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Short communication

The effects of iron injection on blood doping biomarkers in dried blood spots

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Running header: Iron injection impact on doping biomarker in DBS

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Abstract

Iron supplementation is not considered as a doping method; however, it can affect the levels of several biomarkers of the hematologic module of the athlete biological passport (ABP), such as the reticulocyte percentage (%RET) and hemoglobin (HGB) level. Thus, iron injection could be a confounding factor in anti-doping analyses. Previous studies have suggested that the HGB level and the expression levels of reticulocyte-related-mRNAs, such as 5'-aminolevulinate synthase 2 (*ALAS2*) and carbonic anhydrase 1 (*CA1*), could be promising biomarkers for the ABP and detectable in dried blood spots (DBSs). Therefore, in this study, we examined the impact of iron injection on the levels of these potential biomarkers in DBSs. Reticulocyte-related-mRNAs analyses were performed by RT-qPCR. Ferritin level in DBS was measured with ELISA method. Notably, there were no significant effects of iron supplementation on the levels of *ALAS2* and *CA1* mRNAs but by contrast, the %RET and immature reticulocyte fraction (IRF) measured in whole blood increased significantly following iron injection. As expected, iron supplementation increased the ferritin level significantly in both serum and DBS samples. In conclusion, these findings reinforce the specificity of reticulocyte-related mRNAs in DBSs as biomarkers of blood doping to target in anti-doping analyses.

Keywords: Iron supplementation, blood doping, RNA-based biomarkers, dried blood spots

Introduction

The hematological module of the athlete biological passport (ABP) is the main anti-doping tool used to detect blood doping, such as blood transfusion or administration of erythropoiesis-stimulating agents [1]. This module includes the longitudinal monitoring of hematological biomarkers, including the hemoglobin (HGB) level and the reticulocyte percentage (%RET) [1,2], which vary following blood doping. However, confounding factors such as altitude [3] and iron infusion can impact the levels of hematological markers and lead to atypical observations in the longitudinal profile[4], compromising the specificity of the ABP. Iron injection is authorized by the World Anti-Doping Agency (WADA) guidelines, however this should be less than 100 mL in 12 hour period.

Our group has demonstrated that RET-related mRNAs could be useful biomarkers to detect blood doping and to improve the specificity of the hematologic module of the ABP [5,6]. The RET-related mRNAs encoding 5'-aminolevulinate synthase 2 (ALAS2) and carbonic anhydrase (CA1) have demonstrated suitable specificity and sensitivity [5].

The matrix used for analysis is another aspect of the hematological module of the ABP that could be improved. Currently, whole blood is collected in EDTA tubes; however, the collection, transportation, and storage of whole blood samples have some logistical drawbacks, such as temperature and time [7]. Similarly, serum samples, which are used to detect markers of iron metabolism, mainly ferritin, suffer from the same logistical problems [7]. Previous studies have suggested that dried blood spots (DBSs) could provide an alternative approach to overcome these issues [8] and could be used to detect several types of doping [9,10]. For example, we demonstrated in our earlier study the suitability of DBSs to analyze RET-related mRNAs and HGB [5,11].

In this study, we aimed at demonstrating the applicability of the DBSs to monitor hematological markers, and to study the capillary ferritin level in DBS for a marker of iron supplementation. According to our hypothesis, *ALAS2* and *CA1* should remain unaffected by iron infusion and thus, monitoring the levels of RET-related mRNAs in DBSs could increase the specificity of the ABP model in relation to iron uptake.

Materials and methods

Clinical study design

The samples used in this study were collected by the Swiss Laboratory for Doping Analyses in a previous clinical study, for which the randomized, single-blind, placebo-controlled trial was approved by the Human Research Ethics Committee of the canton of Vaud, Switzerland (Protocol: 2016.00324) [12]. Healthy participants were male Caucasians, aged 20–35 years with a ferritin concentration ≤50 µg/l ± 10% and a BMI of 18–30. Sixteen volunteers were randomly allocated to the iron or placebo groups, eight in each. In the iron group, volunteers were perfused with 10 ml of a 50 mg/ml ferric carboxymaltose complex (Ferinject®, Vifor Pharma, Villars-sur-Glâne, Switzerland) combined with 250 ml of 0.9% NaCl (B. Braun Medical AG, Crissier, Switzerland), whereas the volunteers in the placebo group received a 250 ml infusion of 0.9% NaCl. Blood samples were collected at baseline (days 1 and 4 before infusion; D-1 and D-4) and on days 1, 3, 6, and 10 after iron or placebo administration (D+1, D+3, D+6, and D+10). These time points were selected, because peaks of mRNA and RET increase are expected around seven days after EPO administration as previously demonstrated [5]. DBSs were obtained by directly pipetting 20 µl from finger-tip and spotted on Whatman® 903 saver card (Merck, Darmstadt, Germany).

Measurement of ferritin levels in serum and DBS

The level of ferritin in serum samples was measured using an Advia Centaur XP immunoassay system (Siemens Healthineers, Zürich, Switzerland). DBS extraction was performed according to Rocca A et al. [13]. One 20 µl-spot was put in 1 ml ultrapure H_2O + 1% Tween 20 incubated for 15 min on an Eppendorf Thermomixer (37°C, 450 rpm), sonicated for 15 min, and incubated again for 15 min on the Thermomixer. To measure the ferritin level in DBSs, a human ferritin ELISA kit (Ab108698; Abcam®, Cambridge, United Kingdom) was used according to the manufacturer's instructions. Absorbance was read at 450 nm and the analysis was performed with the MyCurveFit® platform, using the best-fit curve through the standard plotted points. The concentrations of the standards were log10 transformed and plotted against absorbance. The 4PL equation, with a factor of 10, was used to assess the sample concentrations.

Measurement of the HGB level

Extraction and analysis of HGB from DBSs were based on the methods developed by our group [11] and Oshiro et al. [14]. Briefly, each spot was excised and transferred into a 2 ml conical polypropylene microcentrifuge tube (Eppendorf, Schönenbuch, Switzerland). Subsequently, 1 ml of a sodium lauryl sulfate solution (0.06%) was added to the tube to extract the blood from the paper. Samples were shaken for 15 min at 450 rpm and 37°C, sonicated for 15 min, and then shaken again using the same conditions. A 200 µl aliquot of each sample was transferred into a well of a 96-well plate, and the absorbance was measured at 540 nm. A standard curve was prepared by using QC samples to transform optical density values into HGB concentrations. The HGB level in blood tubes was analyzed using a Sysmex XN instrument, based on the World Anti-Doping Agency technical document [2].

Measurement of the %RET

The %RET in EDTA tubes was analyzed using a Sysmex XN instrument, based on the World Anti-Doping Agency technical document [2].

Extraction and RT-qPCR analysis of RET-related mRNAs

Extraction of RET-related mRNAs was performed as described previously [15]. Briefly, each DBS was excised and placed into a 2 ml conical polypropylene microcentrifuge tube (Eppendorf), along with 1 ml of QIAzol lysis reagent (Qiagen, Hilden, Germany). The tube was then agitated (450 rpm for 15 min at 37°C), sonicated (15 min), and agitated again under the same conditions. Next, chloroform (250 µl) was added, and the tube was centrifuged for 15 min at 12,000 g. Finally, mRNA was extracted from the aqueous phase (525 µl) using the Maxwell RSC miRNA Plasma and Serum Kit (Promega, Madison, WI, USA) and Maxwell RSC instrument (Promega). The extracted mRNA was diluted in RNase-free water (50 µl).

For RT-qPCR analysis, mRNA was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Life Science, Basel, Switzerland) following the manufacturer's protocol. Subsequently, the cDNA was analyzed by RT-qPCR as described previously [5,15]. Briefly, ten-fold-diluted cDNA (4 µl) was loaded into a well of a 384-well plate (Roche Life Science) and mixed with 6 µl of a master mix comprising 240 µl of SYBR Green Master Mix (Qiagen) and 48 µl of primers (MicroSynth, Balgach, Switzerland). The sequences of the primers used to amplify the reference mRNAs (*GAPDH*, *RGCC L*, *RGCC C*) and target mRNAs (*ALAS2 LC*, *ALAS2 L*, *CA1*) have been published by our group previously [7–9]. A mean of the results of linear and circular version of *ALAS2* has been used in results. Analyses were performed in triplicate. Once loaded, the plate was centrifuged at 2000 rpm for 2 min and then inserted into a LightCycler 480 System (Roche Life Science) for RT-qPCR.

The following amplification conditions were applied: 10 min at 95°C (for denaturation), followed by 45 cycles of 10 s at 95°C and 1 min at 60°C (for amplification), and then 1 min at 55°C and 5 s at 95°C (for melting curve, specificity testing), with a final cool down for 30 s at 40°C. Mean Cq values from the reference mRNAs were used to normalize the mean Cq values from the target mRNAs. Data were analyzed using LightCycler software, version 1.5.0.39.

Statistical analysis

Changes in biomarker levels are expressed as a percentage change from baseline (% baseline). The normality of the data was determined using the Shapiro test and T-tests were used to compare differences between iron-supplemented and placebo samples, with $P < 0.05$ considered statistically significant. Statistical analyses were performed with R software (R Studio Version 1.3.959).

Results

First, the levels of four hematological biomarkers were analyzed in serum or EDTA blood samples, the classical anti-doping matrixes. The serum ferritin level increased significantly at days 1–10 after iron infusion and reached a peak (~1000% of the baseline) on day 6, but did not vary within the placebo group (Figure 1A). By contrast, the whole blood HGB level was unaffected by iron supplementation (Figure 1B). Iron infusion significantly increased the whole blood %RET(~160% of the baseline) on day 6 after administration, whereas infusion of saline had no effect (Figure 1C). The immature reticulocyte fraction (IRF) of whole blood was also affected by iron infusion, with a statistically significant increase (~140% of the baseline) on day 3 after injection, whereas infusion of saline had no effect (Figure 1D).

Next, we examined the levels of ferritin, HGB, and the RET-related *ALAS2* and *CA1* mRNAs in capillary DBSs. Ferritin DBS measurement, it has been done in ELISA due to the lack of sensitivity with Centaur for DBS analysis (data not show). As seen for the serum samples, the ferritin level in the DBSs was increased significantly from day 3 after iron intake and reached a peak (~10,000% of the baseline) on day 10, but did not vary in the placebo group (Figure 2A). As seen in the EDTA blood samples, there was no effect of iron supplementation or placebo on the HGB level in the DBSs (Figure 2B). Similarly, iron supplementation (and placebo) had no effect on the levels of the transcriptomic (RET-related) biomarkers *ALAS2* (Figure 2C) and *CA1* (Figure 2D).

Discussion

The potential use of RET-related mRNAs to improve the sensitivity and specificity of the hematologic module of the ABP has been described previously [5]. Here, we found that the levels of two RET-related mRNAs (*ALAS2* and *CA1*) in DBSs did not vary following iron infusion (Figures 2C and 2D), suggesting that mRNAs may be specifically sensitive to blood manipulation [16] or recombinant human erythropoietin (rhEPO) administration [5,6]. Conversely, the %RET and IRF of whole blood, two components of the ABP hematological module, were increased significantly after iron supplementation (Figures 1C and 1D), confirming a decreased specificity with respect to confounding factors, as demonstrated previously [17].

Iron supplementation increased the ferritin level in the DBSs to a maximum of ~10,000% of the baseline, whereas the serum ferritin level only reached a maximum of ~1000% of the baseline after supplementation (Figures 1A and 2A), these results are in line with the one obtained by Cook et al. [18]. Higher values in DBSs are probably due to ferritin incorporated in erythrocytes. DBSs could also be helpful to monitor iron injection in athlete in anti-doping

field. In the other hand, results obtain in serum and DBSs could not be compared, due to the two different methods used for measurement. Finally, the results presented here confirm that the HGB level can be measured successfully in DBSs, as described previously by our group [11]. However, more study will be done in order to be in line with WADA criteria.

Limitation

This study has been designed to illustrate if iron injection could have an impact on actual marker of the ABP and on new mRNA biomarkers in DBS. Thus, samples were collected only during ten days after iron injection because we expected a possible increase around seven days after iron administration. However, this could be a limitation due to the fact that, increase could be possible more than ten days after specially regarding HGB.

Conclusion

In conclusion, this study demonstrates that measuring the levels of RET-related mRNAs in DBSs could improve the specificity of the hematological module of the ABP in cases where there are confounding factors. Notably, DBSs have several advantages over traditional matrixes, including ease of collection, transport, and storage.

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Figure legends

Figure 1. The effect of iron injection on hematologic biomarkers in serum or EDTA blood samples. Volunteers were given a single infusion of iron or saline (placebo) on day 0, and samples were collected 1 and 4 days prior to injection (D-4P and D-1P), and 1, 3, 6, and 10 days after injection (D+1P, D+3P, D+6P, D+10P). **(A)** The serum ferritin level was measured using an immunoassay system (Centaur). **(B–D)** The HGB level **(B)**, %RET **(C)**, and IRF **(D)** were measured in whole blood using the Sysmex XN instrument. Data are expressed as percentages of the baseline values. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 2. The effect of iron injection on hematologic and transcriptomic biomarkers in DBSs. Volunteers were given a single infusion of iron or saline (placebo) on day 0, and samples were collected 1 and 4 days prior to injection (D-4P and D-1P), and 1, 3, 6, and 10 days after injection (D+1P, D+3P, D+6P, D+10P). **(A)** The level of ferritin in extracts of DBSs was measured by ELISA. **(B)** The HGB level was measured in extracts of DBSs using the Sysmex XN instrument. **(C, D)** The expression levels of the *ALAS2 (mean of ALAS2 LC and ALAS2 L)* **(C)** and *CA1* **(D)** mRNAs were measured in extracts of DBSs via RT-qPCR. Data are expressed as percentages of the baseline values. *P < 0.05.

Figure 1

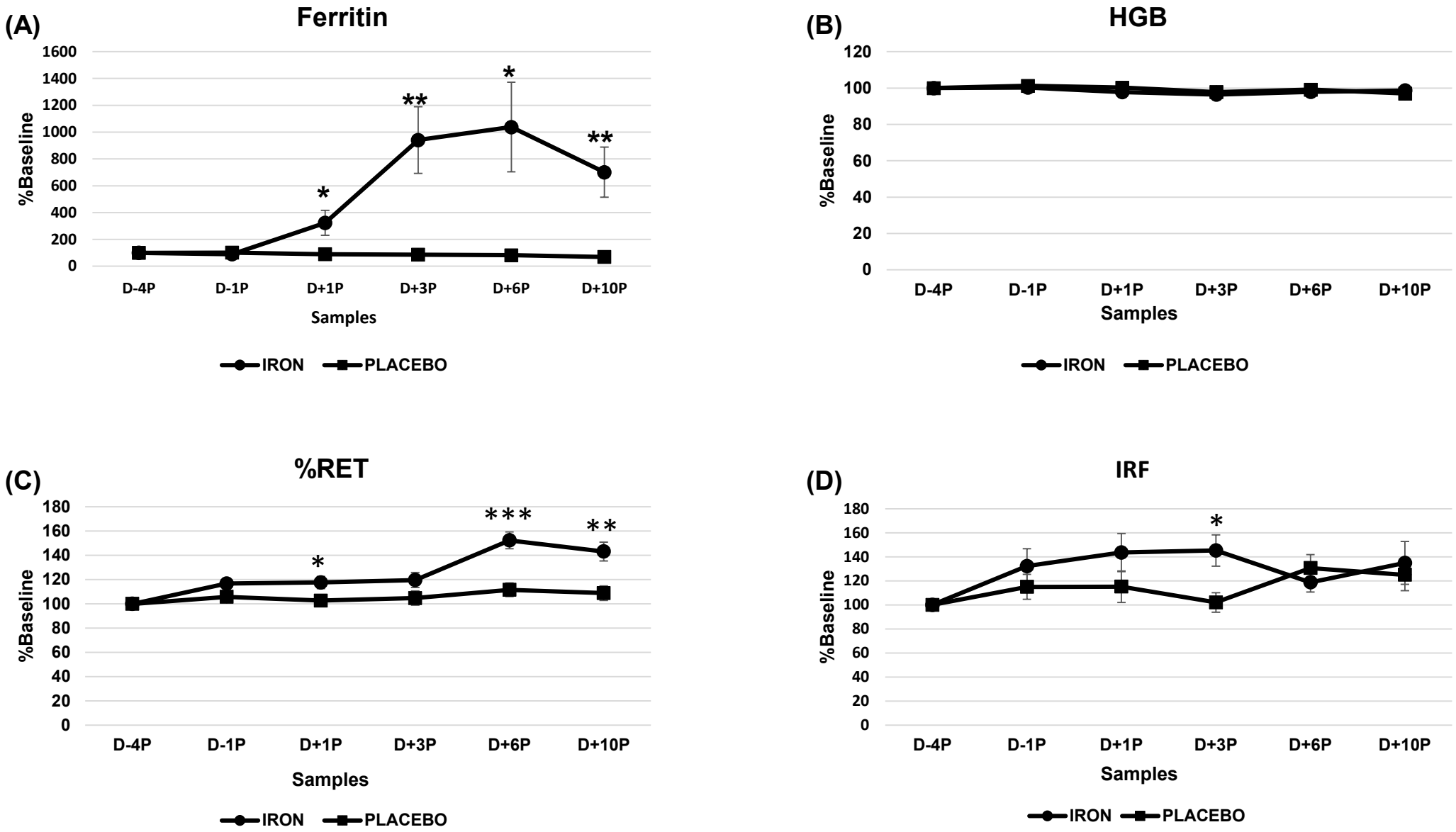
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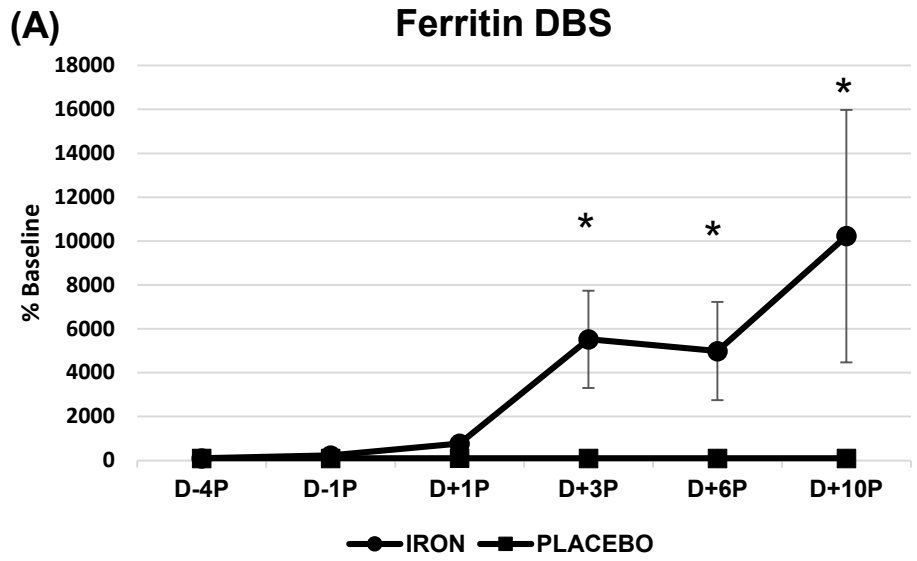
Figure 2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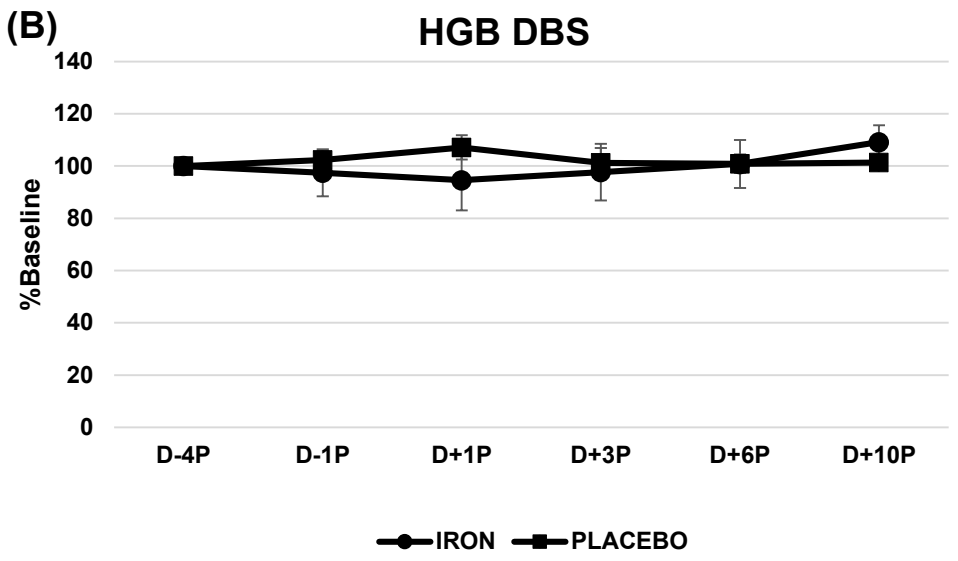
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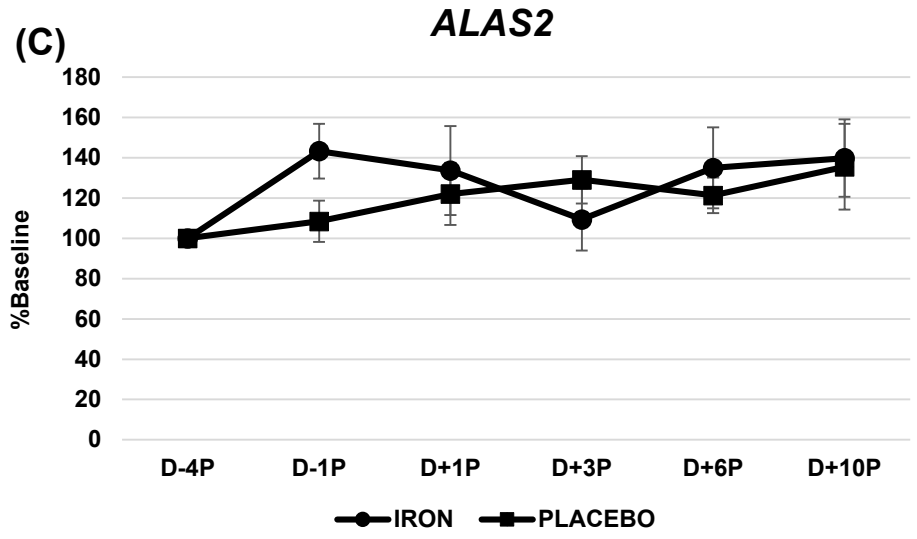
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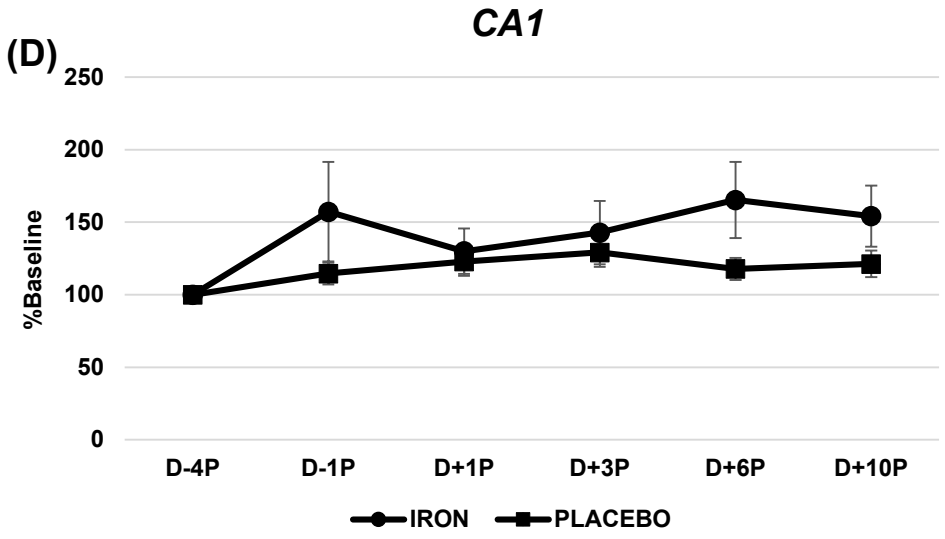
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