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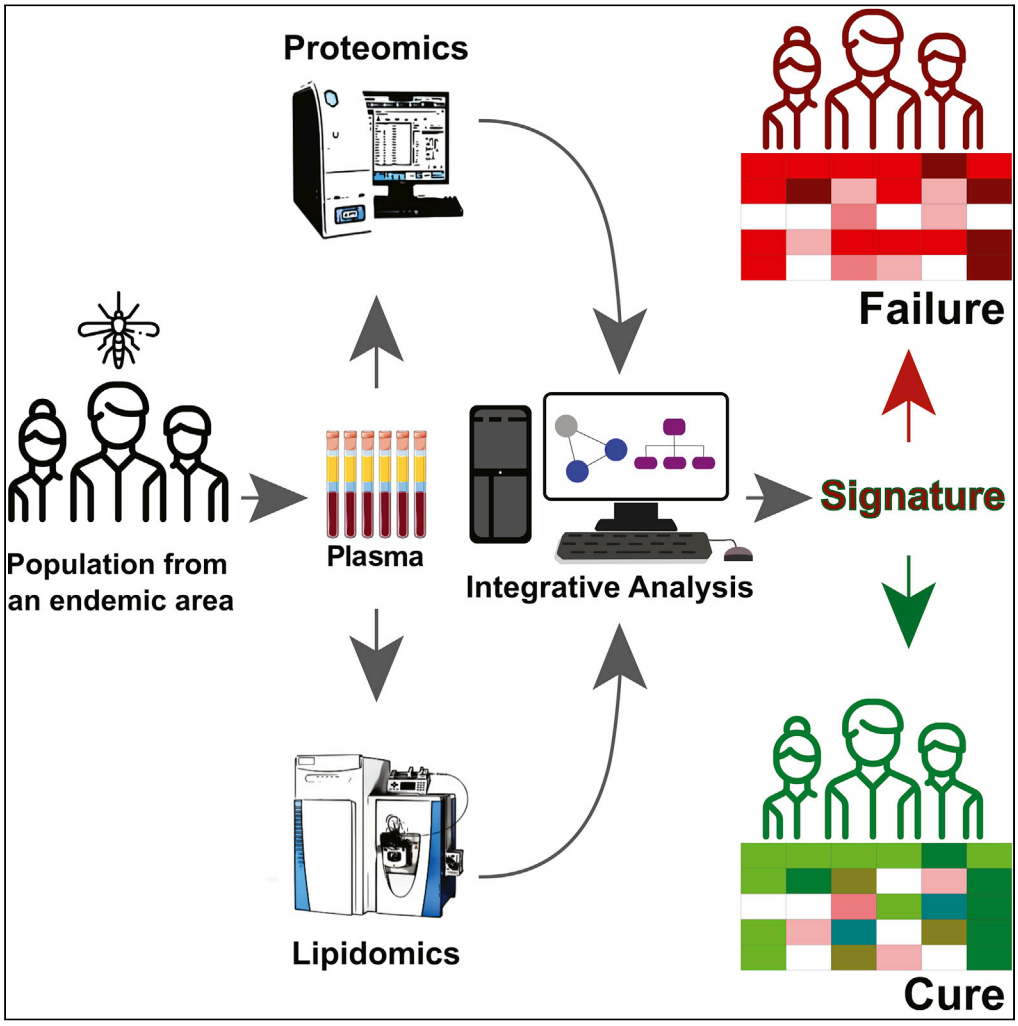

Hayna Malta-Santos, Kiyoshi F. Fukutani, Carlos A. Sorgi, ..., Edgar M. Carvalho, Bruno B. Andrade, Valéria M. Borges

bruno.andrade@fiocruz.br (B.B.A.)
valeria.borges@fiocruz.br (V.M.B.)

HIGHLIGHTS

Plasma markers were tested to predict outcomes of patients with cutaneous leishmaniasis

Patients who failed treatment exhibited distinction in biomarker correlation networks

A biosignature of treatment failure included plasma cytokines and lipid mediators

Levels of eotaxin, TGF-β, and 11-HETE could be used to predict outcomes

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Multi-omic Analyses of Plasma Cytokines, Lipidomics, and Transcriptomics Distinguish Treatment Outcomes in Cutaneous Leishmaniasis

Hayna Malta-Santos,[1,2] Kiyoshi F. Fukutani,[2,3] Carlos A. Sorgi,[4] Artur T.L. Queiroz,[2,3] Viviane Nardini,[4] Juliana Silva,[5] Alex Lago,[5] Lucas P. Carvalho,[2,5] Paulo L.R. Machado,[5] Patrícia T. Bozza,[6] Jaqueline França-Costa,[2,5] Lucia H. Faccioli,[4] Edgar M. Carvalho,[1,2,5] Bruno B. Andrade,[1,2,3,7,8,9,*] and Valéria M. Borges[1,2,9,10,*]

SUMMARY

***Leishmania braziliensis* infection frequently results in cutaneous leishmaniasis (CL). An increase in incidence of drug-resistant CL leading to treatment failure has been reported. Identification of reliable predictors of treatment outcomes is necessary to optimize patient care. Here, we performed a prospective case-control study in which plasma levels of cytokines and lipid mediators were assessed at different time points during antileishmanial therapy in patients with CL from Brazil. Multidimensional analyses were employed to describe a combination of biomarkers able to predict and characterize treatment failure. We found a biosignature influenced mainly by plasma levels of lipid mediators that accurately predicted treatment failure. Furthermore, transcriptomic analysis of a publicly available data set revealed that expression levels of genes related to lipid metabolism measured in skin lesions could distinguish treatment outcomes in CL. Thus, activation of pathways linked to lipid biosynthesis predicts treatment failure in CL. The biomarkers identified may be further explored as therapeutic targets.**

INTRODUCTION

Leishmaniasis is a group of diseases caused by *Leishmania spp* parasites. The World Health Organization (WHO) considers leishmaniasis a serious public health concern (World Health Organization, 2018), with a worldwide incidence reaching as high as 1,2 million new cases every year (Alvar et al., 2012). Individuals infected with *Leishmania* can develop a wide spectrum of clinical manifestations, ranging from localized cutaneous disease (cutaneous leishmaniasis [CL]) to a chronic systemic illness named visceral leishmaniasis (VL) (Dutra et al., 2011). The determinants of disease outcomes are described to involve factors directly linked to parasite species, as well as those associated with the host immune system (Dutra et al., 2011). Brazil is a major endemic region for both cutaneous and visceral leishmaniasis, with a recent geographical spread of disease transmission and increased detection of cases in more urbanized areas (Bustamante et al., 2009; Costa, 2008; Desjeux, 2001; Nascimento et al., 2008). Within this country, *Leishmania braziliensis* accounts for the vast majority of the CL cases (Scorza et al., 2017). This parasite species has been associated with development of different clinical forms such as localized, mucosal, and disseminated leishmaniasis (Queiroz et al., 2012; Scorza et al., 2017), highlighting its contribution to the high burden of this disease. Although there are many different pathophysiologic mechanisms underlying the progression of distinct clinical forms of leishmaniasis, the treatment options are few, with no significant recent advances in the field that have led to implementation of new therapies (Uliana et al., 2018).

Pentavalent antimonials (Sb^{v}) are the first-line drugs used to treat leishmaniasis in Brazil and other countries (World Health Organization, 2018). Other medications, such as amphotericin B, pentamidine, and miltefosine, are often used as alternative treatment options in patients who have failed Sb^{v} therapy or relapsed (Uliana et al., 2018). Treatment failure is reflected by persistence of open ulcers without re-epithelization, whereas relapse is defined as the reactivation of lesions once the therapy is terminated (Ponte-Sucre et al., 2017). In Brazil, studies have shown that occurrence of treatment failure in CL can be as high as 45% (Machado et al., 2010; Prates et al., 2017). Factors that may underlie this high incidence of unfavorable

[1]Faculdade de Medicina da Bahia (FAMED), Universidade Federal da Bahia, Salvador, Brazil

[2]Instituto Gonçalo Moniz (IGM), Fundação Oswaldo Cruz (FIOCRUZ), Salvador, Brazil

[3]Multinational Organization Network Sponsoring Translational and Epidemiological Research (MONSTER), Salvador, Brazil

[4]Faculdade de Ciências Farmacêuticas de Ribeirão Preto (FCFRP-USP), Universidade de São Paulo (USP), São Paulo, Brazil

[5]Serviço de Imunologia, C-HUPES, Universidade Federal da Bahia, Salvador, Brazil

[6]Laboratório de Imunofarmacologia, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

[7]Escola Bahiana de Medicina e Saúde Pública, Salvador, Brazil

[8]Universidade Salvador (UNIFACS), Laureate Universities, Salvador, Brazil

[9]These authors contributed equally

[10]Lead Contact

*Correspondence: bruno.andrade@fiocruz.br (B.B.A.), valeria.borges@fiocruz.br (V.M.B.)

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outcomes are not fully understood. Early identification of patients at high risk of treatment failure can lead to optimization of therapeutic regimens and potential reduction of drug resistance. In fact, a recent study has demonstrated that expression levels of genes related to cytolytic and IL-1 pathways, as well as increased counts of parasite transcripts in skin lesions, are able to predict treatment failure in patients with CL (Amorim et al., 2019). However, such study was focused only on transcriptomic analysis and no evaluation of protein or lipid mediators in similar setting has been performed.

We have previously described that different clinical forms of CL are associated with distinct activation of the eicosanoid pathway (França-Costa et al., 2016). Briefly, among the distinct disease presentations related to CL, patients with localized cutaneous leishmaniasis (LCL) exhibit higher levels of prostaglandin E_2 (PGE_2), whereas those with mucosal cutaneous leishmaniasis (MCL) display augmented levels of leukotriene B_4 (LTB_4) in plasma (França-Costa et al., 2016). Furthermore, a prospective cohort study of patients with VL demonstrated that this disease presentation is associated with heightened levels of both inflammatory proteins and lipid mediators, which significantly diminish after antileishmanial treatment (Araújo-Santos et al., 2017). Whether a prospective change in biomarker signatures, especially in those composed by lipid mediators, among patients with CL undergoing treatment relates to risk of unfavorable outcomes has not been previously described.

Here, we employed systems biology analyses to prospectively examine whether simultaneous assessment of plasma levels of inflammatory proteins and lipid mediators could identify biomarkers able to predict and characterize treatment failure in patients with CL from an endemic region in Brazil. Our findings identified a biosignature highly influenced by unique expression of lipid mediators which is able to accurately predict treatment failure. Such findings, if validated in other settings, may be useful for predicting therapeutic outcomes in CL. In future studies, the molecules identified here as part of the biomarker signature could be explored as potential targets in a host-directed therapy focused on reducing odds of treatment failure.

RESULTS

Patient Characteristics

A total of 63 patients with CL were included in the study. The median age of the study population was 27 years old (interquartile range [IQR]: 19–33), with the majority of the study participants being men (71%). The groups of patients stratified according to treatment outcomes (cure vs. failure) were similar with regard to age and sex (Table 1). In addition, the median disease duration was also similar between the groups ($p = 0.8$, Table 1). At pre-treatment, patients who further experienced treatment failure often presented with increased number of lesions than those who were further cured (Figure S1).

A Unique Profile of Plasma Cytokines and Chemokines Characterizes Patients Who Fail Antileishmanial Treatment

Cryopreserved plasma samples were used for measurements of several biomarkers. The overall design of the analytical plan is described in Figure S2. We prospectively examined changes in plasma concentrations of cytokines, chemokines, and growth factors in patients with CL undergoing antileishmanial treatment. We compared plasma measurements in treatment-naïve patients (day 0) and after treatment (day 60). To do that, we first built a heatmap inputting log-transformed and z-score normalized data on the mean concentration value for each biomarker calculated for each clinical group and time point. An unsupervised

Parameter	Cure	Failure	p-value
N	31	32	
Male, no (%)	21 (67.7%)	24 (75%)	0.5
Age, years	25 (13–52)	27 (16–56)	0.8
Disease duration, days	32 (21–90)	33 (15–70)	0.8

Table 1. Clinical and Epidemiological Characteristics for Patients with Cutaneous Leishmaniasis that Showed Cure or Therapeutic Failure After Antileishmanial Treatment

The variables age and disease duration are presented as median values with range (minimum and maximum values), whereas sex and the number of active lesions were plotted as frequencies.

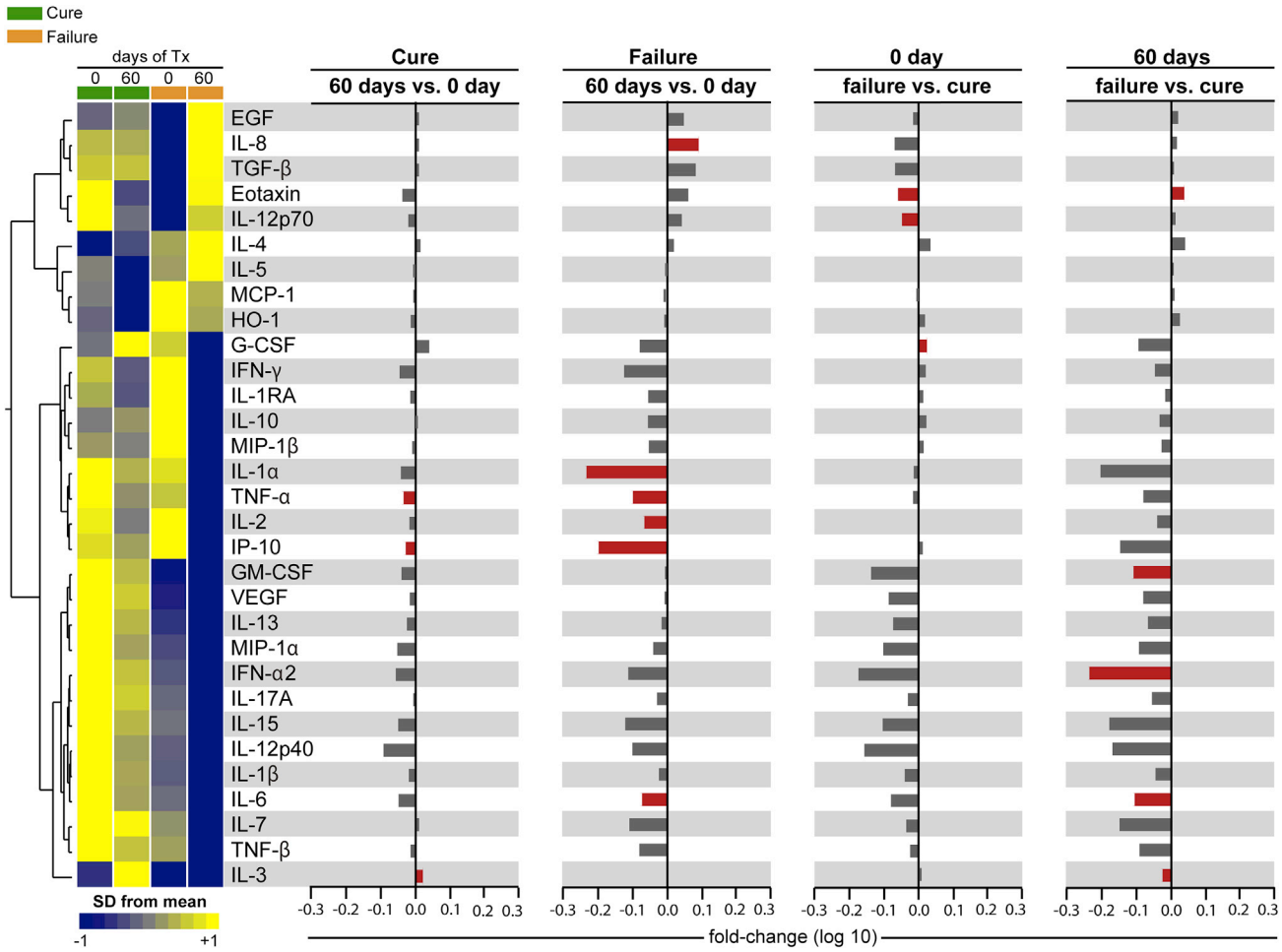

Figure 1. Patients Who Failed Therapy Exhibit a Distinct Profile of Inflammatory Proteins in Plasma

Left panel Data on mean plasma concentration of each indicated marker per patient group and time point were log-transformed and *Z* score normalized, and a heatmap was used to illustrate trends in data variation. A hierarchical cluster analysis (Ward's method with 100X bootstrap) was used to group the biomarkers with similar distribution between clinical groups and time points. Dendrograms represent Euclidean distance. Rigth panel Fold differences between indicated means were calculated, and log10 values were plotted. Differences between day 60 and day 0 within each clinical group were examined using the Wilcoxon matched paired test. Comparisons between the groups of treatment failure and cure at the indicated time points were performed using the Mann-Whitney *U* test. Red bars indicate mediators that were significantly different between groups.

hierarchical clustering (Ward's method) was therefore used to test whether biomarkers could be grouped based on similarity in their profile of expression between the clinical groups. By comparing the two clinical groups, we observed that patients with CL who experienced treatment failure exhibited a unique biosignature characterized by a distinct expression profile of inflammatory cytokines in plasma in both study time points (Figure 1, left panel). Furthermore, we calculated fold differences in concentration values of the cytokines and growth factors between the time points within each clinical group and also between the clinical groups in each time point, as depicted in Figure 1 (right panel). This approach was used to summarize large numbers of comparisons. Details on the distribution of the individual values are shown in Figure 2 (comparisons between time points) and Figure S3 (between the clinical groups at each time point). In the group of patients that were successfully treated, median values of TNF-α and IP-10 substantially decreased whereas, those of IL-3 significantly increased at day 60 (Figure 2). Patients who failed therapy exhibited a significant reduction in concentrations of IL-1α, TNF-α, IL-2, IP-10, and IL-6 with increased levels of IL-8 after therapy (Figure 2). When the two clinical groups were compared at each time point, we observed that, before therapy initiation, patients who would experience treatment failure displayed lower levels of eotaxin and of IL-12p70 and increased concentrations of G-CSF compared to those in individuals who were successfully treated (Figures 1 and S3). At day 60, treatment failure was associated with

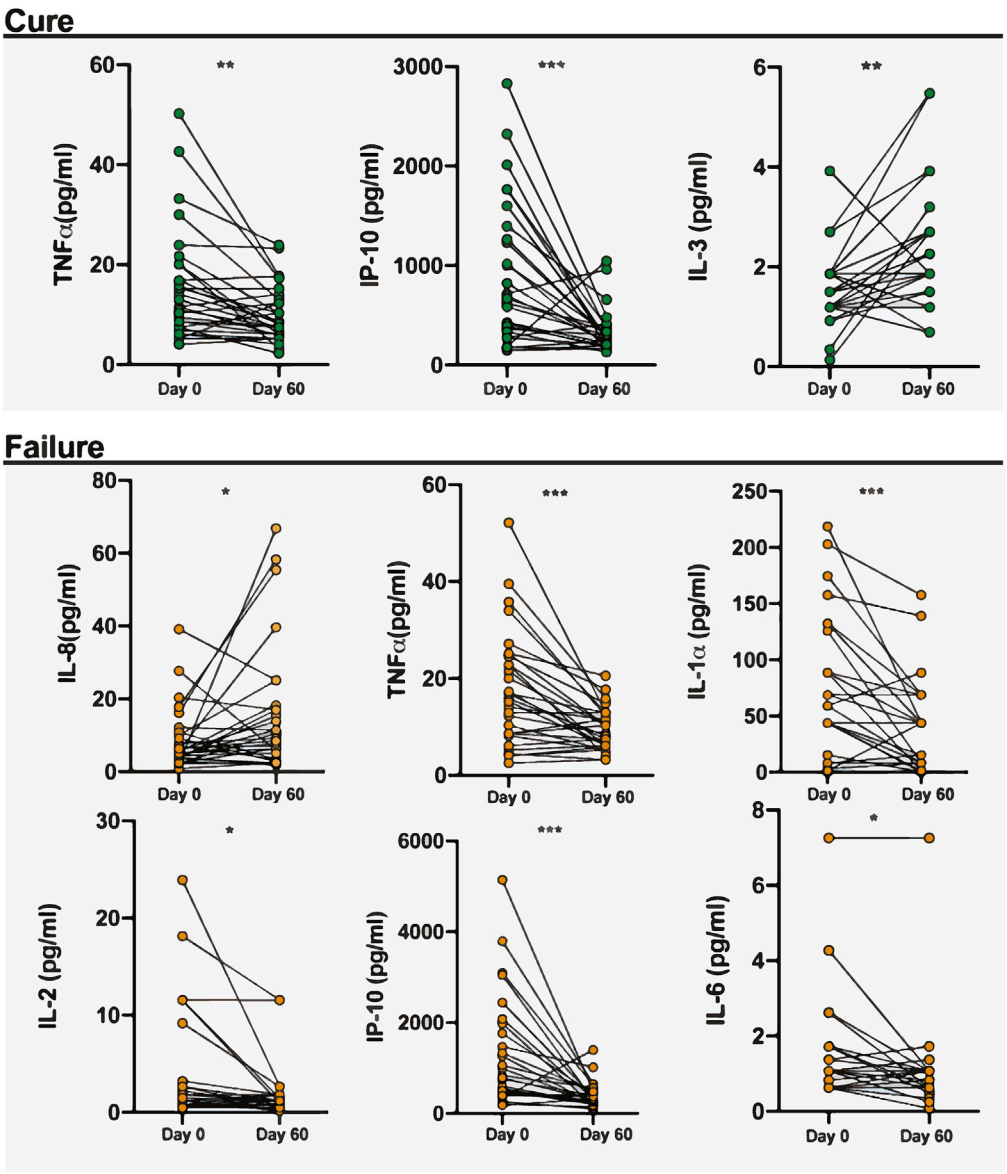

Figure 2. Inflammatory Proteins in Plasma of Patients with Cutaneous Leishmaniasis According To Treatment Outcome

Parameters that displayed statistically significant differences between the time points were tested using the Wilcoxon matched pairs. *p ≤ 0.01; **p ≤ 0.001; ***p ≤ 0.0001.

heightened levels of eotaxin and diminished concentrations of GM-CSF, IFN-α2, IL-6, and of IL-3 (Figures 1 and S3).

Abundance of Lipid Mediators in Patients with Cutaneous Leishmaniasis according to Treatment Outcomes

To gain insights into the association between a specific lipid profile and the treatment outcome of the study population, two sets of analyses were performed. First, we prospectively assessed abundance levels of lipid mediators in plasma and performed hierarchical clustering analysis. This analysis is useful because it considers the representation of each given lipid individually in the total amount of measurable lipids detected in the lipidomics assay. The lipidomics was able to detect lipid mediators from both the inflammatory and resolution pathways, as well metabolites from the cyclooxygenase and lipoxygenase biosynthetic pathways

(Figure 3). Irrespective of the study group or time point, the most abundant lipid mediators in the population were LTB_4, 5-HETE, 5-oxo-HETE, 12-HETE, 11-HETE, PGE_2, and 15-HETE. In addition, the hierarchical clustering revealed that the overall abundance profile of the lipid mediators was distinct between the groups of patients who cured and those who failed treatment (Figure 3A), suggesting that there were differences in relative concentration of several lipids relative to all those measure in the lipidomics. Such difference persisted at day 60 of follow-up. A second analysis of the same lipidomics data set was performed, now considering the raw concentration values in plasma. Using this approach, we found that among all the lipid mediators quantified, plasma concentrations of LTB_4, 5-oxo-HETE, 12-oxo-HETE, 12-HETE, 11-HETE, and of 15-HETE were all significantly reduced at day 60 compared to pre-treatment levels in patients who were successfully treated but not in those from the treatment failure group (Figure 3B).

Correlation Networks between Plasma Proteins and Lipid Mediators

Next, we performed network analyses based on Spearman correlations to evaluate the relationship between lipid mediators and inflammatory proteins in plasma of the subgroups of patients at different time points. The analytical steps leading to design of the correlation networks are illustrated in Figure S2. This kind of analysis is used in systems biology to define statistical relationships between molecules that may suggest regulation or even direct molecular interaction. This approach allowed us to visualize the quality (whether a correlation is positive or negative, indicated in the network by the color of the connecting line) and strength (the thickness of the connecting line being proportional to the Spearman rank coefficient rho value) of the associations in a given network. In such analytical setting, the number of connections (e.g. statistically significant correlations) infers how coordinated a biological process is. Comparing networks thus allow us to estimate the degree or regulation in a given clinical condition. In the context of this study, we observed that, before treatment commencement, there were already major differences in the correlation profiles, which involved bot number and directionality (e.g. positive or negative relationships) of significant correlations (defined here as p-value <0.05 after adjustment for multiple comparisons; Figure 4). In the group of patients who failed treatment later, there were a significantly higher number of relevant correlations than that from the patients who were successfully treated (Figure 4). Moreover, the vast majority of the statistically significant correlations detected in the treatment failure group were composed by positive associations, whereas a higher number of negative correlations were found in those who were further cured. At this time point, GCSF was the most relevant marker exhibiting positive relationships, whereas MIP-1β and TGF-β were the parameters with the highest number of negative correlations in the group of patients who were further cured. In the group who experienced treatment failure, several markers exhibited similar number of correlations, with no clear predominance of any specific parameter. The few negative relationships observed in the group of treatment failure at the study enrollment were between AA and IL-1RA and between EPA and IL-1β or IL-2 (Figure 4).

The findings described above indicated that even before initiation of antileishmanial treatment, patients who are prone to fail therapy already exhibit a distinct profile of associations between concentrations of plasma lipid mediators and inflammatory proteins. Strikingly, such difference in the correlation profiles was even more dramatic at day 60 of follow-up, when patients who were cured exhibited a predominance of negative relationships, whereas those who failed treatment persisted with several positive interactions (Figure 4). In the group constituted by those who were successfully treated at day 60, the most relevant markers were eotaxin, IFNα2, and TGF-β, and the only positive correlation found was between EGF and TXB_2. On the converse, patients with treatment failure exhibited TGF-β, and eotaxin as the most relevant nodes with negative correlations and TNF-β as the most significant marker with positive correlations in the network (Figure 4).

The networks were further explored in more details using node analyses. In such an analytical approach, the markers of a given network are ranked according to the number of connections (e.g. statistically significant correlations) that it is involved with. Each marker is represented by a node in the network. Highly connected markers are thought to be relevant in the regulation of the biological process underlying the network profile. Here, this analysis revealed the top 15 markers in each network that were highly connected. The top highly connected markers were different between the groups and time points. While the biomarker profile network at day 0 was dominated by lipid mediators in the patients who were cured (and this lipid mediator profile remained predominant after treatment), in patients who failed therapy we observed that cytokines represented the most predominant nodes in the networks. Interestingly, after treatment, this highlighted profile has changed (Figure 4, right panels).

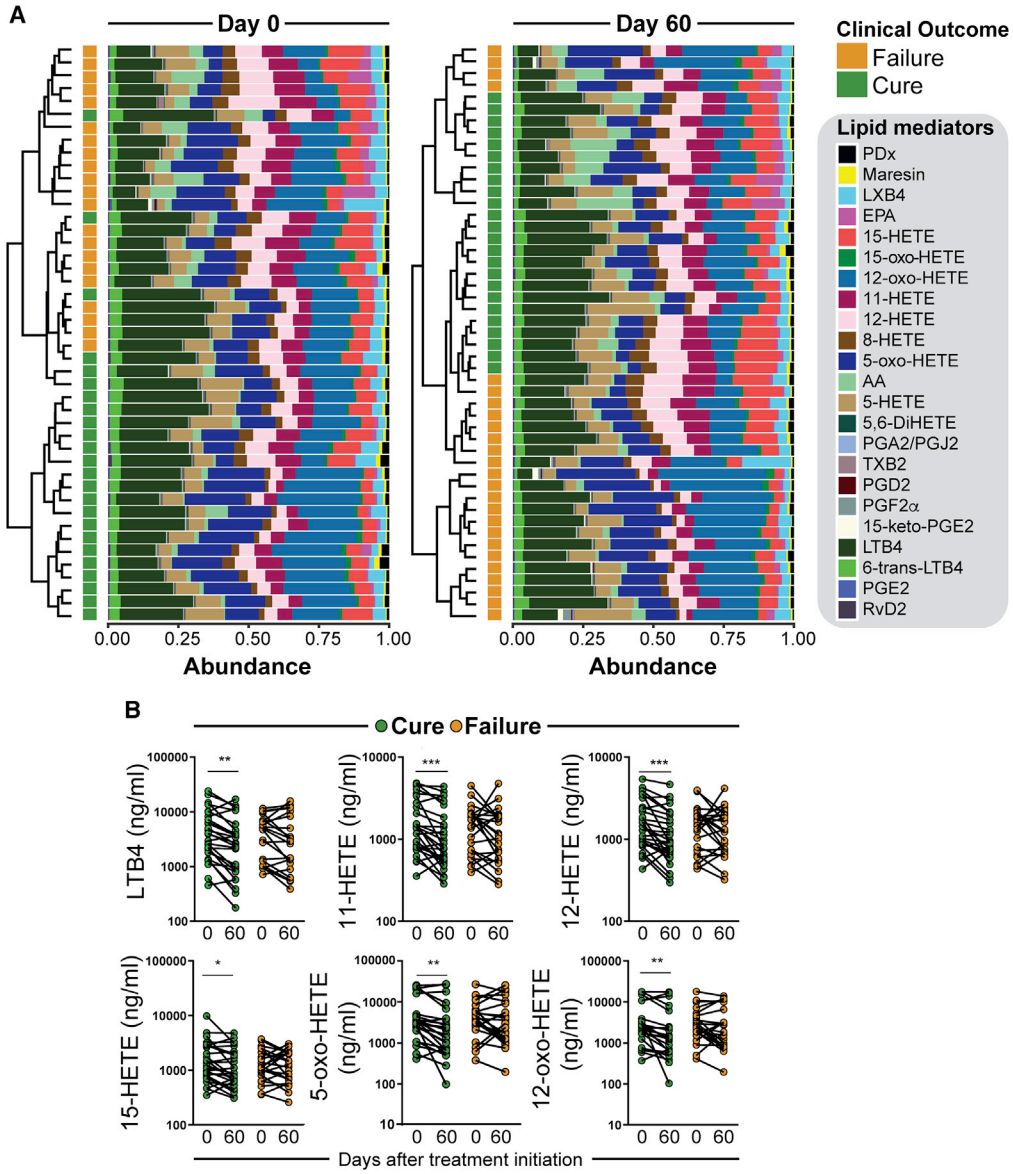

Figure 3. Abundance of lipid mediators in plasma identifies a distinct profile that characterizes patients who failed antileishmanial treatment

(A) Abundance of each lipid mediator was calculated according to Methods. Hierarchical clustering (Ward's method with 100X bootstrap) was performed to test whether the overall profile of lipid mediator abundance could group the patients who failed treatment from those who cured at indicated time points.

(B) Individual parameters that displayed statistically significant differences between the time points tested by Wilcoxon matched pairs test (after log10 transformation) are shown. *$p \leq 0.01$; **$p \leq 0.001$; ***$p \leq 0.0001$.

To test whether the differences in the correlation profiles observed before therapy initiation could be used to predict treatment failure, we employed a discriminant analysis based on canonical correlations (Manabe et al., 2019). The canonical correlation analysis uses the correlation profiles between the markers of a given network, rather than the raw concentration values of each marker, to calculate the prediction performance. In the present study, this analysis was employed to perform a proof of concept that the correlation profiles could be used to characterize and/or predict antileishmanial treatment outcomes. In this approach, we tested three distinct models: one inputting only the inflammatory proteins, a second model inputting data on lipid mediators, and a third model including data on both inflammatory proteins and lipid mediators. Receiver operator characteristics (ROC) curve analysis demonstrated that all the predictive models

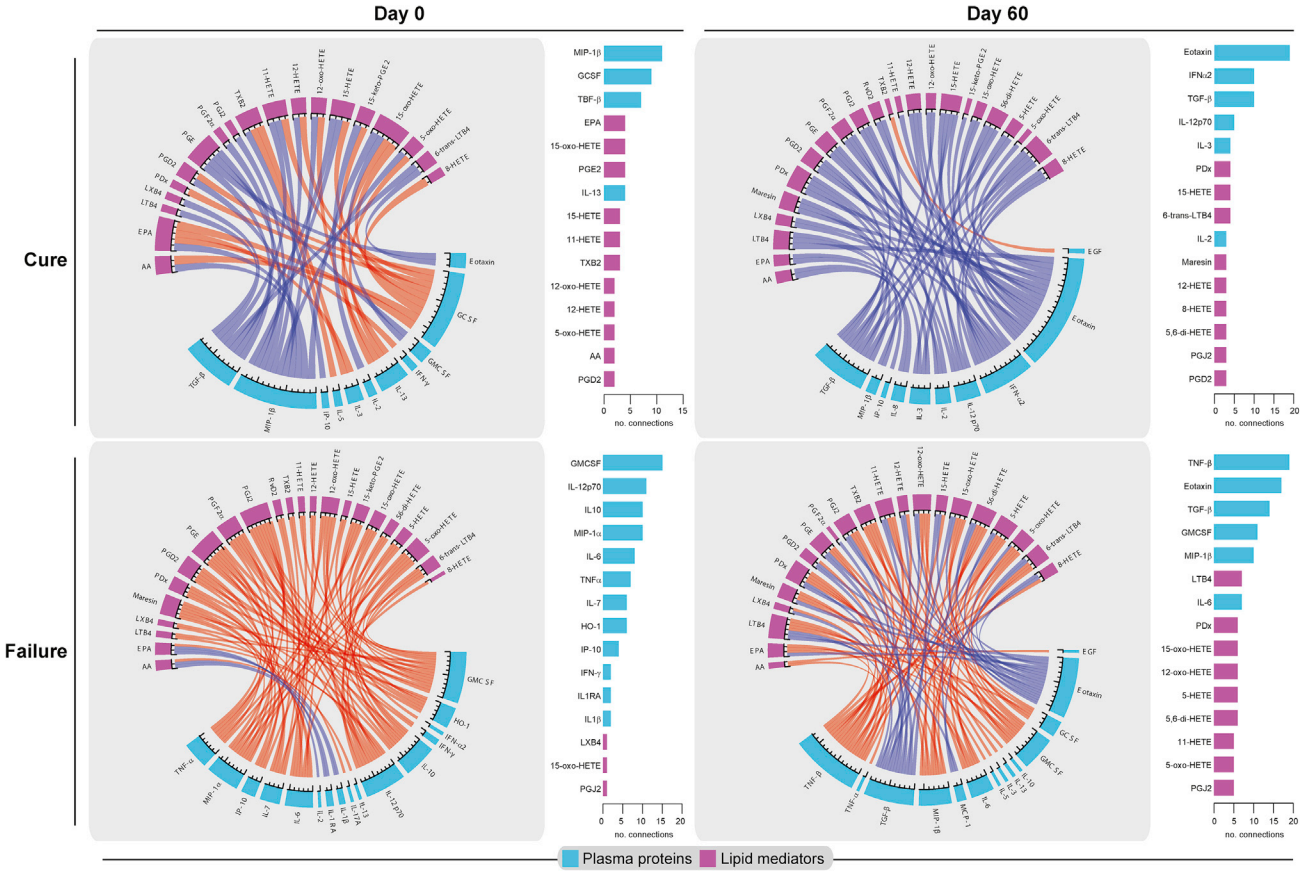

Figure 4. Network analysis of correlations between plasma proteins and lipid mediators in patients undergoing leishmaniasis treatment

Correlations were built using Spearman correlation matrices. Each bar represents a different parameter. The length of each bar is proportional to the number of significant correlations. The connecting lines represent statistically significant correlations ($p < 0.05$ after adjustment for multiple comparisons using the Holm-Bonferroni's method). Red connecting lines represent positive correlations, whereas blue lines infer negative correlations. The thickness of the connecting lines is proportional to the Spearman correlation rank coefficient (rho) value. Markers that did not exhibit statistically significant correlations are not shown. Node analyses shows the top 15 markers highly connected in each network.

were able to discriminate treatment outcomes with high accuracy, with gain in power when data on both proteins and lipid mediators were considered (Figure 5).

Multi-omic Factor Analysis Defined a Signature Enriched in Lipid Mediators that Predicts Treatment Failure

To identify the main factors that were contributing to the prediction of treatment failure in the canonical correlation analysis, we developed a factor analysis integrating the two data sets, one including plasma levels of cytokines and a second including lipidomic measurements, using the Multi-Omic Factor Analysis (MOFA) tool as previously described (Argelaguet et al., 2018). This approach is also focused on the correlation profiles between the biomarkers measured. MOFA generates different combinations of either proteins and/or lipid mediators that compose factors ("latent factors") which correlations more robustly account for discrimination between the clinical groups. The analysis revealed that the latent factor 1 (LF1) was the element that contributed the most for the distinction between treatment cure and failure (Figure 6A). In addition, the lipidomic correlation profile had more relevance in explaining the variance of the LF1 than did the profile of plasma proteins (Figure 6A). Finally, we plotted the loading scores of the LF1 to identify the most relevant lipid mediators and plasma proteins which correlation profiles could explain the distinction of the treatment outcomes. We found that TNF-β was the most important plasma protein (Figure 6B), whereas 12-oxo-HETE, 5-oxo-HETE, and LTB_4 were the top loading parameters in the lipid mediator component of LF1 (Figure 6C).

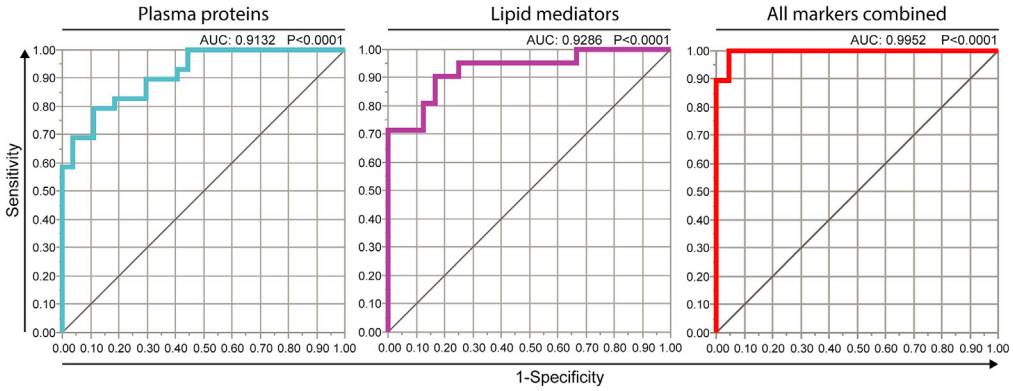

Figure 5. Canonical discriminant analysis of plasma proteins and lipid metiator measures before therapy initiation predicts treatment failure

Receiver operator characteristics (ROC) curve analysis of indicated models inputting data on plasma proteins, lipid mediators, or both, was performed to test power to distinguish treatment cure from failure in patients with leishmaniasis before initiation of antileishmanial therapy.

Machine Learning Decision Tree Using Data from Lipidomics and Proteomics Predicts Treatment Outcomes

The results so far indicated that correlation profiles are so distinct between the groups of patients with CL and different treatment outcomes that could be used in a predictive model. To address the question on whether there is a reasonable format to test prediction using concentration values of biomarkers, rather than correlation profiles, we have employed a stepwise approach using machine learning decision tree (Figure S4). A machine learning conditional tree inference model incorporating values of all the parameters from both Luminex and lipidomics assays assessed at the study baseline (day 0) was designed to answer two main issues: (i) to identify a combination of biomarkers that could best identify treatment failure cases; (ii) to establish cut-off values of the markers that could be used to differentiate between treatment failure and cure. This approach identified three significant splits in the decision tree, including circulating concentrations of eotaxin, 11-HETE, and TGF-β (Figure S4). The results indicate that a rational, stepwise assessment of these three parameters could be used to help identifying patients with high risk of treatment failure.

Analyses from Publicity Available Data Sets Validate the Involvement of the Lipid Biosynthetic Pathway in Discriminating Different Treatment Outcomes in Patients with CL

To investigate the importance of the lipid biosynthetic pathway in patients with treatment failure, we re-analyzed data from a RNAseq experiment recently published (Amorim et al., 2019), from an independent patient cohort of the same endemic region. This analysis included transcriptomes of specimens obtained from skin lesion biopsies collected prior to initiation of antileishmanial treatment. We focused the analysis on genes which were associated with lipid biosynthetic pathway and observed that patients with CL exhibited a completely distinct gene expression profile compared to uninfected healthy controls (HCs) which was able to separate 2 different hierarchical clusters (Figure 7A). Surprisingly, the gene expression profile of the genes related to the lipid pathway was not related to lesion size and parasitic load. Although this approach failed to completely segregate clinical outcomes based on treatment outcomes, we observed that there were two gene clusters which exhibited opposite expression profiles between patients with CL and HCs (Figure 7A). In addition, a principal component analysis (PCA) model including expression values of all genes included in the hierarchical analysis also demonstrated a complete segregation to patients with CL and controls and partial segregation between the CL subgroups with distinct clinical outcomes (Figure 7B). Lastly, a discriminant analysis using ROC curves of such combination of genes resulted in high accuracy in distinguishing *Leishmania* infection and clinical outcomes (Figure 7C).

DISCUSSION

Incidence of treatment failure among patients with CL has been increasing in recent decades (Ponte-Sucre et al., 2017). The identification of markers that can predict treatment outcomes in CL is important not only

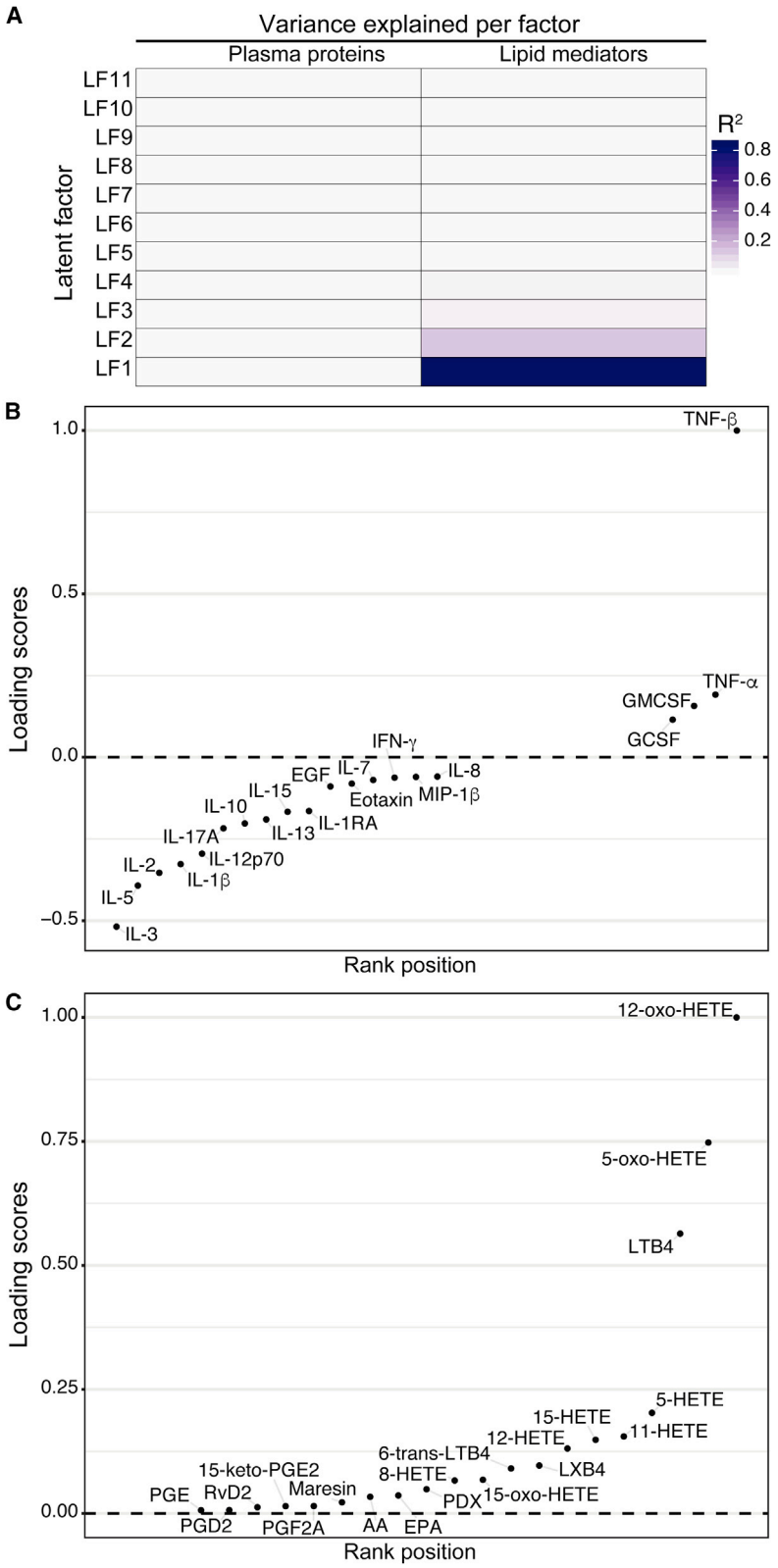
A
Variance explained per factor
Plasma proteins
Lipid mediators
Latent factor
LF11
LF10
LF9
LF8
LF7
LF6
LF5
LF4
LF3
LF2
LF1
R2
0.8
0.6
0.4
0.2
B
Loading scores
1.0
0.5
0.0
−0.5
TNF-β
TNF-α
GMCSF
GCSF
IFN-γ
EGF
IL-7
IL-8
IL-15
Eotaxin
MIP-1β
IL-10
IL-1RA
IL-13
IL-17A
IL-12p70
IL-2
IL-1β
IL-5
IL-3
Rank position
C
Loading scores
1.00
0.75
0.50
0.25
0.00
12-oxo-HETE
5-oxo-HETE
LTB4
5-HETE
15-HETE
11-HETE
12-HETE
6-trans-LTB4
LXB4
8-HETE
15-keto-PGE2
PDX
15-oxo-HETE
PGE
RvD2
Maresin
PGD2
PGF2A
AA
EPA
Rank position

Figure 6. Multi-Omics Factor Analysis Identified Latent Factors Able To Predict Treatment Outcomes in Patients with Tegumentary Leishmaniasis

(A–C) (A) Samples with paired data on plasma cytokines and lipid mediators were analyzed using MOFA as described in Methods. MOFA summarized the protein and lipid mediator data in 11 latent factors (LFs) with different associations (evaluated using the proportion of total variance explained, R^2) with the protein data set, the lipid mediator data set, or both. Each latent factor was inputted as a principal component in a PCA algorithm. Loading scores of the most relevant factor (LF1) in the data set of proteins (B) or lipid mediators (C) were plotted to quantify the contribution of each parameters to the LF1 final score. The most relevant markers are illustrated by the ones with the highest loading score values. PCA, principal component analysis.

to identify patients at higher risk of failure but also to better understand the mechanisms underlying such conditions. In the present study, we show that patients with CL who failed therapy exhibit a very distinct expression profile of plasma proteins and lipid mediators in peripheral blood. Interestingly, the changes are present even before the commencement of therapy, and our analyses reveal that these differences may predict the patients who will experience treatment failure. Furthermore, the results indicate that pharmacological intervention of the specific pathways identified here may serve as adjunct therapy to antileishmanial treatment to optimize clinical management.

The inflammatory response during the CL is characterized by high levels of circulating Th1 lymphocytes, cytokines, and chemokines (França-Costa et al., 2015; Ribeiro-de-Jesus et al., 1998) that significantly reduce after treatment (Brito et al., 2014). Our results indicated that circulating levels of TNF-α and IP-10 substantially reduced at day 60 of therapy compared to that detected at pre-treatment in the group of patients that were successfully treated. Patients who failed therapy exhibited a significant reduction in TNF-α, IP-10, and also in IL-2, IL-1α, and IL-6 at day 60. Interestingly, at day 60, low levels of GM-CSF, IFN-α2, IL-6, and IL-3 were observed in patients who failed therapy, compared to those from patients who cured. The observed profile in treatment failure made us hypothesize that significant reductions on cytokine levels in blood after onset of antileishmanial therapy may be associated with impaired capacity to eliminate the parasite. Indeed, levels of GM-CSF, which is a marker that has been shown to promote protection against *Leishmania* infection (Carvalho et al., 2019), were reduced in patients who failed therapy.

Although several studies have demonstrated the role of cytokines in the pathogenesis of CL and its contribution to treatment outcomes, the potential difference in lipid mediator expression profiles and their metabolites that may contribute to treatment failure is still unknown. Lipid mediators are important modulators of inflammation, being involved in both the initiation and resolution of the inflammatory response (Serhan et al., 2015). While inflammatory lipids such prostaglandins , leukotrienes , and hydroxyeicosatetraenoic acids are derived from arachidonic acid (AA), the specialized pro-resolving mediators (SPMs), including resolvins and maresins, are derived mostly from the eicosapentaenoic acid (EPA) and the docosahexaenoic acid (DHA) (Serhan et al., 2015). We have previously demonstrated important roles of different lipid mediators produced by both AA and DHA pathways in determining the distinct clinical forms of leishmaniasis (Araújo-Santos et al., 2017; França-Costa et al., 2016; Malta-Santos et al., 2017), highlighting the importance of such mediators as biomarkers of cutaneous and visceral leishmaniasis.

In this study, we show the characterization of the abundance profile of lipid mediators in blood of patients with CL undergoing therapy and tested associations with treatment outcomes. We observed that the most abundant lipid mediators were AA derived, such as LTB_4, 5-HETE, 5-oxo-HETE, 12-HETE, 11-HETE, PGE_2, and 15-HETE. Strikingly, the analysis of lipid mediator abundance revealed that patients who failed treatment exhibited a slight but distinct profile from those who were successfully treated even before the initiation of therapy. Thus, the overall composition of lipid mediators in plasma of patients with CL is able to characterize treatment failure. Of note, we and others have demonstrated the balance between PGE_2 and LTB_4, two of the most abundant mediators described here, as a critical factor determining clinical manifestations and/or outcomes in leishmaniasis (França-Costa et al., 2016), as well as in other diseases such as tuberculosis (Mayer-Barber et al., 2014; Shivakoti et al., 2019; Sorgi et al., 2020)and malaria (Abreu-Filho et al., 2019). Interesting, with exception of PGE_2 and 5-HETE, the most abundant inflammatory mediators significantly reduced their levels in patients who were successfully treated (Figure 3B) Supplementary Information, suggesting that the levels of such mediators may be reflecting the degree of immune activation and infection control.

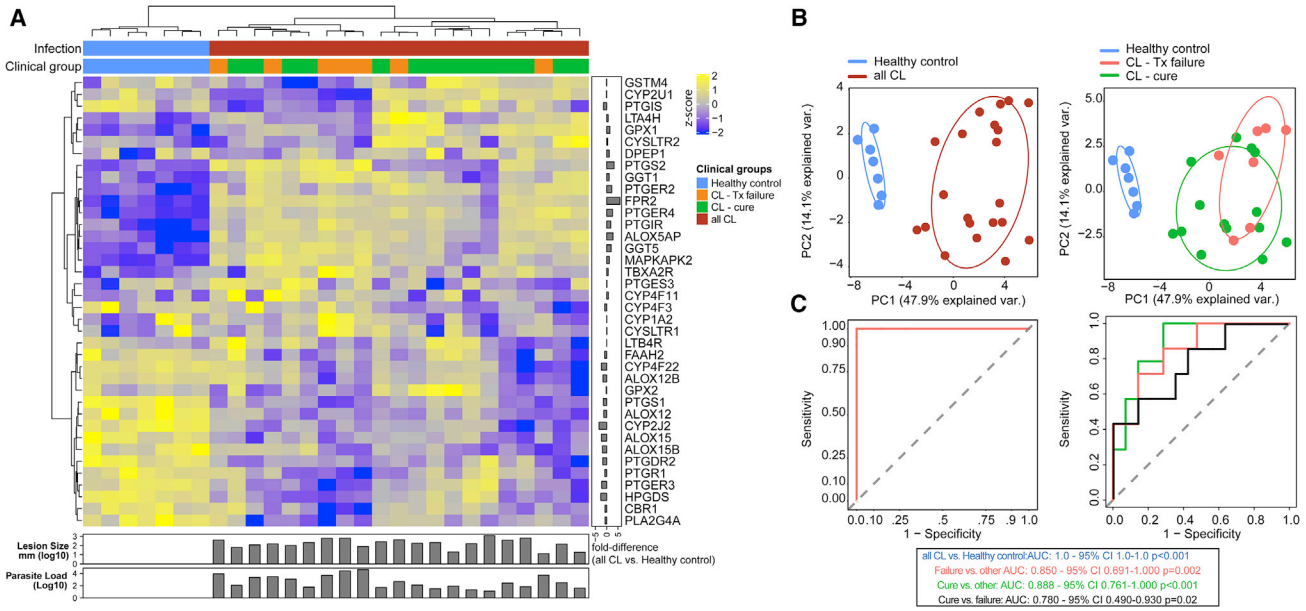

Figure 7. Patients with Cutaneous Leishmaniasis Display a Distinct Profile of Gene Expression from the Lipid Biosynthetic Pathway

(A) A hierarchical clustering analysis (Ward's method) was employed to illustrate the overall profile of genes of the lipid biosynthetic pathway in patients with CL who cured or failed therapy. Each column represents one patient.

(B) A principal component analysis (PCA) model was employed to test whether combination of the genes evaluated could cluster patients with CL separately from controls and the clinical outcomes. A vector analysis was utilized to illustrate the influence of each gene in the distribution of the data of the PCA model.

(C) Lipid gene expression significantly discriminated patients with CL to HCs (area under the curve (AUC) receiving operating characteristic [ROC] curve, 1) and the cure or failure (0.88 and 0.85, respectively).

Our Spearman correlation network analyses of plasma proteins and lipid mediators revealed that there are significant differences in the relationships between plasma levels of the biomarkers that can characterize the distinct clinical outcomes and time points. In addition, the top highly connected markers also were different between the groups and time points, indicating that there is a change in regulation of systemic inflammation induced by treatment, which was distinct between patients who failed and those who were successfully treated. Before treatment, patients who cured exhibited a relative balance hallmarked by a similar number of positive and negative correlations, involving G-CSF and MIP-1β/TGF-β, respectively, with AA-derived metabolites. In general, treatment implementation was associated with reduction in the number of significant interactions, completely changing the network profiles. Thus, at day 60, the most important markers were eotaxin, IFNα2, and TGF-β. Although MIP-1β and eotaxin are chemokines released by monocytes and eosinophil in response to *Leishmania* infection, their marked production is associated with an intense inflammatory response (Matte and Olivier, 2002). TGF-β, another important marker involved in the suppression of immune response favoring *Leishmania* infection (Barral-Netto et al., 1992), was down modulated in patients who were cured throughout the follow-up. Taken together, these data suggest that in patients who were successfully treated, there is a downmodulation of chemokines and mediators involved in persistent inflammation, leading to diminished immune activation, favoring skin healing.

The correlation profile in patients who failed therapy was predominantly marked by positive interactions and the only negative interactions involved IL-1β and IL-1RA. Interestingly, IL-1β is known to be involved in the CL pathogenesis and has been shown to associate with treatment failure (Zamboni and Sacks, 2019). More recently, a transcriptional signature including *IL1B* has been reported to predict clinical outcomes in CL (Amorim et al., 2019). In our study, we found that other cytokines could also characterize treatment outcomes. Interestingly, after treatment implementation in individuals who further experienced treatment failure, TGF-β and eotaxin remained the most relevant markers exhibiting negative connections in the network, whereas TNF-β was the parameter exhibiting the highest number of positive correlations. Polymorphism in the TNF locus, which includes genes encoding TNF-α and TNF-β, is associated with increased susceptibility to infection with either *L. braziliensis* (Cabrera et al., 1995) or *L. infantum* (Karplus et al., 2002). Our results argue that in patients who failed the conventional treatment, there is a potential persistent

interaction between plasma markers. Additional studies are needed to test if polymorphisms in genes of the cytokines reported here could result in increased risk of treatment failure.

Finally, the integrative analysis of protein with lipidomic profiles revealed that the lipid mediators are a major component able to discriminate treatment outcomes. A recently published study used the similar metabolomic approach to identify predictive biomarkers of the treatment outcome in patients with CL caused by *L. viannia* (Vargas et al., 2019). As noted in present study, drug exposure was able to modulate the metabolic products, and this change was associated with immune response and outcome of treatment (Vargas et al., 2019). Here, we demonstrated that among the lipids, the 12-oxo-HETE, 5-oxo-HETE, and LTB_4 stood out as the most robust biomarkers in the predictive model employed. To the best of our knowledge, there are no previously reported studies on describing concentration of oxo-HETEs lipids in CL. Future studies using *in vitro* systems and animal models are warranted to directly elucidate and describe the role to HETEs and oxo-HETEs in CL pathogenesis.

Many of the relevant findings on the present study were based on correlation profiles between biomarker concentrations in plasma. Such an approach is widely used, but it has limited application in clinical practice. In order to come up with a strategy that could be eventually employed in a clinical setting, we built a stepwise approach to predict treatment failure, which was based on assessment of concentration values rather in correlation profiles. This analysis used machine learning to build a decision tree. The results indicate that eotaxin, 11-HETE, and TGF-β levels measured at pre-treatment could be used in sequence to identify individuals who will fail treatment, in case of development of an easily assessable point of care.

The analysis of a publicly available transcriptome data set was an important contribution to the study, due to the fact that reinforced the idea that links expression of genes related to the lipid biosynthesis and odds of antileishmanial treatment failure in an independent cohort (Amorim et al., 2019). Such lipid-related signature was not influenced by lesion size or parasite load. In addition, results presented here are the first formal demonstration that gene expression of targets from the lipid mediator pathway can accurately identify patients with CL. The transcriptional data from normal skin biopsies compared to patients with CL revealed that the gene expression values of targets from the lipid biosynthetic pathway are able to identify samples from *Leishmania*-infected patients, as well as to reliably predict treatment outcomes. Interesting, genes involved in the synthesis of LTs and HETEs (*alox5*), as well PGs (*ptgs2*) and their inflammatory receptors (*ptger2*, *ptger4*, and *ptgir*), presented upregulated values in all patients with CL. These observations are consistent with our findings from plasma lipidomics, where the most abundant lipid mediators were products of the enzymatic activity of the proteins encoded by those genes found to be upregulated in skin lesions from patients with CL. Regardless, our data reveal that a biosignature enriched in lipid mediators is able to reliably predict treatment outcomes in CL, and the markers contributing to such signature may serve as targets in a potential host-directed therapy.

Limitations of the Study

Our study has some limitations. The number of patients was relatively low, although it was similar to other previously published investigations. In addition, patients from only one clinical site, from a single endemic area, were investigated, and thus, larger studies recruiting patients from more diverse clinical and epidemiologic settings are necessary to validate our findings. We performed validation analyses of the hypothesis associating lipid mediator pathways and treatment outcomes, and a more stringent approach measuring the biomarkers in a distinct cohort will be necessary to ultimately confirm the results.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Bruno B. Andrade (bruno.andrade@fiocruz.br).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

Original data sample for Figure 6 in the paper is available GSE127831 (Amorim et al., 2019).

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101840.

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AUTHOR CONTRIBUTIONS

Conceptualization: H.M.S., C.A.S., V.N., P.T.B., J.F.C., B.B.A, and V.M.B. Methodology: H.M.S., C.A.S., and V.N. Software, Formal analysis, Validation, and Data Curation: K.F.F. and A.T.L.Q. Clinical support: J.S., A.L., L.P.C., P.L.R.M., and E.M.C. Writing: H.M.S., C.A.S., P.L.R.M., L.H.F., E.M.C., B.B.A, and V.M.B. Supervision: B.B.A and V.M.B. All authors approved the final manuscript.

DECLARATION OF INTEREST

The author(s) declare no competing interests.

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Supplemental Information

Multi-omic Analyses of Plasma Cytokines, Lipidomics, and Transcriptomics Distinguish Treatment Outcomes in Cutaneous Leishmaniasis

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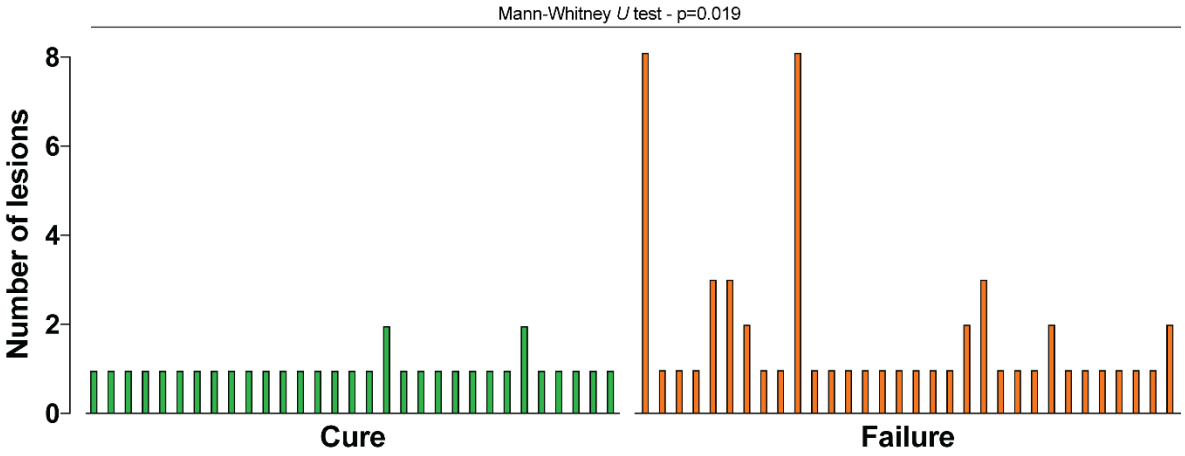

Figure S1. Number of lesions in cutaneous leishmaniasis patients, related to table 1. Number of active lesions per each study participant at study enrollment was plotted in histograms stratified by treatment outcomes. Distribution of values were analyzed using the Mann Whitney *U* test.

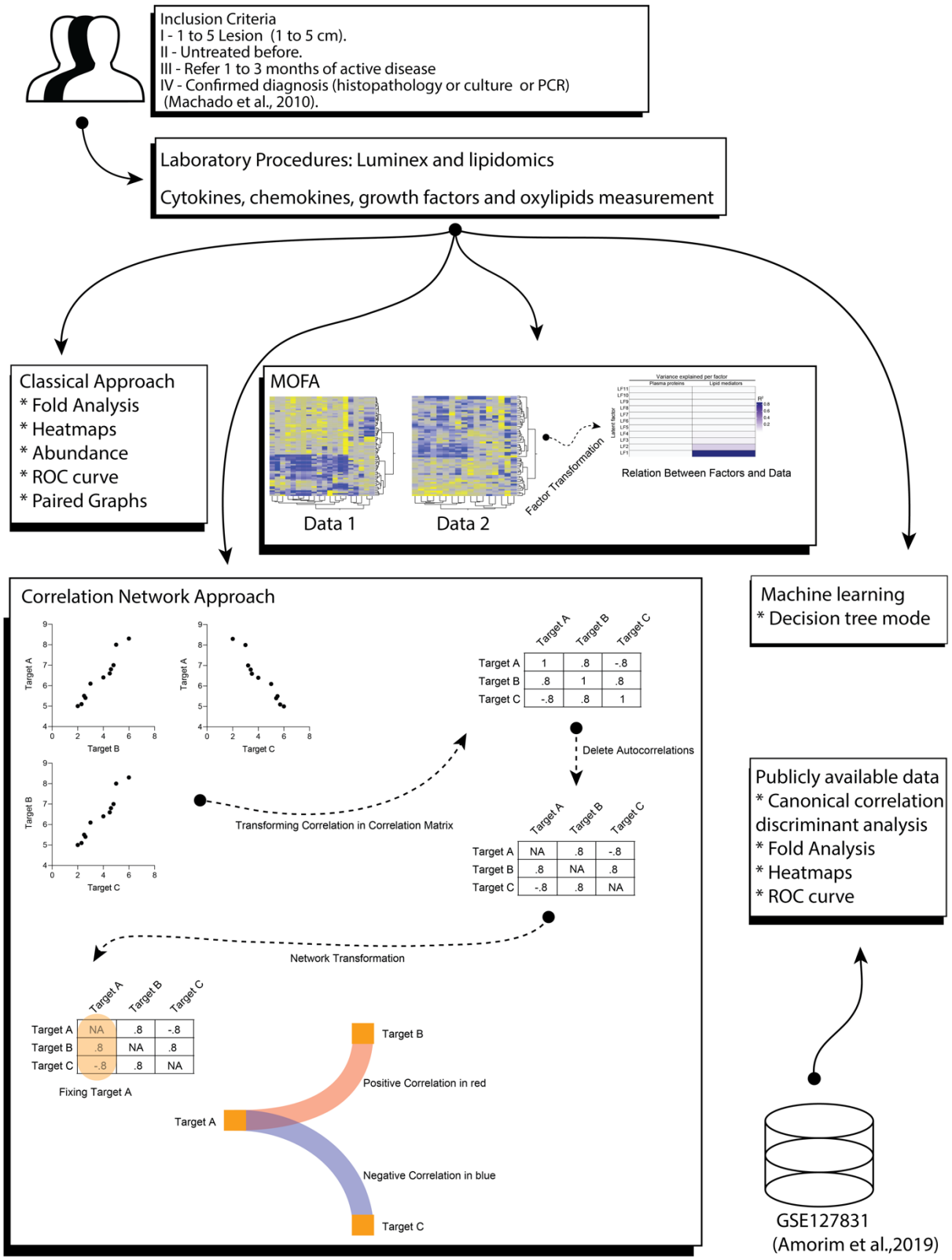

Figure S2. Outline of the analysis plan, related to all figures. Cryopreserved EDTA plasma samples were used for the omics assays. Cytokines and growth factors were measured using a Luminex assay whereas oxylipids were quantified using lipidomics as described in Methods. Analyses were divided in two main portions. In the first batch of analyses, classical analytical approach, which included calculations of fold-differences, design of heatmaps and abundance color maps, estimation of accuracy in predicting treatment outcomes by using Receiver Operator Characteristics (ROC) curves and dynamic changes using paired analysis. In addition,

Correlation matrices were calculated and used to build networks with the objective of describing the overall profile of relationships between plasma lipids and proteins in each clinical group and study timepoint. Data from Luminex and lipidomics were also integrated in a Multi-Omic Factor Analysis (MOFA) to identify major contributor of the regulatory networks driving the distinctions between the clinical groups that could predict treatment outcomes. Furthermore, a machine learning strategy using decision trees was designed to identify combination of markers with their respective cut-off values which were able to predict treatment failure. Finally, Transcriptome data from a publicly available dataset was used to validate the hypothesis that gene expression values of targets from the lipid biosynthesis pathway could be used to identify characterize patients with leishmaniasis who developed different treatment outcomes.

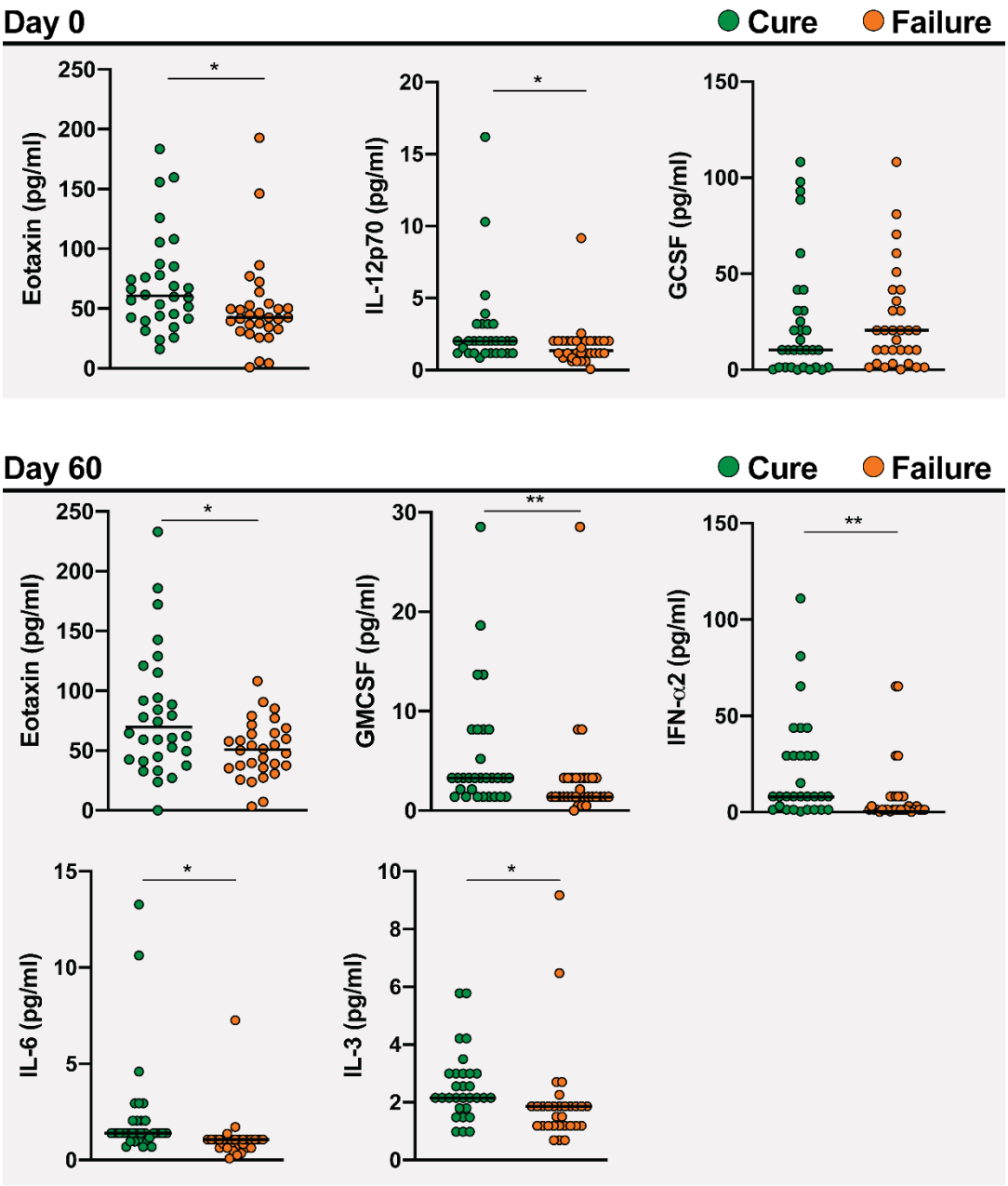

Figure S3. Inflammatory proteins in plasma of cutaneous leishmaniasis patients according to timepoint and treatment outcome, related to Figure 1. Parameters that displayed statistically significant differences between the study groups at each timepoint were tested using the Mann-Whitney *U* test. *$P \leq 0.01$; **$P \leq 0.001$.

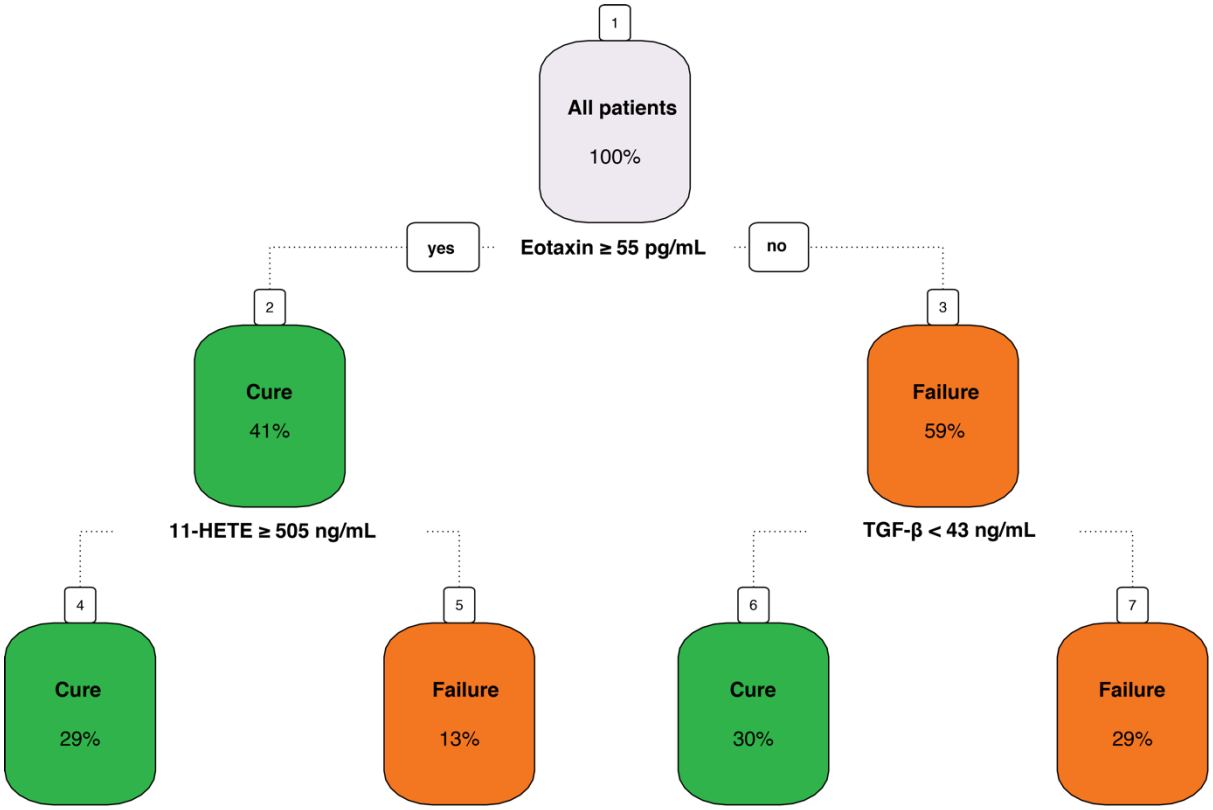

Figure S4. Machine learning decision tree model to predict treatment failure using pre-treatment levels of plasma biomarkers, related to Figure 3. All markers from both the Luminex and the lipidomic assays were included in the model. Measurements from study baseline (day 0) were considered. The p-value of the combined performance was 0.001. The area under the curve (AUC) of the receiver operator characteristics (ROC) curve was 0.7 (95% confidence interval: 0.6-0.8).

Transparent Methods

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki and approved by the Ethics Committee of the Hospital Universitário Prof. Edgard Santos of the University Federal da Bahia (number 2.471.184). Written informed consent was obtained from all participants or legal guardians, and all data analyzed were anonymized.

Clinical study design

A prospective case control study was performed in eligible patients that spontaneously sought medical treatment at a referral center in Corte de Pedra, Brazil, an endemic area for CL caused by *L. braziliensis*. The present study was nested with a clinical investigation in which all patients were evaluated at days 0, 15, 30, 60, 90 and 210 after recruitment, however blood specimens were available only at day 0 (pre-treatment) and day 60 (for monitoring of biochemical parameters). All patients were treated with intravenous 20 mg Sb^V/kg for 20 days. Patients were followed up for 210 days to define the main outcomes, cure and treatment failure. EDTA plasma samples were cryopreserved at -80ºC for the laboratory assessments. Clinical, epidemiological and therapeutic outcome data were captured in standardized clinical report forms by trained physicians who are also part of the research team (the authors P.R.L.M. and E.M.C.). The major aim of the study was to describe biomarkers able to predict treatment failure. To estimate the total sample size for a study power greater than 90% with a Type 1 error of less than 5% and considering the incidence of treatment failure of 40% (Machado et al., 2010) and loss to follow up of 30% (for whatever reason), the sample calculation revealed that we would need to recruit a total of 60 treatment-naïve CL patients. We recruited a total of 63 patients, with individuals 32 developing treatment failure.

Patients

Inclusion criteria were: (i) to present with one to five ulcerated lesions with sizes varying between 1 and 5 cm in diameter; (ii) to be anti-*Leishmania* treatment naïve; (iii) to refer 1 to 3 months of active disease; and (iv) to have a confirmed diagnosis by positive identification of amastigotes in histopathological examination, positive *L. braziliensis* culture or positive polymerase chain reaction for *L. braziliensis*, as previously described (Machado et al., 2010). Exclusion criteria were: (i) pregnant or breastfeeding women; childbearing-age women unwilling to adhere to contraceptive measures during treatment and until 2 months after the

end of treatment; (ii) previous history of leishmaniasis treatment; (iii) malnutrition; (iv) referred or confirmed concomitant diseases such as cardiac, pulmonary, hepatic, cancer, tuberculosis, Hansen disease, malaria, HIV/AIDS or any other infectious disease; (v) laboratory evidence of chronic liver or kidney disease (Machado et al., 2018).

Outcome definition

Treatment outcomes were reported as the following: cure was defined by complete re-epithelization of lesion(s) and absence of infiltration whereas treatment failure denoted persistence of ulceration at up to 60 days after the end of treatment. Patients who failed treatment received an additional cycle of Sb^v or Amphotericin B.

Cytokines, chemokines and growth factors measurement

Plasma levels of EGF (epidermal growth factor), Eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-α2, IFN-γ, interleukin (IL)-1β, IL-1RA, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, interferon inducible protein (IP)-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, tumor necrosis factor (TNF)-α, TNF-β and vascular endothelial growth factor (VEGF) were measured in cryopreserved EDTA plasma samples using a commercially available Luminex kit (Merck, Darmstadt, Germany) according to the manufacturer instructions. Levels of transforming growth factor β (TGF-β) and heme oxygenase-1 (HO-1) were measured using enzyme-linked immunosorbent assays (ELISA) (R&D Systems, Minneapolis, Minnesota).

Oxylipids extraction

Oxylipids extraction was performed from plasma samples using the SPE (Solid Phase Extraction) method according to a previously described protocol (Machado et al., 2010; Sorgi et al., 2018). In brief, each plasma sample (150 μL) was spiked with internal deuterated standard (IS) solution (Cayman Chemical, Ann Arbor, Michigan) before being extracted. The samples were then submitted to protein precipitation with 1.5 mL of methanol/acetonitrile (1:1, v/v) at 4 °C, which was left to denature overnight. Furthermore, plasma samples were centrifuged for at 800x *g* for 10 min at 4 °C. The denatured proteins were quantified using the Bradford protein assay (Sigma-Aldrich, St. Louis, MO) to normalize the lipid concentration for each sample, and the resulting supernatants were diluted with Milli-Q water to decrease the organic solvent to a maximum concentration of 10-15 %. For the SPE extraction protocol, the cartridge (Hypersep C18-500 mg, 3 mL, Thermo Scientific, Bellefonte, Pennsylvania) was

washed with 4 mL of MeOH and equilibrated with 4 mL of H_2O using an extraction manifold (Waters, Milford, Connecticut). After loading the diluted samples, the cartridges were again flushed with 4 mL of Milli-Q water to remove hydrophilic impurities. The analytes were eluted with 1 mL of MeOH. The solvent was removed in vacuum (Concentrator Plus, Eppendorf, Hamburg, Germany) at room temperature and re-dissolved in 50 µL of MeOH/H_2O (7:3, *v/v*) for LC-MS/MS analysis.

Oxylipids identification and quantification

After lipid extraction, specimens were transferred to autosampler vials and 10 µL of each sample were injected on the TripleTOF® 5600+ Target Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) system (Sciex, Foster City, California), as previously described (Sorgi et al., 2018). The method employed a High-Performance Liquid Chromatography (HPLC) system (Nexera X2, Shimadzu, Kyoto, Japan) using an Ascentis Express C18 column (Supelco, St. Louis, Missouri) with the following specifications: 100 × 4.6 mm and particle size of 2.7 µm. Elution was conducted under a binary gradient system with Phase A constituted by H_2O/ACN/acetic acid (69.98:30:0.02, *v/v/v*) at pH 5.8, and Phase B composed by ACN/isopropanol (70:30, *v/v*). Gradient elution was carried out for 25 min at a flow rate of 0.6 mL.min−1. An electrospray ionization (ESI) source in the negative ion mode was used for high-resolution multiple-reaction monitoring (MRMHR) scanning. The mass range of the product ion from the experiments varied from 50 to 700 m/z; the dwell time was 10 ms at a mass resolution of 35,000. Additional instrumental parameters were: nebulizer gas (GS1), 50 psi; turbo-gas (GS2), 50 psi; curtain gas (CUR), 25 psi; electrospray voltage (ISVF), −4.0 kV; and turbo ion spray source temperature, 550°C. Data acquisitions were performed using AnalystTM Software (Sciex, Foster, California). Data processing proceeded through multiple steps, including filtering, feature detection, alignment, and normalization. The PeakView 2.1 (Sciex, Foster, California) software was used for identification of the lipid species and MultiQuant™ (Sciex, Foster, California) software was utilized for quantitative analysis. The final oxylipids concentration in plasma samples was normalized by protein concentration.

Statistical analysis

Median and interquartile ranges (IQR) were used as measured of central tendency and dispersion, respectively. Percentage was used to describe categorical variables such as sex and number of active lesions. Continuous variables were compared using the Mann–Whitney *U* test (between cure and failure groups at each time point) or the Wilcoxon matched pairs test (the

same patient group between two different timepoints). The Fisher's exact test was used to compare frequencies. In some analyses, data on each biomarker was log-transformed and z-score normalized to build heatmaps to illustrate overall trends of data variation between the study groups. A hierarchical cluster analyses (Ward's method) were used to group the biomarkers with similar distribution between clinical groups and time points. In such analyses, dendrograms represent Euclidean distance. In addition, abundance of each lipid mediator was calculated as the following: the concentrations of all lipid mediators detected were summed and considered 100% abundance of lipid mediators in plasma, as previously described (Shivakoti et al., 2019). Then, abundance of the total lipid mediators was calculated for each individual marker relative to the total value considered 100% abundance (Shivakoti et al., 2019). Moreover, a second hierarchical clustering analysis was performed to test whether the overall profile of lipid mediator abundance could group the patients who failed treatment from those who were cured at indicated timepoints. In further analyses, a machine-learning based conditional tree including the values of all the biomarkers measured at study baseline (day 0) was designed to identify the best biomarker or combination of markers that were able to discriminate treatment outcomes. All analyses were pre-specified. To account for multiple measurements, the p-values were adjusted using the Holm-Bonferroni's method. Differences with adjusted p-values < 0.05 were considered statistically significant. All data is available in supplementary data.

Network analysis

Profiles of correlations between inflammatory proteins and lipid mediators at different timepoints in the groups of patients with distinct clinical outcomes were examined using network analysis of the Spearman correlation matrices (with 100X bootstrap). Only statistically significant correlations (adjusted p values < 0.05) were included in the network visualization (lines represent statistically significant correlations). This model is based in quality of correlations (whether a correlation is positive or negative). *Circos* plots were used to illustrate the networks as previously reported (Vinhaes et al., 2019). In additional analysis, number of correlations were quantified per each node (marker) in patients stratified based on timepoint and treatment outcome.

Canonical correlation discriminant analysis

The discriminant analysis model using sparse canonical correlations (canonical correlation analysis, CCA) was employed to test if different combinations of plasma and/or lipid mediators

measured at pre-treatment could distinguish patients who failed anti-*Leishmania* treatment from those who were cured. The CCA algorithm was chosen because many variables were studied. This approach reduces dimensionality for two co-dependent data sets (biomarker profile and baseline characteristics profile, which were sex and age) simultaneously so that the discrimination of the clinical endpoints (treatment failure or cure) represents a combination of variables that are maximally correlated. Thus, trends of correlations between parameters in different clinical groups rather than their respective distribution within each group are the key components driving the discrimination outcome. In our CCA algorithm, simplified and adapted from previously reported investigations of biomarkers for diagnosis of infectious diseases (Mayer-Barber et al., 2014), linear regression graphs represent coefficients from different combinations of plasma factors and baseline characteristics. In addition, investigating statistical relationships between the markers rather than just concentrations allow us to infer about regulatory immune networks (Mayer-Barber et al., 2014). The overall accuracy of each canonical model was tested using C-statistics, with Receiver Operator Characteristics (ROC) curves resulting in calculation of area under the curves (AUC), sensitivity and specificity using the pROC package of the R software as previously described (Manabe et al., 2019; Robin et al., 2011).

Multi-Omics factor analysis

Multi-omics factor analysis (MOFA) enables to analyze biological multidimensional data, ranging from genome, transcriptome, proteome, lipidome and metabolome, integrating all these layers across a more comprehensive result (Argelaguet et al., 2018). The MOFA model used here integrated data from plasma inflammatory proteins and lipidomic analysis, defined by several parameters: (i) the logical scale and paired samples were selected by the functions #scaleViews set as “FALSE” and #removeIncompleteSamples set as “TRUE”; (ii) the number of factors was selected by default with #likelihood = “gaussian” (log2 transformed) and #sparsity set as “TRUE”; (iii) the #tolerance was selected as recommend = 0.01. After selecting all the parameters and preparing the datasets, we used as an input the corrected bath effect count table of the plasma proteins and lipid mediators obtained as the formal analytical merged dataset. All the data were paired by study, individual and platform. Two models of principal component analyses (PCA) were used to identify which plasma proteins and lipid mediators were contributing to separation of the different study groups based on treatment outcomes. In all analyses, a p-value < 0.05 after the 5% FDR adjustment was considered statistically significant.

Decision tree analysis

The metabolite abundance values were used as input to perform a supervised machine-learning approach, based on the outcome definition. Thus, a decision tree algorithm was applied to identify the minimal variable (metabolite) set which exhibit the higher classification power to describe the groups using the rpart package.

Transcriptomic analyses of skin lesions from publicly available datasets

Data samples were downloaded from NCBI GEO: GSE127831 (Amorim et al., 2019) and labeled according to the informed metadata (21 samples infected to *L. braziliensis* before treatment sequenced with *Illumina NexSeq 500,* and 7 uninfected endemic controls). Changes in gene expression levels were considered significant when statistical test values (FDR adjusted p-value) were lower than 0.05 and the fold-difference higher than ±1.5. A heatmap of including expression values of genes identified by our group as being part of lipid biosynthetic pathways was plotted using the *Complexheatmap* package (Gu et al., 2016). A PCA algorithm was performed using cpm log-transformed data of the indicated genes using the *plotPCA* function from *Deseq2* package from R software (Gu et al., 2016; Love et al., 2014).

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