A model to predict the response to therapy against hepatitis C virus (HCV) including low-density lipoprotein receptor genotype in HIV/HCV-coinfected patients

Karin Neukam1, Carmen Almeida2, Antonio Caruz3, Antonio Rivero-Juárez4, Norma I. Rallón5, Federico A. Di Lello3, Rocio Herrero3, Angela Camacho4, José M. Benito5, Juan Macías1, Antonio Rivero4, Vicente Soriano5 and Juan A. Pineda1*

1Unit of Infectious Diseases and Microbiology, Hospital Universitario de Valme, Avenida de Bellavista S/N, 41014 Seville, Spain; 2Unit of Investigation, Hospital Universitario de Valme, Avenida de Bellavista S/N, 41014 Seville, Spain; 3Immunogenetics Unit, Faculty of Sciences, Universidad de Jaén, Campus Las Lagunillas S/N, 23071 Jaen, Spain; 4Unit of Infectious Diseases, Maimónides Institute for Biomedical Research (IMIBIC), Hospital Universitario Reina Sofia, Avd. Menéndez Pidal S/N, 14004 Córdoba, Spain; 5Department of Infectious Diseases, Hospital Carlos III, Calle Sinesio Delgado 10, 28029 Madrid, Spain

*Corresponding author. Tel: +34955015684; Fax: +34955015795; E-mail: japineda@telefonica.net

Received 26 July 2012; returned 8 September 2012; revised 16 October 2012; accepted 11 November 2012

Objectives: Accurate prediction of sustained virological response (SVR) to pegylated interferon-α (Peg-IFN) plus ribavirin in HIV/hepatitis C virus (HCV)-coinfected patients could improve the management of these patients. We aimed to develop a model to predict SVR to Peg-IFN/ribavirin in HIV/HCV-coinfected individuals combining HCV genotype and baseline HCV RNA load with interleukin 28B and low-density lipoprotein receptor genetic variations.

Methods: Three hundred and twelve treatment-naive HIV/HCV-coinfected patients receiving Peg-IFN/ribavirin were analysed in an on-treatment approach. One hundred and eighty-one of them were included in the development group and 131 in the validation population. The predictive model was obtained from a logistic regression equation including the above-mentioned variables. The areas under the receiver operating characteristic (AUROC) curves (95% CI), sensitivity and specificity, as well as negative and positive predictive values, were calculated.

Results: SVR was achieved by 88 (48.6%) patients from the development group and 68 (51.9%) individuals from the validation group. The AUROC curve values (95% asymptotic CI) were 0.83 (0.77–0.89) for the development group and 0.84 (0.77–0.91) for the validation group. Using two cut-off values, maximum specificity and sensitivity were 89.7% and 96.6%, respectively, with a negative predictive value for SVR of 88.9% and a positive predictive value of 83.6%. Thirteen (7.2%) individuals were misclassified using these cut-off values.

Conclusions: This model represents a reliable and easily applicable tool to individually evaluate the probability of achieving an SVR to Peg-IFN/ribavirin among HIV/HCV-coinfected patients.

Keywords: interferon-α, ribavirin, interleukin 28B, viruses, HCV genotype, HCV viral load

Introduction

The estimation of the probability of response to treatment against hepatitis C virus (HCV) with pegylated interferon-α (Peg-IFN) plus ribavirin can have a high clinical impact. In particular, special populations with lower response rates could benefit from better selection of candidates for HCV therapy. HIV/HCV-coinfected patients achieve sustained virological response (SVR) rates of <50%.1–3 Precise identification of individuals with a very low likelihood of achieving SVR could help us to more properly manage a treatment with frequent and sometimes severe side effects. Thus, in patients without advanced fibrosis and classified as very unlikely responders, treatment against HCV could be deferred until more effective combinations, including new direct-acting antivirals (DAAs), become approved in HIV/HCV co-infection. Conversely, patients with a high probability of SVR with Peg-IFN/ribavirin should be treated with this drug regimen, given that SVR is associated...
with decreases in the incidence of hepatic decompensations and in liver-related mortality in HIV/HCV-coinfected individuals. Thus, a reliable tool to easily predict the probability of SVR to Peg-IFN/ribavirin could be very useful in current clinical practice. Among the pre-treatment predictors of SVR that have been identified, HCV genotype, baseline plasma HCV RNA load and host genetic variations are highly predictive of response. In this context, the single-nucleotide polymorphism (SNP) rs12979860 near the interleukin 28B (IL28B) gene and the SNP rs14158 at the low-density lipoprotein receptor (LDLR) gene have recently been independently associated with SVR in HIV/HCV-coinfected patients. In both SNPs, the C allele exerts a beneficial effect and lower response rates are observed among carriers of the homozygous TT or the heterozygous CT genotype compared with CC genotype carriers. Interestingly, a synergistic effect between these SNPs has been observed in HCV genotype 1/4 carriers. Thus, HIV/HCV-coinfected individuals with both favourable IL28B and LDLR genotypes show a response rate of ~70%, while only 14% of those who present a combination of both genotypes other than CC achieve SVR. One approach to enhancing the predictive capacity of isolated factors is the combination of several predictors. However, the use of the predictive tools currently available is limited due to incomplete accuracy or because they use parameters not accessible everywhere. It is therefore important to develop a predictive model that allows reliable determination of the probability of response to Peg-IFN/ribavirin in HIV/HCV-coinfected patients.

To overcome the drawbacks of the currently available predictive tools to forecast SVR to Peg-IFN/ribavirin in HIV/HCV-coinfected patients, we aimed to develop a new model using novel predictors of SVR, such as the genotype of LDLR, the determination of which is both simple and inexpensive, in order to facilitate HCV therapy decisions in such a population.

**Patients and methods**

**Study patients**

HIV/HCV-coinfected patients who started treatment between May 2000 and May 2010 were included in this prospective study. Data to develop the model were collected from a cohort followed in two university hospitals in southern Spain. To validate the model, data derived from patients who were seen at a hospital in Madrid were acquired. The inclusion criteria of the cohorts were: (i) older than 18 years; (ii) written informed consent given; (iii) coinfection with HIV; (iv) previously naive for therapy against chronic hepatitis C; (v) initiation of therapy with Peg-IFN/ribavirin; and (vi) collection and cryopreservation at −70°C of a whole blood sample for genetic determinations. Those who voluntarily dropped out or discontinued therapy due to adverse events were excluded from the study population in order to analyse data using an on-treatment approach. Likewise, patients in whom HCV, IL28B or LDLR genotype could not be determined were excluded from the analysis. Clinical visits were scheduled every 4 weeks during the first 24 weeks of treatment and every 8–12 weeks afterwards, according to a pre-defined protocol that included a visit 24 weeks after completing therapy in order to evaluate SVR. All patients were prospectively followed and data were collected in real time.

**Drug therapy**

The daily doses of Peg-IFN 2α and Peg-IFN 2β were 180 µg once per week and 1.5 µg/kg once per week, respectively. Peg-IFN was administered in combination with 800–1200 mg of ribavirin per day. Those patients bearing HCV genotype 2 or 3 received HCV therapy for 24 weeks if they showed undetectable plasma HCV RNA load at week 4, and 48 weeks otherwise. Carriers of HCV genotype 1 or 4 were treated for 48 weeks. Treatment duration and stopping rules were applied according to international guidelines in force at the moment of treatment. SVR was defined as undetectable plasma HCV RNA 24 weeks after discontinuation of scheduled HCV therapy.

**Plasma HCV RNA determination**

The plasma HCV RNA was determined by a quantitative PCR assay according to the technique available at the time when the patient was treated (Cobas Amplicor HCV Monitor (Roche Diagnostic Systems Inc., Branchburg, NJ, USA), detection limit of 600 IU/mL; Cobas AmpliPrep-Cobas TaqMan (Roche Diagnostic Systems Inc., Meylan, France), detection limit of 50 IU/mL; Cobas TaqMan (Roche Diagnostic Systems Inc., Pleasanton, CA, USA), detection limit of 10 IU/mL).

**rs12979860 and rs14158 SNP genotyping**

DNA was extracted from cryopreserved whole blood using the automated MagNA Pure DNA extraction method (Roche Diagnostics Corporation, Indianapolis, IN, USA). The SNP rs14158 in the 3’ untranslated region of the LDLR gene was genotyped. Likewise, variations in rs12979860, an SNP located 3 kb upstream of the IL28B gene, were analysed. Genotyping was carried out using a custom TaqMan assay (Applied Biosystems, Foster City, CA, USA), following the manufacturer’s instructions, on a Stratagene MX3005 thermocycler using MXpro software (Stratagene, La Jolla, CA, USA) as described previously. The researchers responsible for genotyping were blinded to the treatment outcome of the patients.

**Statistical analysis**

The study population parameters were characterized in a descriptive analysis. Baseline characteristics of the groups for development and validation of the model were compared using the χ² test or Fisher’s test for categorical variables and Student’s t-test or the Mann–Whitney U-test for continuous variables. The Hardy–Weinberg equilibrium was analysed for both rs12979860 and rs14158 using Haploview software v.4.2.12 For development of the predictive model, the host genetic profiles were classified into four categories according to the risk alleles (TT = TC < CC): IL28B CC/LDLR CC, IL28B non-CC/LDLR CC, IL28B CC/LDLR non-CC and IL28B non-CC/LDLR non-CC. Likewise, patients were categorized by HCV genotype as 2/3 or 1/4 carriers. The outcome variable was SVR. The diagnostic performance of the isolated predictors was analysed by binary logistic regression analysis. Multivariate logistic regression analysis adjusted for age and sex was performed in order to confirm that the host genetic profile, the HCV genotype and the baseline HCV RNA load were independently associated with SVR in the study population. Clinically relevant variables for SVR were entered into the model in order to optimize its diagnostic performance. The formula resulting from the logistic regression equation represented the predictive model. The 95% CIs of the SRV rates were calculated. The predictive value of the logistic regression model was assessed via receiver operating characteristic curves, where 1.0 indicates perfect discrimination and 0.5 indicates random prediction. Because receiver operating characteristic curves are only able to incorporate binary outcomes, two cut-off values were selected, a high one and a low one, derived from the formula that maximized the negative predictive value (NPV) and positive predictive value (PPV), as well as sensitivity and specificity in predicting SVR versus non-SVR. The CIs for the predictive values were calculated and diagnostic accuracy was determined on the basis of these values. Statistical analysis was carried out using the SPSS statistical software package release 19.0 (IBM Corporation, Somers, NY, USA) and STATA 9.0 (StataCorp LP, College Station, TX, USA) and Fisterra.com (Elsevier 2012; http://www.fisterra.com/mbe/investiga/pruebas_diagnosticsas/pruebas_diagnosticsas.asp).
Ethical aspects

The study was designed and performed according to the Helsinki declaration and was approved by the Ethics Committees of the participating hospitals.

Results

Main characteristics of the study population

Three hundred and twelve patients were included in this study, 181 of them constituting the development group and 131 the validation group. Thirty-six (9.9%) and 17 (4.7%) patients had been excluded due to voluntary drop-out and discontinuation caused by adverse events. In the overall population, 131 (42.0%) of the patients bore $IL28B$ genotype CC, 140 (44.9%) genotype CT and 41 (13.1%) genotype TT. The distribution of $LDLR$ genotypes was: 178 (57.1%) CC, 120 (38.5%) CT and 14 (4.5%) TT. Both SNPs were in Hardy–Weinberg equilibrium ($P=0.709$ for rs12979860 and $P=0.266$ for rs14158). The main baseline characteristics of the two study groups are presented in Table 1.

Response to HCV therapy

SVR was achieved by 88 (48.6%; 95% CI 41.1%–56.1%) patients in the model development group and by 68 (51.9%; 95% CI 43%–60.7%) individuals in the validation group. In the development group, 49 (65.3%) of those patients with $IL28B$ genotype CC versus 39 (36.8%) patients with $IL28B$ genotype CT or TT ($P=2.8\times10^{-4}$) achieved SVR. Likewise, 60 (54.5%) individuals with $LDLR$ genotype CC versus 28 (39.4%) with genotype CT or TT attained SVR ($P=4.7\times10^{-2}$). The numbers of patients who achieved SVR according to HCV genotype in the development group were 29 (30.5%) for genotype 1, 49 (81.7%) for genotype 2/3 and 10 (38.5%) for genotype 4 ($P=3.2\times10^{-9}$). In the development group, patients who achieved SVR showed a median (IQR) HCV RNA load at baseline of 5.8 (5.2–6.5) log10 IU/mL compared with 6.2 (5.7–6.7) log10 IU/mL for those who did not reach SVR ($P=5\times10^{-3}$). The univariate and multivariate associations of the variables entered into the model are presented in Table 2.

Model development

On the basis of the logistic regression equation, the following formula to determine the individual probability of response to treatment against HCV with Peg-IFN/ribavirin was elaborated:

$$\text{Probability}_{SVR} = \frac{1}{1 + e^{-z}}$$

where $GT$ is HCV genotype (1 or 4 = 1 and 2 or 3 = 0), $VL$ is baseline plasma HCV RNA load (log10 IU/mL), $A$ is $IL28B/LDLR$ genetic group, $B$ is $IL28B/LDLR$ genetic group, $C$ is $IL28B/LDLR$ genetic group, and $D$ is $IL28B/LDLR$ genetic group.
profile (IL28B non-CC/LDLR CC = 1 and other = 0), B is IL28B/LDLR genetic profile (IL28B CC/LDLR non-CC = 1 and other = 0) and C is IL28B/LDLR genetic profile (IL28B non-CC/LDLR non-CC = 1 and other = 0).

Model validation

The areas under the receiver operating characteristic (AUROC) curve values (95% asymptotic CI) were 0.83 (0.77–0.89) for the development group and 0.84 (0.77–0.91) for the validation group (Figure 1). Maximum predictive values were yielded by applying cut-off values of 18.8 and 62. The predictive values obtained are shown in Table 3.

Discussion

We report herein a useful and innovative model for the prediction of response to Peg-IFN/ribavirin in HIV/HCV-coinfected patients based on the combination of two well-known predictors of SVR—HCV genotype and baseline plasma HCV-RNA load—plus the genetic pattern defined by IL28B and LDLR genotype. Its application allows reliable determination of the probability of achieving SVR by combining host genetic and viral parameters.

Table 2. Associations between SVR and host genetic profile, HCV genotype and baseline HCV RNA load in the univariate and multivariate analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Model development group, n = 181</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SVR, n (%)</td>
</tr>
<tr>
<td>IL28B/LDLR genetic profile</td>
<td></td>
</tr>
<tr>
<td>non-CC/non-CC</td>
<td>13 (31.0)</td>
</tr>
<tr>
<td>non-CC/CC</td>
<td>26 (40.6)</td>
</tr>
<tr>
<td>CC/non-CC</td>
<td>15 (51.7)</td>
</tr>
<tr>
<td>CC/CC</td>
<td>34 (73.9)</td>
</tr>
<tr>
<td>HCV genotype</td>
<td></td>
</tr>
<tr>
<td>1/4</td>
<td>39 (32.2)</td>
</tr>
<tr>
<td>2/3</td>
<td>49 (81.7)</td>
</tr>
<tr>
<td>Baseline HCV RNA load (IU/mL)</td>
<td></td>
</tr>
<tr>
<td>≥600000</td>
<td>46 (40.4)</td>
</tr>
<tr>
<td>&lt;600000</td>
<td>42 (62.7)</td>
</tr>
</tbody>
</table>

Table 3. Predictive values of the model within the two groups

<table>
<thead>
<tr>
<th>Cut-off value</th>
<th>Development group (n=181)</th>
<th>Validation group (n=131)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sensitivity, % (95% CI)</td>
<td>96.6 (89.7–99.1)</td>
<td>95.4 (86.1–98.9)</td>
</tr>
<tr>
<td>specificity, % (95% CI)</td>
<td>25.2 (17.5–36.1)</td>
<td>33.3 (22.3–46.4)</td>
</tr>
<tr>
<td>PPV, % (95% CI)</td>
<td>55.2 (47.0–63.1)</td>
<td>60.7 (50.8–69.9)</td>
</tr>
<tr>
<td>NPV, % (95% CI)</td>
<td>88.9 (69.7–97.1)</td>
<td>87.5 (66.5–96.7)</td>
</tr>
<tr>
<td>misclassified patients, n (%)</td>
<td>3 (1.7)</td>
<td>3 (2.3)</td>
</tr>
<tr>
<td>sensitivity, % (95% CI)</td>
<td>58 (47.0–68.3)</td>
<td>62.5 (50.6–74.4)</td>
</tr>
<tr>
<td>specificity, % (95% CI)</td>
<td>89.7 (80.7–94.4)</td>
<td>92.1 (81.7–97.0)</td>
</tr>
<tr>
<td>PPV, % (95% CI)</td>
<td>83.6 (71.5–91.5)</td>
<td>89.6 (76.6–96.1)</td>
</tr>
<tr>
<td>NPV, % (95% CI)</td>
<td>69.2 (60.0–77.1)</td>
<td>69.9 (58.7–79.2)</td>
</tr>
<tr>
<td>misclassified patients, n (%)</td>
<td>10 (5.5)</td>
<td>5 (3.8)</td>
</tr>
</tbody>
</table>

*Conducted with the baseline HCV RNA load expressed as a continuous variable (log10 IU/mL); the Nagelkerke r² value was 0.38.
Predicting success of treatment against HCV in the setting of HIV coinfection is of the highest relevance in current clinical practice. Although DAAs, such as the protease inhibitors (PIs) boceprevir and telaprevir, are currently available for HIV/HCV-coinfected individuals, their use in the coinfected population has not been licensed yet and therapy with Peg-IFN/ribavirin still represents the standard of care (SOC) for this population. Unfortunately, the overall response rates that are achieved with SOC are low for these patients.\(^1\)\(^-\)\(^3\) Treatment combinations with new PIs have yielded higher response rates compared with SOC in HCV-mono-infected patients.\(^13\)\(^,\)\(^14\) Likewise, preliminary results of clinical trials carried out with combinations including PIs in the HIV/HCV genotype 1-coinfected population are promising.\(^15\)\(^,\)\(^16\) However, the 24 week preliminary results reported in clinical trials give reason to expect that the SVR rates will reach 70% at the most; hence, a considerable percentage of patients may not benefit from triple therapy.\(^17\)\(^,\)\(^18\) Furthermore, boceprevir and telaprevir will not be applicable to all coinfect patients. Drug-drug interactions between boceprevir or telaprevir and antiretroviral therapy will limit the use of these PIs in HIV/HCV-coinfected patients.\(^17\)\(^,\)\(^18\) and there is a higher probability of side effects with triple therapy.\(^15\)\(^,\)\(^16\) Besides these limitations of the use of PIs that are specific for HIV/HCV coinfection, the first-generation PIs will be limited to patients infected by HCV genotype 1 and the treatment costs will increase considerably. This may result in lack of availability of DAAs in some economically depressed areas, where, as a consequence, bitherapy would remain the only treatment option. Because of these reasons, accurate identification of the likelihood of SVR to SOC among HIV/HCV-coinfected patients could improve their management. Individuals with a very high probability of SVR to SOC could be treated without delay. In the case of HCV genotype 1-infected patients, this would also imply an important saving of costly PIs. On the other hand, treatment could be deferred in individuals with a very low probability of response to SOC until more effective regimens are available. Finally, the first regimens including a PI will be based on Peg-IFN/ribavirin and it is likely that the predictive value of predictors of SVR to standard therapy will be preserved at least partially.

A number of predictors of SVR have been identified so far. In fact, the determinations of baseline HCV viral load, HCV genotype and IL28B genotype are among the standard procedures recommended in recent updates of international guidelines in order to evaluate whether a patient is a candidate for treatment.\(^19\)\(^,\)\(^20\) Furthermore, there is growing evidence of the usefulness of LDLR genetic variants to predict SVR to SOC and it can be speculated that the determination of LDLR genotype, which is inexpensive and comparable to the cost of IL28B genotype determination, will be incorporated in clinical practice in the future. However, a single predictor usually provides less accurate information about the probability of response than more than one predictor considered together. In the model reported here, parameters that are mostly available in routine practice are combined in order to achieve maximal predictive performance. The model yielded an NPV of 89% and a PPV of 84%, respectively, with considerably high specificity and sensitivity. Importantly, the misclassification rates were low and only 5.5% were erroneously classified as likely to achieve SVR. In contrast, the use of the same parameters as in this study but without including the LDLR genotype leads to a considerably higher rate of misclassification.\(^9\) Finally, AUROC curve values of both the elaboration and validation groups indicate that the diagnostic performance of the model was good and similar to that described in the Prometheus index, which showed an AUROC value (95% CI) of 0.85 (0.76–0.93) in the validation group.\(^8\)

The model presented in this work is not the first approach to the prediction of the treatment response of HIV/HCV-coinfected patients. For example, the Prometheus index allows the calculation of the probability of SVR to Peg-IFN/ribavirin in HIV/HCV-coinfected patients as a function of HCV genotype (1/4 versus 2/3), IL28B genotype (CC or non-CC), baseline HCV RNA load and baseline liver stiffness as assessed by transient elastometry.\(^8\) However, this approach has the limitation that transient elastometry is not available as a standard procedure and many sites do not have this method at their disposal. In a more recently published model, patients were classified into anticipated responders and unlikely responders according to HCV genotype, baseline plasma HCV RNA concentration above or below 600000 IU/mL and IL28B genotype.\(^9\) Nevertheless, a considerably high percentage of patients remained unclassified when this algorithm was applied, specifically 42% of genotype 1 carriers and 38% of genotype 4 carriers.\(^9\) In contrast, the model reported here is applicable in all patients since it enables the calculation of a precise probability for each individual and thus represents a clear advantage over treatment algorithms. In this way, the contribution to individual treatment decisions using this application is higher.

This study has limitations. First, the determination of the LDLR genotype is not a standard procedure in clinical practice. However, the determination of LDLR SNPs may be incorporated in clinical practice without difficulties wherever a PCR procedure can be carried out. Second, only one SNP of the LDLR gene was incorporated in this model. Further investigation is required in order to evaluate whether there is an LDLR SNP associated with higher SVR rates or whether the combination of several SNPs in this region results in better diagnostic performance. Finally, the model was not validated in HCV-mono-infected patients. Critical differences in the characteristics of HIV/HCV-coinfected patients may lead to a different response profile and the effectiveness of the model in a mono-infected population may not be satisfactory. Further studies to validate this model in HCV monoinfection are required.

In conclusion, this model allows reliable determination of the probability of SVR to Peg-IFN/ribavirin in HIV/HCV-coinfected patients using simple laboratory determinations. Its application in clinical practice could aid treatment decisions and represents a further step towards individualization of therapy against HCV.

**Acknowledgements**

These data were presented at the Nineteenth Conference on Retroviruses and Opportunistic Infections, Seattle, WA, USA, 2012 (Abstract 764; Themed Discussion #16).

We would like to thank Eugenia Víspio and Pablo Barreiro for their support.

**Funding**

This work was supported in parts by the Red de Investigación en SIDA (ISCIII-RETIC RD06/006), the Fundación Progreso y Salud, Consejería de
Transparency declarations

J. M. has been an investigator in clinical trials supported by Roche, Bristol-Myers Squibb and Abbott Pharmaceuticals. He has received lecture fees from Roche, Gilead, Boehringer Ingelheim and Bristol-Myers Squibb, and consulting fees from Boehringer Ingelheim, Bristol-Myers Squibb, Merck Sharp & Dohme and Schering-Plough. A. R. has received consulting fees from Bristol-Myers Squibb, Abbott, Gilead, Janssen-Cilag, Merck Sharp & Dohme and Boehringer Ingelheim, has received research support from Abbott, GlaxoSmithKline, Roche, Bristol-Myers Squibb, Abbott and BoehringerIngelheim, and has received lecture fees from GlaxoSmithKline, Abbott, Bristol-Myers Squibb, Merck Sharp & Dohme, Janssen-Cilag, Gilead, Boehringer Ingelheim and Schering-Plough. J. A. P. reports having received consulting fees from GlaxoSmithKline, Bristol-Myers Squibb, Abbott Pharmaceuticals, Gilead, Merck Sharp & Dohme, Schering-Plough, Janssen-Cilag and Boehringer Ingelheim. He has received research support from GlaxoSmithKline, Roche, Bristol-Myers Squibb, Schering-Plough, Abbott Pharmaceuticals and Boehringer Ingelheim, and has received lecture fees from GlaxoSmithKline, Roche, Abbott Pharmaceuticals, Bristol-Myers Squibb, Gilead, Merck Sharp & Dohme, Janssen-Cilag, Boehringer Ingelheim and Schering-Plough. All other authors: none to declare.

Author contributions

K. N.: planning and conducting the study, collecting and interpreting data and drafting the manuscript.
C. A.: interpreting data.
A. Caruz: collecting and interpreting data.
A. R. J.: collecting and interpreting data.
N. I. R.: collecting and interpreting data.
F. A. D. L.: collecting and interpreting data and drafting the manuscript.
F. A. D. L.: collecting and interpreting data and drafting the manuscript.
R. H.: collecting and interpreting data.
A. Camacho: collecting and interpreting data.
J. M. B.: collecting and interpreting data.
J. M.: collecting and interpreting data and drafting the manuscript.
A. R.: planning and conducting the study, interpreting data and drafting the manuscript.
V. S.: planning and conducting the study, interpreting data and drafting the manuscript.
J. A. P.: planning and conducting the study, interpreting data and drafting the manuscript.

References
