic molecular subsets that break down this heterogeneity have been identified across multiple cohorts and organ systems using whole genome expression profiling (3-6). These are the inflammatory subset, characterized by increased immune and fibrotic processes, the fibroproliferative subset, with increased fibrotic, immune and cell-cycle-related processes, the normal-like subset, which more closely resembles gene expression patterns of healthy controls, and lastly, the limited subset which is comprised primarily of patients with lcSSc. These gene expression differences observed between patients may lead to the differential response to therapies. When results from clinical trials are analyzed in aggregate without considering molecular subsets, the differing responses may lead to a report of overall therapy failure, however, retrospective data analyses identified that some molecular SSc subsets respond better to particular drugs (7). These data suggest that molecular intrinsic subsets should be taken into consideration prior to clinical trials. This marks an important step toward precision medicine in SSc.

Here we report a detailed analysis of the gene expression data from skin biopsies of patients treated with abatacept, which is a fusion protein comprised of the extracellular region of CTLA-4 and the
Fc region of IgG1. Abatacept binds to CD80/86 and outcompetes CD28 binding, thus preventing T cell activation (8). A pilot study of eight SSc participants performed a 24-week, placebo-controlled trial that identified key changes in patients that improved on abatacept, in comparison to those that did not (9). The study demonstrated a reduction in the inflammatory signature and CD28-dependent signaling, suggesting that abatacept may be more beneficial to patients with SSc who are in the inflammatory molecular subset.

Following this, in a randomized, double-blind, placebo-control, Phase 2 trial of 88 participants, the Abatacept in Systemic Sclerosis Trial (ASSET), it was shown that while abatacept was well tolerated, the change in mRSS in the abatacept group was greater but not statistically significant compared to the placebo group (10). The data from this study showed that patients from the abatacept treatment group who were classified into the inflammatory intrinsic subset at baseline showed the largest and statistically significant decrease in mRSS in comparison to patients in the placebo treatment group.

We identify gene expression changes in skin occurring in the abatacept Phase 2 clinical trial that relate to the clinical improvement in patients exhibiting an elevated inflammatory molecular signature at baseline. We find that patients in the inflammatory intrinsic gene expression subset have higher expression of the Costimulation of the CD28 family pathway, a signature related to abatacept’s mechanism of action, when compared to proliferative or normal-like patients. Furthermore, though the expression of this pathway decreases in all patients on abatacept, the inflammatory patient subset is the only population that demonstrates a statistically significant decrease with abatacept treatment. We also find that the baseline expression of Costimulation of
the CD28 family is significantly correlated to changes in mRSS in inflammatory patients. Inflammatory and proliferative individuals move toward a normal-like subset when compared to placebo arm individuals of the same molecular subset, who largely remain in the molecular subtype they were at baseline. We therefore conclude that inflammatory patients have elevated expression of pathways targeted by abatacept, which decreases upon treatment, and these patients are thus most likely to clinically respond to this therapy, where they would otherwise remain in their molecular subset assignment and not show signs of disease quiescence or management.

Results

Participants’ clinical and demographic characteristics

Eligible participants with early dcSSc (≤3 years from onset of 1st non-Raynaud’s sign or symptom) were randomized in a 1:1 ratio to either ABA (125 mg subcutaneous) or matching placebo (PLA), stratified by duration of dcSSc. The co-primary endpoints were change in mRSS and safety over 12 months. Escape therapy with immunomodulatory agents was permitted as add-on therapy to study medications due to worsening of dcSSc starting at month 6 (Protocol section of the Supplementary Text). The decision to initiate escape therapy was based on investigator discretion. No biologic agents were allowed as escape therapy.

ABA was well tolerated. Among 88 participants (44 in each treatment group), the adjusted mean change in mRSS at 12 months was -6.24 in the ABA group and -4.49 in the PLA group, with a least-squares mean treatment difference of -1.75 (95% CI -4.93, 1.43; \( P = 0.28 \)) and marked individual variability. Secondary efficacy outcome measures were statistically significant, favoring ABA.
Skin biopsies were collected at baseline, 3 and 6 months from 84 of the 88 patients enrolled in the ASSET clinical trial. The RNA was prepared and sequenced by Illumina RNA sequencing (RNA-Seq). Samples reads were parsed for quality, repeats, and missing information resulting in 140 biospecimens that were used in the initial analysis (Supplementary Figure 3). This data cleaning was done stepwise. First, duplicate patient samples, those with low quality sequence reads, and those missing either their baseline or 6 month biopsies were removed (level 1, representing 36 skin biopsies from 20 participants). In order to allow clinical improvement to be determined accurately, patients that escaped prior to 12 months or had missing mRSS data at 12 months were also removed (Level 2 filtering, representing 57 skin biopsies from 21 participants). The remaining 140 skin biopsies from 47 patients were analyzed (Supplementary Figure 3). Improvement was classified by patients that had a reduction in their baseline mRSS by 20% or 5 points. There were no significant differences between abatacept and placebo arms after biospecimen curation with regards to clinical characteristics including sex, age, and race. (Table 1). Key analyses were subsequently repeated on the larger set of 197 biospecimens from 68 patients that passed level 1 filtering.

**Gene Expression in baseline skin biopsies is associated with molecular subsets**

Analyses of baseline skin biopsies recapitulates the previously defined molecular subsets (Figure 1A). Assessment for batch effect raw counts of RNA-seq transcripts with the gPCA package in R revealed no detectable differences due to batch (Supplementary figure 4; P-value = 0.612). Similar analyses for batch effect post-RPKM normalization similarly revealed no significant variation explained by batch (Supplementary figure 5; P-value = 0.594). Genes and pathways associated
with molecular subset calls assigned using the previously trained SVM classifier (11) are show in figure 1B (10). We find that the patients assigned to the inflammatory subset have an enrichment in pathways consistent with an active immune response, patients assigned to the fibroproliferative subset have active pathways related to cell differentiation and keratinization, and patients assigned to the normal-like subset have elevations in pathways for lipid processes. Of note, the hierarchical clustering of baseline samples does not show patterns in clustering based on treatment arm (abatacept or placebo) or improvement status (Figure 1C). The main driver of the clustering of baseline samples is intrinsic molecular subtype.

The genes and pathways with increased expression in the inflammatory molecular subset (Figure 1B) show enrichment in key immune-related genes that are implicated in abatacept’s mechanism of action (e.g. CD80). These data are consistent with the pilot study, which suggested that the inflammatory subset might be most responsive to treatment, encouraging further analysis (9).

**Change in subtype over time between treatment arms**

In order to understand if patients in the inflammatory or fibroproliferative subtypes become more normal-like over time, we analyzed the SVM subtype classifications at baseline, 3 months, and 6 months post-treatment., (Figure 1D). We observe that the molecular subsets to which the samples were assigned were largely stable over time when considering biopsies from the same patient, with 30 of 47 (63%) participants’ molecular subsets remaining unchanged (Figure 1D). Participants in the abatacept arm that were in the inflammatory subset demonstrated a shift toward a more normal-like signature with 5 of 9 (56%) participants shifting from inflammatory to normal-like and 3 of 9 (33%) remaining inflammatory. In contrast, 6 of 7 (86%) participants who were inflammatory at
baseline in the placebo arm were also inflammatory at their 6 month time point (Figure 1D Fisher’s Exact Test; Inflammatory; P=0.09). Of the 6 participants classified as fibroproliferative in abatacept arm, 4 of 6 (67%) were normal-like at 6 months, while one remained fibroproliferative and one was classified as inflammatory. In contrast, of the 3 participants classified as fibroproliferative at baseline in the placebo arm, all remained in that subset at 3 and 6 months (Figure 1D). Although there is an increase in the number of patients in the fibroproliferative subset that change to normal-like on abatacept relative to placebo, these differences did not reach statistical significance (Figure 1D; Fisher’s Exact Test; Fibroproliferative; P=0.214). Participants that were normal-like at baseline did not show a significant change in subtype regardless of treatment (Figure 1D and Supplementary Table 5; Fisher’s Exact Test; Normal-like; P=0.385). Interestingly, a small subset of normal-like patients became inflammatory (3 participants) or fibroproliferative (3 participants) at 6 months. Therefore, this suggest that some number of patients may move from normal-like, back to a more pathogenic gene expression signature.

**GSEA reveals therapeutic modulation of pathways consistent with abatacept treatment**

GSEA was performed to interrogate and compare enriched pathways across baseline and 6-month biopsies in response to treatment. We performed GSEA on all participant biopsy gene expression profiles and parsed by treatment and improvement status to identify potential associations between therapy-mediated improvement and molecular signatures. As a result, there were 64 pathways enriched in baseline biopsies and 2 pathways enriched in 6-month time point biospecimens. We find that immune-related pathways are enriched in the baseline biopsies of improvers on abatacept but not in 6-month samples, suggesting that these pathways have decreased in the 6-month biopsies (Supplementary Table 1). As in the pilot investigation (9), we identify Costimulation by the CD28
family as one of top pathways enriched in baseline biopsies (0.006% FDR) and subsequently modulated by abatacept in improvers as demonstrated by the directionality of regulation (Table 2). The False Discovery Rate (FDR) acts as a contextualizing statistic, reporting the chances of false discovery (which we prefer to be low) of the genes enriched in this pathway. We do not find this pathway enriched in the baseline of abatacept-treated non-improvers (Supplementary Table 1C), placebo-treated improvers (Supplementary Table 1B), or placebo-treated non-improvers (Supplementary Table 1D). These data suggest that only in improvers on abatacept do we see molecular modulation of pathways directly linked to abatacept’s mechanism of action.

The analysis of ASSET clinical outcomes reported that the inflammatory subset of patients showed the largest clinical improvement, as determined by a decrease in mRSS (10). Accordingly, we divided our dataset by intrinsic subset to identify molecular pathways specific to the subsets in the GSEA analysis. The top 10 pathways for inflammatory (Supplementary Table 2), fibroproliferative (Supplementary Table 3), and normal-like (Supplementary Table 4) can be found among the supplementary materials. Importantly, we find that in inflammatory patients that improved on abatacept, there is an enrichment of the Costimulation by the CD28 family pathway in the pre-treatment biopsies (Table 2). This pathway is not significantly changed in patients on placebo or patients of the proliferative subset treated with abatacept (Table 2). Interestingly, we see that non-improving patients of the inflammatory subset on abatacept still show modulation of the Costimulation by the CD28 family pathway, albeit at the lower pathway rank (Table 2). This suggests that while these patients might not be clinically improving, we still observe effective targeting of the pathway at the molecular level. Taken together, these data demonstrate a measurable decrease in pathways related to abatacept treatment that is specific to inflammatory
patients. The change in pathway expression occurs regardless of clinical improvement, as shown through the enrichment of therapy-relevant pathways in baseline samples from a specific molecular subset of patients. It also reinforces the application of precision medicine to identify patients which could potentially benefit the most from a specific therapy.

Costimulation by the CD28 family pathway is elevated in inflammatory patients and decreases when patients are treated with abatacept

With Costimulation by the CD28 family being implicated in the pilot study and in this work, we sought to take a more granular look and investigate the expression trends of the genes driving the enrichment of this pathway. We find that the core enrichment genes (n = 23) show a decreasing trend between baseline and 6-month time points of improvers treated with abatacept (Figure 2A, (p = 0.064)), with no significant changes in abatacept treated non-improvers (Figure 2B), placebo improvers (Figure 2C), or placebo non-improvers (Figure 2D).

This trend is further clarified when the baseline biopsies are stratified by intrinsic subset. While eight of nine inflammatory patients on abatacept show a decrease in the expression of the core enrichment genes between baseline and 6 months (Figures 3A and 3B), only the improvers show a significant decrease (Figure 3A, p = 0.047). Inflammatory patients on placebo do not show significant changes in the core enrichment genes from Costimulation by the CD28 family (Figures 3C and 3D). Of note, this pathway does not show any significant change in fibroproliferative (Supplementary Figure 1) or normal-like patients (Supplementary Figure 2).
Unsurprisingly, at baseline, the inflammatory subset of patients has significantly higher expression of core enrichment genes from *Costimulation by the CD28 family*, compared to the baseline expression of this pathway in patients that were proliferative (p = 0.0026) or normal-like (p<0.0001) (Figure 4). We repeated this analysis adding back the patients removed by level 2 filtering and find this difference remains when the set of 197 skin biopsies (68 patients) is analyzed (Supplemental Figure 7). Thus, we postulate that inflammatory patients are more likely to improve on abatacept because genes and pathways relevant to its mechanism of action have increased expression at baseline, and thus inflammatory patients are more responsive to therapeutic modulation by abatacept.

**Expression of the Costimulation by the CD28 family at baseline in inflammatory patients is significantly related to change in skin thickness score at 12 months**

To determine if the *Costimulation by the CD28 family* signature was associated with clinical improvement on abatacept, we calculated change in mRSS correlations between baseline and 12 months and the original baseline expression. When considering the 47 patients that passed filtering, there are no significant correlations in the abatacept (Figure 5A) or placebo (Figure 5B) arms between baseline mRSS and total change across time points. In conducting this analysis parsed by molecular subtype, we observed significant correlation between baseline expression of core enrichment genes of the *Costimulation by the CD28 family* pathway and decrease in mRSS for inflammatory patients on abatacept (Figure 5A, Pearson R = -0.76, p = 0.018). This correlation was not significant for inflammatory patients on placebo (Figure 5B), proliferative patients on abatacept (Figure 5A) or placebo (Figure 5B), or normal-like patients on abatacept (Figure 5A). Conversely, we do observe the opposite association between change in mRSS and normal-like
patients on placebo (Figure 5B, Pearson R = 0.67, p=0.016). We repeated this analysis in the 68 patients obtained when only level 1 filtering is applied and find the result is confirmed in the larger cohort (Supplemental Figure 8).

Inclusion of all inflammatory patients at baseline is significantly related to the expression of the Costimulation by the CD28 family

To identify pathways modulated between baseline and post-treatment timepoints, prior analyses required removal of samples that were missing RNA-seq measurements or clinical data at the post-treatment timepoint (level 1 and 2 filtering, Supplementary Figure 3). However, using only baseline gene expression for subsequent analyses means that patients for whom RNA-seq data was missing can now be added to analyses and in addition, linear mixed models can be used to estimate mRSS in post-treatment time points for patients who escaped therapy (10). We find that our results remain true when all inflammatory patients on abatacept (regardless of filtering) in the trial are considered (Figure 6, Pearson R= -0.62, p=0.02) relative to change in MRSS from baseline to 12 months. We also analyzed the correlation between average expression centroid of the core enrichment genes from Costimulation by the CD28 family pathway in the 6 month biopsy compared to the change in mRSS from baseline to 6 months. We find that the overall relationship holds at the 6-month time point although the slope of the line has decreased, likely reflecting the treatment associated decrease in the pathway expression (Supplementary Figure 6 A and B).
Discussion

The rarity and heterogeneity in SSc often lead to statistically underpowered clinical trials that result in outcomes that are difficult to interpret. Retrospective analyses of clinical trials have previously suggested that assessing patient heterogeneity can help identify patients that are most likely to benefit from specific therapies. Gene expression analyses of a pilot study of abatacept suggested that inflammatory pathways were highly expressed in patients that improved, and that the expression of these pathways decreased with improvement (9). A meta-analysis of clinical trials in SSc came to a similar conclusion using data from five clinical studies including mycophenolate mofetil, rituximab, abatacept, nilotinib, and fresolimumab. This study showed that abrogation of inflammatory pathways, regardless of treatment, was most significantly associated with clinical improvement (7). This meta-analysis also showed that patients with a high TGF-β signature at baseline were most likely to benefit in a trial of fresolimumab, which targets TGF-β-1 (7).

In this work we analyze the gene expression at baseline of patients in the abatacept clinical trial and its changes with clinical improvement across molecular subtypes in the context of therapy. Consistent with previous work, we find that inflammatory patients with SSc have an increased expression of pathways targeted by abatacept at baseline and are the most likely group to benefit from treatment. We find that the Costimulation by the CD28 family pathway, which is directly related to mechanism of action of abatacept, is elevated in inflammatory patients at baseline, and that the decrease in expression of this pathway correlates with clinical improvement and resolution of skin fibrosis as represented by decrease in skin severity score. Interestingly, in the placebo arm, normal-like patients seem to worsen if they have high expression of the CD28 family genes at
baseline. While this data may be explained by a select few samples, we note that normal-like patients who improved on abatacept showed modulation of *Costimulation by the CD28 family* and that the expression of these genes seemed to decrease in most normal-like patients treated with the drug. As with the clinical study (10) we see no clear impact on the fibroproliferative subset of patients on abatacept, except for a non-statistically significant increase in the number of fibroproliferative patients that move to the normal-like subset.

These data suggest that intrinsic subsets may provide a powerful platform to stratify the patient population with SSC prior to assessing the clinical efficacy of a therapeutic agent. In participants treated with abatacept who showed clinical improvement, we observe a consistent and statistically significant shift from the inflammatory to the normal-like subset by month 6. Interestingly, a similar shift was observed for a subset of fibroproliferative participants, but the change did not reach statistical significance. Participants in the placebo arm of the study were more likely to maintain a stable subset assignment from baseline to 6 months although we note several exceptions in the inflammatory subset that included one biopsy classification as fibroproliferative at the 3 month point and one as normal-like at the 3 month point, which were both bounded by inflammatory subset assignments at baseline and 6 months. One patient is observed to shift from inflammatory to fibroproliferative at 6 months. These changes represent 3 biopsies of 21 (14%) for the inflammatory patients in the placebo arm, which is within 15% classification error rate. Since individuals in the placebo arm predominantly remained in their baseline molecular subtype, largely unchanged, reproducing prior observations (4) suggesting that the subtypes are stable through 6 months in the absence of disease modifying treatment. These data provide an approach by which we can use precision medicine in SSC to identify those patients most likely improve on
therapy, determine target engagement, and track an individuals’ molecular changes in disease state over time. This and our previously published analyses suggest that clinical trialists should acknowledge molecular heterogeneity in early SSc and should enrich for pharmacologic target specific subset (such as inflammatory intrinsic subset for abatacept, mycophenolate mofetil, etc) or stratify randomization based on these subsets. In addition, our recent published meta-analysis highlights the role of scleroderma autoantibodies as an enrichment criterion and the overlap with the intrinsic gene expression sets (12, 13).

This study has a number of limitations. RNA-sequencing of skin biopsies from participants followed by subset classification using our machine learning algorithm confirmed the a priori hypothesis that the inflammatory subset would improve. Although this is very promising, this result needs to be confirmed in a prospective phase 3 clinical trial. The study population remains relatively small and these results need to be confirmed in a larger cohort of patients. Some individuals escaped therapy due to clinically worsening disease during the trial including mRSS increase, pulmonary hypertension or renal crisis, which could potentially introduce biases into our analyses. Those that escaped therapy included 10 individuals in the inflammatory subset, which could reduce the generalizability of our findings.

While the use of RNA-sequencing to further investigate the tissue biology of these individuals brought many benefits, the filtering for data quality and escape events resulted in a reduction in samples analyzed here. We addressed this by adding back samples that were removed in level 2 filtering and repeating key analyses. We find that the exclusions increased signal to noise but did not change the main findings of the analysis, as similar trends in co-stimulation by CD28 Pathway
expression among molecular subtypes was still observed (Supplementary Figure 7), with highest expression difference noted among participants in the inflammatory (P<0.05). Correlations between the CD28 pathway and mRSS demonstrated the same trends when all biopsies were added back in from those individuals were removed in level 2 filtering (Supplementary figure 8).

In summary, we demonstrate that measuring molecular heterogeneity in SSc patients can assist in the interpretation of clinical trial results and may be valuable tool that can be used in future studies to identify the patients most likely to improve on a given therapy.
Methods

Participants in the ASSET Clinical Trial

Participants in this study were from a Phase 2, investigator-initiated, randomized, double-blind, placebo-controlled trial of abatacept in patients with early-stage dcSSc, meaning disease duration at less than 36 months at enrollment (clinicaltrials.gov NCT02161406). DcSSc was defined as skin thickening, proximal as well as distal, to the elbows or knees with or without involvement of the face and neck. Study participants were treated for 12 months on double-blind study medication and were offered an additional six months of open-label SC abatacept therapy as previously (14). Key inclusion criteria and have been described in the clinical study (10). Oral corticosteroids (≤10 mg/day of prednisone or equivalent) and NSAIDs were permitted if the patient was on a stable dose regimen for ≥2 weeks prior to and including the baseline visit, but no background immunomodulatory therapies were allowed. Written informed consent was obtained from each participant. Improvement was defined as a 5 point or >20% change in modified Rodnan skin score (mRSS) between baseline and 12 months as used in the primary ASSET clinical trial analyses (10, 15).

Collection and RNA-sequencing

Skin biopsies were collected (3mm) from the forearm of participants enrolled in the trial. Biopsies were collected at baseline, 3 months, and 6 months. Samples were stored in RNALater and subsequently homogenized using a Qiagen TissueLyser II. RNA was purified using Qiagen’s RNeasy Fibrous Tissue minikit. Quality and concentration of RNA was assessed on a TapeStation 4400 (Agilent). 100ng of total RNA was used for library preparation using the TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA). Libraries were prepared manually or automated on an epMotion 5075t (Eppendorf, Hauppauge, NY). Libraries were assessed for size
and concentration using the TapeStation 4400. Sequencing libraries were quantified on a Qubit fluorometer 3.0 (Thermo Fisher Scientific, Waltham, MA) prior to normalization for equimolar pooling. Single-index paired-end sequencing was performed on Illumina’s NextSeq 500 to achieve >40 million reads per sample. Sequencing runs for samples were kept according to experiment and patient specific factors outlined in Supplementary Figure 3.

**Subset Classification**

RNA-seq data was first normalized using Feature-specific Quantile Normalization (FSQN) (16) to allow subset classification of RNA-seq data. Molecular subset classifications assigned in the ASSET Phase II clinical trial paper were used for purposes of these analyses. The SVM classifier to assign SSc intrinsic molecular subsets was previously developed and its construction (including cross-validation of training and test sets), internal, and external validation in independent datasets can be found in Franks et al 2019 (11).

**Data processing and visualization**

233 biospecimens were prepared and sequenced. An in-home RNA-seq pipeline was applied to align the paired-end sequencing data. First, we applied the cutadapt (version 1.15) to remove the adapter sequence (in our case it is “AGATCGGAAGAGC”) for the FASTQ files. Then, STAR (version 2.5.3a) was applied to align the reads to the hg19 human genome(17). Next, gene abundance was quantified using RSEM (version 3.3.9)(18). Lastly, normalized RPKM was calculated using TMM function (edgeR package)(19). The dataset is available on NCBI’s Gene Expression Omnibus (GEO) under accession number GSE217067.
RPKM data was log2 and median-centered in R prior to data being visualized via Cluster 3.0 and Java Treeview. Pathway enrichment in gene lists was determined using gProfiler. Data visualization for subtype progression between baseline through month 6 was assessed for statistical significance by Fisher’s Exact Test for each molecular subtype.

**Gene Set Enrichment Analysis (GSEA)**

To identify differentially enriched molecular pathways, GSEA was run as a Gene Pattern module using the gene set permutation option. The C2:Reactome gene set database from Molecular Signatures Database was used (v6.1 MSigDB) (Subramanian, Tamayo et al. 2005, Liberzon, Subramanian et al. 2011)(20, 21). GSEA was performed on various comparisons and only results <FDR 10% are reported. The GSEA .html output files defined core enrichment.

**Author Contributions**

MLW, DK, DF and CS designed the research study. BKM, JMF, YY, YW, and TW conducted experiments. BKM, TW, DK and MLW acquired data. BKM, MEE, JMF, YY, YW, TW, JG, CS, DF, DK and MLW analyzed data. BKM, MEE, DK and MLW wrote the manuscript. All authors approved the manuscript. BKM and MEE are co-first author. BKM performed all initial RNA-seq of samples, bioinformatic analyses and drafted the manuscript. MEE completed the bioinformatic analyses and the manuscript.

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**Role of the funding source**

This was an investigator-initiated trial designed by the Sponsor (Dinesh Khanna, MD, MSc) and the steering committee. The industry funder, Bristol-Myer Squibb (BMS), had no role in collecting, analyzing, and interpreting the data. Mechanistic studies, including analysis of gene expression in skin biopsies, was funded by the NIH/NIAID through the University of Michigan Clinical Autoimmunity Center of Excellence. The clinical data were stored at the University of Michigan DCC. Gene expression data were generated, stored and analyzed at Dartmouth College.
## References


Figure 1. Baseline skin biopsies recapitulate intrinsic subset biology. A. Hierarchical clustering of Log2 median centered data, with the most variable genes (2179 genes) from 2fold-2array on Cluster 3.0. Color bars represent intrinsic subset by SVM classifier, and treatment-improvement status. B. Expansion of gene clusters from (A) that correlate with intrinsic subset SVM call. Selected genes are shown. Pathways are top 10 from g:profiler (FDR < 5%). C. Dendrogram
showing color bars for SVM calls at baseline, 3 and 6 months skin biopsies. The bar on the far right shows abatacept and placebo treatment, color-coded by improver and non-improver status.

D. graphic describing progression of subtypes from baseline to month 6. Counts of individuals with each progression are to the right.
Figure 2. Comparison of the *Costimulation by the CD28 family* average expression for patients between baseline and 6-month time points. A. Average gene expression for core enrichment genes in *Costimulation by the CD28 family* pathway in patients on abatacept that improved. Data is log2 and median centered. B. Average gene expression for core enrichment genes in *Costimulation by the CD28 family* pathway in patients on abatacept that did not improve. Data is log2 and median centered. C. Average gene expression for core enrichment genes in *Costimulation by the CD28 family* pathway in patients on placebo that improved. Data is log2 and median centered. D. Average gene expression for core enrichment genes in *Costimulation of CD28*
family pathway in patients on placebo that did not improve. Data is log2 and median centered.

Paired T-test p-values shown.
Figure 3: Comparison by the Costimulation of CD28 family average expression for patients in the inflammatory subset between baseline and 6-month time points. A. Average gene expression for core enrichment genes in Costimulation by the CD28 family pathway in inflammatory patients on abatacept that improved. Data is log2 and median centered. B. Average gene expression for core enrichment genes in Costimulation by the CD28 family pathway in inflammatory patients on abatacept that did not improve. Data is log2 and median centered. C. Average gene expression for core enrichment genes in Costimulation by the CD28 family pathway in inflammatory patients on placebo that improved. Data is log2 and median centered. D. Average gene expression for core enrichment genes in Costimulation by the CD28 family pathway in
inflammatory patients on placebo that did not improve. Data is log2 and median centered. Paired T-test pvalues shown.

Figure 4: Baseline skin biopsies classified as the inflammatory intrinsic subset have elevated expression of core enrichment genes from Costimulation by the CD28 family. Baseline skin biopsies (n = 47) were log2 and median centered. ANOVA found the means to be significantly different (p<0.001). P-values shown are using Tukey’s test for multiple comparisons. B. Hierarchical clustering of core enrichment genes from Costimulation by the CD28 family and baseline biospecimens.
Figure 5: Baseline expression of core enrichment genes from the Co-stimulation by the CD28 family is associated with patient improvement on abatacept and on placebo therapy in the inflammatory subset. A) All patients on Abatacept (n = 25) then split by intrinsic subset: Inflammatory, Proliferative, and Normal-like. B) All patients on placebo (n = 22) then patients split by intrinsic subset: Inflammatory, Proliferative, and Normal-like.
Figure 6: Baseline expression of core enrichment genes from *Costimulation by the CD28 family* is associated with estimated change in skin severity on abatacept in the inflammatory subset (n = 18).
<table>
<thead>
<tr>
<th></th>
<th>All (n=47)</th>
<th>Placebo (n=22)</th>
<th>Abatacept (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal-like (n=22)</td>
<td>Inflammatory (n=16)</td>
<td>Proliferative (n=9)</td>
</tr>
<tr>
<td>Age, Years, mean (SD)</td>
<td>51(13.2)</td>
<td>54(8.9)</td>
<td>46(13.9)</td>
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<tr>
<td>Sex</td>
<td>Female, N (%)</td>
<td>21(95)</td>
<td>11(69)</td>
</tr>
<tr>
<td></td>
<td>Male, N (%)</td>
<td>1(5)</td>
<td>5(39)</td>
</tr>
<tr>
<td>Race/Ethnicity</td>
<td>White, N(%)</td>
<td>18(82)</td>
<td>14(88)</td>
</tr>
<tr>
<td></td>
<td>Black, N(%)</td>
<td>1(4)</td>
<td>1(6)</td>
</tr>
<tr>
<td></td>
<td>Asian, N(%)</td>
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<td>0(0)</td>
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<tr>
<td></td>
<td>Hispanic, N(%)</td>
<td>3(14)</td>
<td>1(6)</td>
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<tr>
<td>Disease Duration, Years, mean (SD)</td>
<td>1.5(0.9)</td>
<td>1.8(0.8)</td>
<td>1.5(0.7)</td>
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<tr>
<td>mRSS, mean(SD)</td>
<td>17(4.6)</td>
<td>26(7.1)</td>
<td>22(5.2)</td>
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</table>

Table 1. Demographic and Baseline characteristics for all (N=47) individuals, also reported by treatment arm: Placebo (N=22) and Abatacept(N=25). Continuous variables reported as mean (SD); categorical values reported as count (percentage).
<table>
<thead>
<tr>
<th></th>
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<th>Proliferative</th>
<th>Normal-like</th>
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<tr>
<td>FDR Base/6month</td>
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<td>0.451</td>
<td>0.0153</td>
<td>0.659</td>
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<tr>
<td></td>
<td>(Base)</td>
<td>(Base)</td>
<td>(Base)</td>
<td>(Base)</td>
</tr>
<tr>
<td></td>
<td>0 (Base)</td>
<td>-</td>
<td>0.0561</td>
<td>0.337</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(Base)</td>
<td>(6Month)</td>
</tr>
<tr>
<td>FDR 6 month</td>
<td>Downregulated</td>
<td>Downregulated</td>
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<tr>
<td></td>
<td>-</td>
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<tr>
<td><strong>Placebo</strong></td>
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<tr>
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<td></td>
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<td>0.987</td>
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<td>FDR 6 month</td>
<td>-</td>
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</table>

Table 2. False Discovery rates of CD28 and resulting global directionality of expression in Abatacept vs Placebo treatment arm participants. FDR Base/6Month: False Discovery Rate (FDR) of CD28 costimulatory pathway in Abatacept and Placebo individuals plus timepoint where significant enrichment of the pathway is noted in trial participants (Base or 6Month). FDR 6 Month: Resulting pathway directionality at 6 Month time point of ASSET clinical trial (Downregulated/Upregulated/No Change). No change in pathway at 6 months is denoted by “-“.