Disruption of the Blood-Brain Barrier by Extracellular Vesicles From Preeclampsia Plasma and Hypoxic Placentae: Attenuation by Magnesium Sulfate

José León; Jesenia Acurio; Lina Bergman; Juán López, Anna Karin Wikström; Pablo Torres-Vergara; Felipe Troncoso; Fidel Ovidio Castro; Manu Vatish; Carlos Escudero

ABSTRACT: Preeclampsia, a pregnancy-related endothelial disorder, is associated with both cardiovascular and cerebrovascular complications. Preeclampsia requires the presence of a placenta as part of its pathophysiology, yet the role of this organ in the cerebrovascular complications remains unclear. Research has shown that circulating small extracellular vesicles (also known as exosomes) present in preeclampsia plasma can generate endothelial dysfunction, but it is unclear whether the impairment of function of brain endothelial cells at the blood-brain barrier is secondary to plasma-derived or placental-derived exosomes. In this study, we evaluated the effect of small extracellular vesicles isolated from plasma samples of women with preeclampsia (n=12) and women with normal pregnancy (n=11) as well as from human placental explants subjected to hypoxia (1% oxygen) on the integrity of the blood-brain barrier, using both in vitro and animal models. Exposure of human-derived brain endothelial cell monolayers to plasma and plasma-derived small extracellular vesicles from preeclamptic pregnancies increased the permeability and reduced the transendothelial electrical resistance. A similar outcome was observed with hypoxic placental-derived small extracellular vesicles, which also increased the permeability to Evan's blue in the brain of C57BL6 nonpregnant mice. Cotreatment with magnesium sulfate reversed the effects elicited by plasma, plasma-derived, and hypoxic placental-derived small extracellular vesicles in the employed models. Thus, circulating small extracellular vesicles in plasma from women with preeclampsia or from hypoxic placentae disrupt the blood-brain barrier, which can be prevented using magnesium sulfate. These findings provide new insights into the pathophysiology of cerebral complications associated with preeclampsia. (Hypertension. 2021;78:1423–1433. DOI: 10.1161/HYPERTENSIONAHA.121.17744.) • Data Supplement

Key Words: blood-brain barrier • exosomes • extracellular vesicles • placenta • pregnancy

Preeclampsia is a syndrome affecting 2% to 8% of pregnant women and remains a major global cause of fetal and maternal morbidity and mortality. It is estimated to account for 15% of maternal deaths worldwide. It is characterized by widespread endothelial dysfunction and currently diagnosed by new-onset hypertension and proteinuria or other end-organ dysfunction in the second half of pregnancy. The disease is progressive and can lead to multisystem disorders including short (eg, eclampsia) and long-term cerebral complications, which in turn are major causes of maternal death and long-term morbidity. The majority of women with eclampsia, and some women with preeclampsia, exhibit neuro-radiological evidence of brain alterations including edema known as posterior reversible encephalopathy syndrome, thought to be caused by an injured blood-brain barrier (BBB). The BBB is a highly restrictive and specialized neurovascular network that isolates and protects the brain.
parenchyma from potentially harmful molecules in the systemic circulation.

Animal models of preeclampsia characterized by placental dysfunction can cause abnormal cerebral vascular reactivity, impaired autoregulation of cerebral blood flow, increased BBB permeability, cerebral edema, neuroinflammation, and decreased seizure threshold. Also, in vitro studies have demonstrated an increased permeability of brain vasculature exposed to plasma from women with preeclampsia. However, it is unclear which factors are responsible for the disruption of the BBB in preeclampsia, although candidates such as proinflammatory cytokines or vascular modulators have been suggested to play a role.

Importantly, circulating exosomes (or small extracellular vesicles), including placental-derived extracellular vesicles (PDsEVs) and sEVs, can be released in greater numbers and have been suggested to have a role in the pathogenesis of preeclampsia. They are carriers of harmful molecules and may have important signaling functions. However, whether circulating sEVs or PDsEVs found in plasma from women with preeclampsia can disrupt the BBB is unknown.

Magnesium sulfate is a well-established treatment for severe preeclampsia and manifest eclampsia to avoid recurrent seizures and prevent maternal death. Although the mechanism of action is not completely understood, it may decrease BBB permeability, reduce neuroinflammation, and improve endothelial dysfunction. No information is available regarding the potential role of magnesium sulfate in preventing the effect of sEVs or PDsEVs on endothelial dysfunction or BBB permeability.

In this study, we aimed to investigate whether sEVs isolated from the plasma of women with preeclampsia or PDsEVs from hypoxic placentas (as an in vitro model of preeclampsia) disrupt the BBB in both in vitro and in vivo models. Also, we sought to investigate whether magnesium sulfate could influence the disruption of the BBB generated in our preeclampsia-like experimental conditions.

**Nonstandard Abbreviations and Acronyms**

- **BBB** blood-brain barrier
- **FITC** fluorescein-5-isothiocyanate
- **oxLDL** oxidized low-density lipoprotein
- **PDsEVs** placental-derived extracellular vesicles
- **sEVs** small extracellular vesicles or exosomes
- **TEER** transendothelial electrical resistance
- **TNF-α** tumor necrosis factor-α
- **VEGF** vascular endothelial growth factor

**METHODS**

Note that additional detailed methods are included in the online-only Data Supplement.

**Participants**

The plasma samples were obtained between 2013 and 2016 from women with preeclampsia and women with normal pregnancies recruited from the obstetric ward or the outpatient clinic at Uppsala University Hospital in Sweden, and the population has been previously extensively characterized. For this study, we randomly chose plasma samples from 12 women with late-onset preeclampsia (from a whole group of 28 women) because late-onset preeclampsia has previously been found to have a larger effect on BBB than early-onset preeclampsia and 11 from the normal pregnant group (from a whole group of 28 women). Women with preeclampsia were diagnosed, according to the International Society for Studies on Hypertension in Pregnancy guidelines from 2014.
The research was performed in accordance with the principles expressed in the Declaration of Helsinki and under the authorization of the respective Ethical Review Boards. All participants gave their informed consent before sample collection.

### Placental Explants

Placental explants (10 g) were transferred into culture plates with culture medium 200 (Thermo Fisher Scientific, Hampton, NH) supplemented with 2% FBS, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin and placed in an incubator at 37 °C with 21% oxygen and 5% CO₂. After 2 hours of stabilization, cultured explants were washed and culture medium was replaced with medium 200 previously depleted of nanoparticles by ultracentrifugation (120,000 × g for 18 hours; Hitachi, CP 80 NX, Hitachi Koki, Co, Ltd, Tokyo, Japan) and microfiltration (filter 0.22 μm). For experimentation, placental explants were cultured for 18 hours at 8% O₂ (normoxia) or 1% O₂ (hypoxia) in a hypoxia chamber (BioSpherix C-Chamber, Proox Model 110, Parish, NY) as previously reported. After the incubation period, the conditioned media from each explant were collected and frozen (−80 °C) until isolation of extracellular vesicles was performed.

### sEV Isolation and Characterization

Plasma and culture media collected from the placental explants were used to isolate sEVs using differential centrifugation and microfiltration, as described elsewhere. The NanoSight NS300 equipment (Malvern Instruments, Ltd, Malvern, United Kingdom) was used to count and to measure the size of sEVs. Also, proteins (70 μg) from plasma-sEVs or PDsEVs were separated by SDS-PAGE (10%), transferred to nitrocellulose membranes, and probed with primary antibodies for specific markers of sEVs included in Table S1 I in the Data Supplement.

### Transendothelial Electrical Resistance and Cell Permeability

Monolayers of the female human brain endothelial cell line human cerebral microvascular endothelial cell line (Merck Millipore, Darmstadt, Germany) were used as a model of BBB for in vitro experiments. Measurements of transendothelial electrical resistance (TEER) and cell permeability to high molecular weight fluorescent dye (fluorescein-5-isothiocyanate [FITC]–dextran 70 kDa) were performed as previously reported. Although TEER values are directly correlated to the tightness of a cell monolayer and are an unspecific measure of its integrity, the permeability is inversely correlated to the tightness of a cell monolayer. Permeability can be carried out with a suitable probe to obtain more detailed information about the mechanisms by which a molecule is transported across a cell monolayer. Human cerebral microvascular endothelial cell line cell monolayers were exposed for 12 hours to plasma (1:10 v/v) or plasma-sEVs or PDsEVs (total small-EVs protein concentration 100 μg per well), following previous publications and in house experimental setups (see Data Supplement). PDsEVs were derived from explants cultured either in normoxia or hypoxia conditions. Total protein quantification was performed using Pierce BCA protein assay kit following manufacture instructions (Thermo Fisher Scientific, Waltham, MA).

### Treatment With Magnesium Sulfate

Parallel in vitro experiments were performed in cells exposed to plasma, plasma-sEVs, or PDsEVs in presence (12 hours) of magnesium sulfate (MgSO₄, 80 mg/L). For in vivo experiments, the dose of magnesium sulfate was 98 mg/L (or 8 mEq/L; similar to concentrations used in humans).

### Animal Model

Nonpregnant C57BL6 mice were purchased from the animal facility of the Universidad de Valparaíso, Chile. All experiments were performed in compliance with the 3R principles for animal experimentation, and according to the recommendations of the guidelines for the Care and Use of Laboratory Animals published by the US National Institute of Health. The Ethical Committee from the Universidad del Bio-Bio and the Agencia Nacional de Investigacion (Fondecyt grant 1200250, Chile) approved the protocol.

The mouse models were used to verify whether PDsEVs altered the permeability of the cerebral microvasculature using the analysis of the Evan blue extravasation over the BBB as reported elsewhere. Two groups of mice were analyzed side-by-side. One received a single injection via dorsal tail vein of solution containing PDsEVs (200 μg of proteins/70 μL) whereas another group received vehicle (phosphate buffer, pH 7.4, room temperature), as indicated previously. For in vivo experiments, the sample size for detecting a difference between the means was calculated considering the TEER values obtained in brain endothelial cells exposed to sEVs from plasma of women with normal pregnancy and preeclampsia (see Data Supplement).

### Statistical Analyses

Background characteristics are presented as mean±SD and percentage as appropriate and were compared between groups by Student t test or χ² test. Time course TEER experiments were analyzed using 2-way ANOVA with a Holm-Sidak post-test for multiple comparisons. In experiments using magnesium sulfate, pair t test was used. For the BBB permeability analyses, values are presented as mean and SD, and differences between normal pregnancy and preeclampsia (or normoxia and hypoxia) were compared by nonparametric analysis. Data and statistical analyses were performed using SPSS version 25, GraphPad Prism 6.00 (GraphPad Software, CA).

### RESULTS

#### Population Characteristics

Clinical characteristics of the women have been described in previous studies. Subsets of women were included in the current study (Table S2). Women with preeclamp sia displayed increased plasma concentrations of soluble fms-like tyrosine kinase-1 and decreased concentrations of placental growth factor compared with both control groups (Table S2) providing biochemical support for the clinical diagnosis.

#### Preeclampsia Plasma Impairs the Permeability and TEER of Brain Endothelial Cells

Plasma from women with preeclampsia exerted a reduction in TEER values (Figure 1A) and increased permeability (Figure 1B) in brain endothelial cell monolayers...
compared with normal pregnancy plasma, confirming previous findings.\(^\text{14}\)

The reduction in TEER induced by plasma from women with preeclampsia started as early as 30 minutes postincubation and remained decreased during the whole period of evaluation (12 hours), compared with respective basal condition or compared with cells exposed to plasma from women with normal pregnancies (Figure 1C). The latter also showed a decrease compared with control, but this was not as marked as that seen in plasma from women with preeclampsia.

**Isolation of sEVs From Plasma**

A summary of the process for isolation of sEVs from plasma (Figure S1) with respective distribution of particle size and concentration (Figure 2A), and identification of protein markers for endocytic pathway and exosomes, microvesicles, and placenta is presented in Figure 2A through 2D. These data confirmed that sEVs had been successfully isolated.

Plasma from women with preeclampsia had a higher concentration of sEVs than plasma from women with normal pregnancies (\(6.7\times10^8\pm9.9\times10^7\) versus \(5.4\times10^8\pm5.4\times10^7\) particles/ml, respectively, \(P=0.05\); Figure 2C), confirming previous reports.\(^\text{38}\) In addition, the size of sEVs detected in plasma from women with preeclampsia showed a nonsignificant trend to be larger than those in normal pregnancy (\(114\pm1.1\) versus \(109\pm2.8\) nm, respectively, \(P=0.16\)).

**Preeclampsia Plasma-sEVs Disrupt Brain Endothelial Cell Monolayers**

Neither plasma nor sEVs (from normal pregnancy or preeclampsia) impacted cell viability (Figure S2A and S2B). sEVs from women with preeclampsia generated a greater reduction in TEER (Figure 3B, \(P=0.006\)) and higher
permeability (Figure 3D, P=0.03) than sEVs from women with normal pregnancies. The reduction in TEER elicited by sEVs from women with preeclampsia was similar to the reduction generated using the plasma where the sEVs originated from (−32.7±0.6 versus −32.4±3.6 % of reduction, respectively; Figure 3A). Similar results were found when comparing cell permeability (Figure 3C).

**Hypoxic PDsEVs Disrupt the Brain Endothelial Cell Monolayer**

Initial characterization in particle count and size (Figure 4A), as well as protein markers (Figure 4B), confirmed positive identification of PDsEVs. In this case, PDsEVs from placental explants cultured in hypoxia were larger (Figure 4C, P=0.03) than those isolated from placentas cultured in normoxia.

Hypoxic PDsEVs induced a greater reduction in TEER values (Figure 4D; P=0.02) while simultaneously increasing the permeability (Figure 4E; P=0.0003) than PDsEVs isolated from normoxic placentas. Neither PDsEVs from hypoxic or normoxic placentae affected cell viability (Figure S2C).

**Effect of PDsEVs on BBB Permeability in C57BL6 Nonpregnant Mice**

The injection of hypoxic-derived PDsEVs induced a 3-fold increase in the extravasation of Evan blue into the brain tissue compared to injection of normoxic derived PDsEVs (P=0.01; Figure 4F and Figure S3).

**Effect of Magnesium Sulfate on Disruption of the Employed BBB Models**

The significant reduction in TEER values induced by plasma from women with preeclampsia compared with normal pregnancy was no longer significant in the presence of magnesium sulfate at 6 and 12 hours of coincubation (Figure S4). However, the effect of magnesium sulfate was not consistent in all samples, as shown in before and after treatment experiments at 12 hours (Figure 5A). In contrast, magnesium sulfate did not have an effect on reduction of the TEER in cells exposed to plasma from women with normal pregnancies.

In regard to the effects of sEVs isolated from plasmas, cotreatment with magnesium sulfate exhibited a tendency to counteract the effects on TEER elicited by exposure to sEVs from women with preeclampsia, but this outcome was nonsignificant (Figure 5B). However, the addition of magnesium sulfate induced a significant reduction in the TEER values when brain endothelial cells were exposed to sEVs from women with normal pregnancies (P=0.002; Figure 5B).

Also, the higher reduction in TEER and increased permeability induced with PDsEVs from hypoxic placentae compared with PDsEVs from normoxic placentae were completely attenuated after treatment with...
magnesium sulfate (Figure 5C and 5D). When this was further analyzed in vivo with the rodent model, the already above reported hypoxic PDsEVs induced extravasation of Evan blue in brain tissue was completely abrogated when pretreated with magnesium sulfate (Figure 5E and 5F).

**DISCUSSION**

**Main Findings**

In this study, we bring new insights into the pathogenesis of cerebral complications in preeclampsia arguing for a potential direct harmful effect of circulating sEVs. Specifically, we show that (1) sEVs from plasma from women with preeclampsia disrupt the human BBB in an in vitro model based on brain endothelial cell monolayers; (2) hypoxic-derived PDsEVs from placental explants disrupt both the human and rodent BBB as demonstrated in vitro with brain endothelial cell monolayers and in vivo with C57BL6 mice, respectively; (3) magnesium sulfate attenuates the effects of both plasma-derived preeclampsia sEVs and hypoxic-derived PDsEVs on the BBB.

**Results in Context**

A majority of women with eclampsia and some women with preeclampsia present with posterior reversible encephalopathy syndrome, a vasogenic cerebral edema, which is seen as an indirect sign of BBB injury. Although little information is available about the potential circulating mediators causing the BBB injury, at least 3 candidates have been studied, mainly in animal and in vitro models, including VEGF (vascular endothelial growth factor), TNF-α (tumor necrosis factor-α), and oxLDL (oxidized low-density lipoprotein). These have all shown the potential to impair BBB function with increased permeability and neuroinflammation that is partly reversed by inhibitors. The origin of the elevated circulating concentrations of these potential harmful molecules in preeclampsia is not well understood, and potentially, other factors might also contribute to the increased BBB permeability and neuroinflammation in preeclampsia with neurological complications. For instance, all these molecules could be transported in sEVs. Our findings regarding increased circulating concentrations of sEVs in women with preeclampsia are in agreement with the literature. Additionally, previous
publications demonstrate that the sEVs in preeclampsia contain not only a different surface chemistry\(^\text{20,44}\) but also different microRNA compared to sEVs from normotensive pregnancies.\(^\text{38}\) Our manuscript provides new data regarding the potential negative impact of plasma-sEVs of women with preeclampsia and PDsEVs from hypoxic placentae on the integrity of the BBB.

Previous studies have shown that plasma-sEVs from women with preeclampsia induce endothelial dysfunction in other organs using in vitro models.\(^\text{45,46}\) In this study, we specifically investigated the BBB, using both in vitro and in vivo models. In agreement with previous studies,\(^\text{13,42}\) we demonstrate that plasma from women with preeclampsia was able to negatively impact BBB permeability in vitro. Our novel findings show that sEVs are also able to contribute to disrupted BBB function.

PDsEVs comprise 5% to 10% of total sEVs in pregnancy and this percentage is increased in women with preeclampsia.\(^\text{38,47,48}\) The precise delineation of effects caused by PDsEVs in plasma is difficult to distinguish because plasma carries other sEVs from different tissues/organs. To overcome this limitation, we used an experimental setting combining isolation of PDsEVs from placental explants (either in normoxia or hypoxia— as a well-described model of preeclampsia). Hypoxic PDsEVs enhanced the BBB permeability both in vitro and in vivo (using nonpregnant mice). There are previous studies using nonhuman PDsEVs to demonstrate a preeclampsia-like syndrome with or without focus on the brain.\(^\text{49,50}\) Han et al.\(^\text{49}\) used PDsEVs isolated from injured murine placentae and injected them in pregnant mice to generate a preeclampsia-like syndrome. These PDsEVs disrupted the integrity of a cultured mouse brain endothelial cell line (bEnd.3) and generated a reduction in the cerebral blood flow in nonpregnant mice.\(^\text{49}\) In contrast, another study indicated that sEVs may not participate in endothelial dysfunction using human umbilical vein endothelial cells as an in vitro model exposed to PDsEVs extracted from conditioned medium of placental explants of women with preeclampsia.\(^\text{51}\) Murine placenta has a different structure compared to the human placenta and thus the results may not be directly comparable. In
the latter study, it was not clear what concentration of sEVs was used, and a relatively modest characterization of the EVs was performed. Additionally, PDsEVs are derived from the syncytiotrophoblast cells (part of the chorionic villus in direct contact with maternal blood) in multivesicular bodies. These are secreted directly into the maternal circulation and thus not only anatomically remote from the umbilical vein but also functionally in the wrong direction.

We were able to replicate our findings from sEVs in plasma using PDsEVs derived from hypoxic human placentae, suggesting that PDsEVs are contributing to the BBB changes. PDsEVs have been reported in the circulation from early pregnancy and thus have the capacity to have sustained effects on both generalized endothelial cells as well as those in the BBB. There are documented increases in sEVs in preeclampsia, and these sEVs may affect the BBB. Finally, the sEVs carry genetic cargo, and disruption of the BBB could bring these vesicles into direct contact with neuronal tissues. Our own data with Evan blue suggests that this sEVs exposure might be substantial. This could provide a possible mechanistic explanation for changes in long-term cerebrovascular function.

Our results also indicate that magnesium sulfate can partially revert the disruption of the BBB mediated by plasma, sEVs in plasma from women with preeclampsia, and PDsEVs from hypoxic conditions. Magnesium sulfate is the drug of choice for both prevention and treatment of eclampsia and has been shown to reduce maternal deaths. Suggested mechanisms include protection of BBB, decrease in neuroinflammation, or modulation of
gamma aminobutyric acid receptor activation.12 Our data adds to existing knowledge by proposing that magnesium sulfate may prevent the PDsEVs-mediated disruption of the BBB. Accordingly, a recent study demonstrated that magnesium sulfate reduces the permeability to FITC-dextran 40 kDa in human brain endothelial cells, an effect that was related to the functional expression of both TRPM7 (transient receptor potential melastatin 7) and MagT1 (magnesium transporter subtype 1),25 2 proteins involved in the uptake of magnesium within the cell. Because TRPM7 and MagT1 have been scarcely studied in the context of preeclampsia (and its cerebrovascular complications),26 these proteins and other magnesium transporters are interesting targets to assess their role in the effects elicited by magnesium sulfate on the BBB of preeclamptic women. Mechanisms by which the BBB permeability is improved by magnesium are currently being investigated by our group.

A major limitation in our study is that we do not provide information about the cellular mechanisms by which sEVs from plasma of women with preeclampsia may disrupt the BBB. However, we combine in vitro and in vivo experiments using PDsEVs from hypoxic placenta to suggest that these particles may participate in this disruption using a well-validated and previously published human model of BBB.14

In conclusion, plasma-derived sEVs, potentially originating from the placenta in women with preeclampsia, play a role in BBB impairment, potentially contributing to cerebral complications in preeclampsia. Our data provide insight into the physiological process of BBB injury generated by PDsEVs. Importantly, sEVs-mediated disruption of the BBB can be prevented using magnesium sulfate.

Perspectives

We demonstrate that BBB disruption in preeclampsia can be mediated partly by plasma-sEVs, and specifically, we propose that PDsEVs may account for those alterations. Further research should focus on sEVs and not only how they interact with the BBB but also the effect of their cargo on neuronal tissues. Whether BBB impairment generates long-lasting consequences for the maternal brain needs to be further investigated. The underlying mechanisms associated with magnesium sulfate’s abrogation of the effect of sEVs on the BBB warrant further exploration. These findings raise the possibility of PDsEVs’ effect on longer-term cerebral function in women with preeclampsia.

ARTICLE INFORMATION

Received May 19, 2021; accepted August 23, 2021.

Affiliations

Vascular Physiology Laboratory, Department of Basic Sciences, Universidad del Bio-Bio, Chillán, Chile (J.A. Leon, J.A., J. Lopez, F.T., C.E.); Escuela de Enfermería, Facultad de Salud, Universidad Santo Tomás, Los Ángeles, Chile (J. Leon), Group of Research and Innovation in Vascular Health (Group of Research and Innovation in Vascular Health), Chillán, Chile (J.A., F.T., C.E., P.T.-V.); Department of Women’s and Children’s Health, Uppsala University, Sweden (L.B., A.K.W.); Department of Clinical Sciences, Sahlgrenska Academy, Gothenburg University, Sweden (L.B.); Department of Obstetrics and Gynecology, Stellenbosch University, South Africa (L.B.); Department of Pharmacy, Faculty of Pharmacy, University of Concepción, Chile (P.T.-V.); Department of Animal Science, Faculty of Veterinary Sciences, Universidad de Concepción, Chillán, Chile (F.D.C.); Nuffield Department of Women’s & Reproductive Health, University of Oxford, Women’s Centre, John Radcliffe Hospital, United Kingdom (M.V.).

Acknowledgments

We thank the researchers belonging to GrIVAS health for their valuable input and the Comision Nacional de Investigación, Ciencia y Tecnología (Chile) grant REDI170373. Also, we thank Hermes Sandovol and Belen Ibáñez for their contribution in the confirmatory experiments asked during the review process. C. Escudero conceptualized the study and conducted the statistical analyses. J. León performed most of the experiments in this article. J. Acurio, J. López, F. Troncoso perform selected experiments in both in vitro and in vivo approaches. L. Berghman and A.K. Wikström were responsible for patient recruitment. F.O. Castro and M. Vats is were consultants in exosome/small extracellular vesicles (sEVs) characterization. P. Torres-Vergara was a consultant in experiments related to the blood-brain barrier. C. Escudero, L. Bergman, M. Vats is, and P. Torres-Vergara edited the article. All coauthors approved the final version of this article.

Sources of Funding

This study was funded by Fondecyt 1200250 (Chile).

Disclosures

None.

REFERENCES

5. ACOG TfoHP. Hypertension in Pregnancy; 2013.

Preeclampsia

Hypertension. 2021;78:1423–1433. DOI: 10.1161/HYPERTENSIONAHA.121.17744

November 2021 1431


32. Mellito EA, Velasquez AE, Nuñez MJ, Cabezas JG, Cueto JA, Fader C, Castro PO, Rodríguez-Álvarez L. Identification and characteristics of