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Abstract: High density lipoproteins (HDLs) are pivotal in innate immunity and decrease in serum during inflammation. Several studies have been done about lipoprotein changes in adult transition cows but little is known about their changes in newborn calves. The aim of this study is to provide information about HDLs in newborn calves, by defining the possible age-related changes in healthy calves compared with adults and by assessing the possible differences in calves with inflammation. Lipoprotein electrophoresis and colorimetric measurement of HDL (HDL-C) were performed on healthy cows and calves in order to identify possible differences in the lipoprotein profile due to the age. Then, calves of the same ages affected by inflammatory conditions were also evaluated. Results showed that in calves HDL% and VLDL% were lower (mean values ± SD: 77.6 % ± 8.6% and 2.6% ± 2.5%, respectively) and LDL% was higher (19.7% ± 7.4%) than in adults (89.0% ± 3.9%; 5.2 ± 2.1% and 5.8% ± 3.1%, respectively). Sick calves revealed a decrease of both HDL% (mean values ± SD: 61.0% ± 22.1%) and HDL-C (22.8 ± 11.6 mg/dL) and an increase of VLDL% (12.1% ±13.1%) compared with controls (77.6 % ± 8.6%; 41.5 ± 11.2 mg/dL and 2.6% ± 2.5%, respectively). Paraoxonase-1 activity, influenced by inflammation and oxidation, was measured, and it appeared correlated with HDL% and HDL-C in sick calves. In conclusion, this study revealed that HDLs concentration in healthy calves is lower than in adults, and further decreases in calves with inflammation, likely due to oxidation.

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Knowledge and skills about inflammation in dairy cows and calves

Highlights:

- Electrophoretic and colorimetric lipoprotein measurement were performed in calves
- Good correlation between the two methods was found
- Healthy calves showed lower HDL% and VLDL% and higher LDL% compared to adult cows
- HDL decreases early during inflammation in sick calves
- PON-1 activity was positively correlated with HDL-C in both sick and healthy calves

Original Research Paper

Biochemical and electrophoretic evaluation of lipoprotein fractions in healthy neonatal calves: comparison with results from adult cows and from calves with inflammatory conditions

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Abstract

High density lipoproteins (HDLs) are pivotal in innate immunity and decrease in serum during inflammation. Several studies have been done about lipoprotein changes in adult transition cows but little is known about their changes in newborn calves. The aim of this study is to provide information about HDLs in newborn calves, by defining the possible age-related changes in healthy calves compared with adults and by assessing the possible differences in calves with inflammation. Lipoprotein electrophoresis and colorimetric measurement of HDL (HDL-C) were performed on healthy cows and calves in order to identify possible differences in the lipoprotein profile due to the age. Then, calves of the same ages affected by inflammatory conditions were also evaluated. Results showed that in calves HDL% and VLDL% were lower (mean values ± SD: 77.6 % ± 8.6% and 2.6% ± 2.5%, respectively) and LDL% was higher (19.7% ± 7.4%) than in adults (89.0% ± 3.9%; 5.2 ± 2.1% and 5.8% ± 3.1%, respectively). Sick calves revealed a decrease of both HDL% (mean values ± SD: 61.0% ± 22.1%) and HDL-C (22.8 ± 11.6 mg/dL) and an increase of VLDL% (12.1% ±13.1%) compared with controls (77.6 % ± 8.6%; 41.5 ± 11.2 mg/dL and 2.6% ± 2.5%, respectively). Paraoxonase-1 activity, influenced by inflammation and oxidation, was measured, and it appeared correlated with HDL% and HDL-C in sick calves. In conclusion, this study revealed that HDLs concentration in healthy calves is lower than in adults, and further decreases in calves with inflammation, likely due to oxidation.

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- PON-1 activity was positively correlated with HDL-C in both sick and healthy calves

1. Introduction

During the acute phase response, besides quantitative and structural variations of acute phase proteins (APPs), changes in lipids and lipoproteins have been also reported (Carpentier and Scruel, 2002). Specifically, hepatic triglyceride production increases after the lipolysis mediated by pro-inflammatory cytokines (e.g. Tumor Necrosis Factor-α, Interleukin-1, Interferon), leading to an increase of very low density lipoproteins (VLDL) levels in plasma (Hardardóttir et al., 1995). In contrast, plasma cholesterol usually decreases in many acute conditions due to a decrease of both low and high density lipoproteins (LDL and HDL), with some differences among animal species (Carpentier and Scruel, 2002; Khovidhunkit et al., 2004).

HDLs are pivotal in innate immunity (Van Lenten et al., 2006) and besides their plasma level, also their composition changes during inflammation in order to better sustain their protective and antioxidant properties. Specifically during infection and inflammation, a reduction of plasma proteins (cholesterol acyltransferase, cholesterol ester transfer protein, phospholipids transfer protein) involved in the HDL-mediated reverse cholesterol transport and in inhibition of plasma lipid oxidation has been recorded (Navab et al., 2011). Moreover, the lipid composition of the HDL is altered during inflammation: the huge increase in serum amyloid A induces the displacement of the apolipoprotein A-I particle, which contains the antioxidant enzyme paraoxonase-1 (PON-1), from HDLs, thus reducing the HDL anti-inflammatory properties (Hyka et al., 2001). PON-1 is an anti-oxidative enzyme and a negative acute phase reactant that is related with HDL, and decreases in serum due to both a reduced production and to an increased peripheral consumption associated with oxidation (Feingold et al., 1998).

There are several reports in veterinary literature describing changes in lipoprotein metabolism in cows, but the majority of them is focused on the relationship between lipid mobilisation and oxidation during transition period (Ileri-Büyükoğlu et al., 1998; Kurpinska et al., 2015; Newman et al., 2016; Turk et al., 2013). In contrast, very few information is reported about lipoprotein changes

in newborn calves (Herosimczyk et al., 2013). In a previous study (Giordano et al., 2013) it has been demonstrated that newborn calves show a different pattern in paraoxonase-1 activity compared to older animals, suggesting that a similar trend may be expected also for lipoproteins, due to the strict association between the metabolism of PON-1 and HDLs. The electrophoretic fractionation of lipoproteins on agarose gels, which is an easy-to-perform method useful to investigate possible variations in lipid profile, has not been widely investigated in cattle, and very few observations have been done on calves.

The newborn calf is frequently prone to inflammatory conditions especially in case of failure of passive transfer (Murray et al., 2013). Therefore, information regarding analytical methods (electrophoretic and colorimetric) for measuring serum lipoproteins in healthy and diseased calves will help to better clarify the changes in circulating levels of lipoproteins during inflammation, and possibly improve the knowledge about pathogenesis, early diagnosis and monitoring of neonatal diseases of calves.

2. Material and methods

2.1 Animals

Adults group: the study involved twenty healthy pluriparous Holstein dairy cows during the first week after parturition. Specifically, samples were collected at day 3 post-.partum. All the cows were kept in free-stall barns with free access to food and water. The age of cows ranged from four to six years (2^{nd}-5^{th} lactation). After parturition all cows underwent a gynaecological and a complete general examination as well as a complete laboratory profile, and only cows considered clinically healthy and with all the haematological and biochemical values within species-specific reference intervals adopted in the Laboratory were included in this study. Adult cows were sampled at the same hour in the morning at milking.

Control calves group: thirty-eight Holstein newborn calves were enrolled in the study. The calves were 21 males and 17 females with a median weight at birth of 35 kilograms (31-40). Immediately after birth all calves were artificially fed with pooled colostrum, while from the 3rd to the 14th day after birth they were fed twice a day with milk powder. Free access to a calf starter was allowed from day 14 after birth. All the calves received a complete physical examination immediately after birth (rectal temperature, maturity, measurement of a modified Apgar score) (Mee, 2004; Probo et al., 2012). A single blood sample was then collected from each calf, with calves ages ranging from 7 to 20 days. Blood samples were collected at different day times, but at least one hour far from food administration. Basic biochemical and hematological profile were performed, and only those calves with all the clinical and laboratory parameters within the reference intervals were considered as healthy, and thus included in the control group.

Sick calves: twelve Holstein calves (from the same herd of healthy controls) affected by inflammatory conditions were also enrolled. Calves included were positive for systemic inflammatory response syndrome (SIRS) criteria [two out of four criteria, (a) or (b) obligatory]: (a) fever or hypothermia, (b) increased or decreased leucocyte counts, (c) tachycardia, (d) tachypnoea or need for mechanical ventilation (Hofer et al., 2010). Pathological conditions were represented by acute diarrhoea (n=4) caused by *E.coli*, or by respiratory diseases (n=8) such as pneumonia or bronchopneumonia caused by *M. hemolytica* and *P. multocida*. Diagnosis was achieved based on a thorough clinical examination, on performance of a complete blood cells count and on isolation of colonies with bacteriology. A single blood sample was collected for each sick calf at different ages depending on the appearance of the pathological event, but always ranging from 7 to 20 days after birth.

Blood samplings were performed as a part of the health monitoring after parturition for adult cows, and as part of the wellness assessment for calves (control group) or for routine diagnostic purposes (sick calves) under informed consent of the breeders. Therefore, according to the guidelines of our

Institution, a formal approval from the Ethical Committee is not required since samplings were performed for routine diagnostic purposes.

2.2 Samples and methods

Twenty mL of venous blood were collected from the jugular vein; ten mL were placed in plain tubes (Venoject, Terumo, Italia S.r.l) while 10 mL were placed in tubes with EDTA (Venosafe plastic tubes for hematology, Terumo, Europe), both immediately transported to the laboratory (Clinical Pathology Laboratory of the Large Animal Teaching Hospital of the University of Milan, Lodi, Italy). Whole blood samples were used to perform routine hematology using the laser counter ADVIA 120 with multispecies software (Siemens Healthcare Diagnostics, Deerfield, IL, USA), while samples in tubes without anticoagulant were centrifuged (2500g x 10 min) to obtain serum to perform the routine diagnostic/check-up biochemistry mentioned above (Ilab 300 plus, Instrumentation Laboratory, Milan, Italy). The remaining serum was then transferred to plain tubes and aliquots were frozen at -20°C until analyses, that were performed within one month. None of the specimens had evident hemolysis and/or lipemia: all the haematological and biochemical results were within the species-specific reference ranges adopted in the laboratory (adult cattle and newborn calves), and therefore all the samples were included in the study.

Since no information on age-related changes of lipidograms are reported, the first step of the study was to determine the normal electrophoretic pattern of lipoproteins in calves compared to that of adult cows. Therefore, we enrolled in the study a larger number of clinically healthy animals, compared with the number of sick calves, in order to conform with the current guidelines on establishment of reference intervals that recommend not to use small groups of animals for this purpose (Friedrichs et al., 2012). Once the lipidogram of newborn calves had been determined as above, the possible changes detectable in sick calves were investigated using the same methodological approach.

One aliquot of serum was thawed and lipoprotein electrophoresis was performed in an automated apparatus (Hydrasis, Sebia Italia S.r.l.), using kits produced by the manufacturer of the instrument (Hydragel 15 lipoproteins). After migration (160 V, 25 minutes), agarose gels (8 g/L) were stained with Sudan black, washed with ethanol (45%), dried, and placed on the gel scanner. Scanned images were analyzed using the software Phoresis (Sebia Italia S.r.l., Bagno a Ripoli, Italy) that calculates the area under the peaks corresponding to HDL, VLDL and LDL, and expresses the results as a percentage of the total area (HDL%, VLDL%, LDL%).

Moreover, the concentration of HDL in serum of sick and clinically healthy calves was measured colorimetrically (HDL-C) using a commercially available assay (Cholesterol esterase/oxidase reaction after precipitation of LDL and VLDL) for automated spectrophotometers (Ilab 300 plus, Instrumentation Laboratory), to assess whether this method may provide additional information compared with lipoprotein electrophoresis in calves.

Finally, in order to draw preliminary information on the relationship between HDL and oxidative stress in calves, the activity of paraoxonase-1 (PON-1) was measured in serum of healthy and sick calves using an automated spectrophotometer (Cobas Mira, Roche Diagnostics) and an enzymatic method validated in calves (Herosimczyk et al., 2013). Serum samples (6 μL) were incubated at 37 °C with 89 μL distilled water and 100 μL 0.05 M glycine buffer (pH 10.5) containing 1 mM paraoxon-ethyl (purity > 90%, Sigma-Aldrich) and 1 mM $CaCl_2$. The rate of hydrolysis of paraoxon to p-nitrophenol was measured by recording the increase in absorbance at 504 nm using a molar extinction coefficient of 18,050 L/mol/cm. PON activity, expressed as U/mL, is defined as 1 nmol of p-nitrophenol formed per minute.

2.3 Statistical analysis

Statistical analysis was performed in an Excel (Microsoft Corp, Redmond, WA, USA) spreadsheet using the Analyse-it software (Analyse-it Software Ltd, Leeds, UK). For each of the investigated parameters, a non-parametric *t*-test for independent samples (Mann Whitney *U* test) was used to

compare results obtained in healthy calves with those of adult cows as well as to compare values recorded in healthy with those obtained in sick calves. In both healthy and sick calves, the possible presence of correlations between electrophoretic (HDL%) and enzymatic (HDL-C) HDL values and between HDL values obtained with the two methods and PON-1 activity was assessed using the Spearman's correlation test. Reference Intervals in healthy neonatal calves were determined using an Excel (Excel, Microsoft Corp, Redmond, Washington, USA) spreadsheet with the Reference Value Advisor (version 2.0) set of macroinstructions (Friedrichs et al., 2012) that performs computations following the IFCC-CLSI recommendations as also suggested by ASVCP (American Society of Veterinary Clinical Pathology) guidelines (Geffré et al., 2011). Results yielding a *P*-value <0.05 were considered as statistically significant.

3. Results

The reference intervals calculated in the group of healthy neonatal calves are reported in Table 1. The comparison between results obtained in the present study in neonatal calves and those obtained in adult cows revealed that HDL% and VLDL% were significantly lower ($P < 0.001$ for both) in calves (mean values ± SD: 77.6 % ± 8.6% and 2.6% ± 2.5%, respectively) than in adults (89.0% ± 3.9% and 5.2 ± 2.1%), while LDL% was significantly higher ($P < 0.001$) in calves (19.7% ± 7.4%) than in adults (5.8%± 3.1%). Therefore, LDL peaks were more pronounced in lipidograms from calves than in those from adults (Fig. 1A-B).

Lipidograms from sick calves were visually different from those of clinically healthy animals (Fig. 1C-D) showing higher peaks in LDL and especially in VLDL fractions in sick calves compared with controls. The visual analysis of lipidograms did not constantly reveal different profiles between calves with diarrhoea and with respiratory diseases.

The comparison of results from the two groups of calves (clinically healthy vs. sick) revealed a significant decrease of PON-1 activity ($P < 0.05$) and of both HDL% ($P < 0.01$) and HDL-C ($P <$

0.001), which were highly correlated to each other ($P < 0.001$; $r = 0.96$), and a significant increase of VLDL% ($P < 0.01$) in sick calves compared with clinically healthy age-matched controls (median values and data distribution are reported in Fig. 2). No significant differences were found between calves with diarrhoea and with respiratory diseases.

The Spearman's correlation test showed that PON-1 activity was positively correlated with HDL-C either in sick ($P = 0.043$, $r = 0.43$) and in healthy calves ($P < 0.001$; $r = 0.54$), whilst a positive correlation with HDL% was found only in sick calves ($P = 0.013$, $r = 0.69$).

4. Discussion

The relationship between inflammation and lipid metabolism has been confirmed in both human and veterinary medicine among different animal species (Carpentier and Scruel, 2002). Different methods are reported to measure lipoproteins in cows (Feingold et al., 1998; Kushibiki et al., 2002) but usually they require ultracentrifugation of the samples which is not routinely performed in veterinary diagnostic laboratories. Most of the literature concerning lipoprotein changes in cows deals with the transition period, while little is known about possible changes of lipoproteins in newborn calves (Turk et al., 2013).

The first step of the present study was to compare electrophoretic lipidograms obtained in adult cows with those from healthy calves. This electrophoretic method has the advantage of being performed using an automated technique, widely diffused in veterinary laboratories, relatively cheap and rapid. Moreover, the high correlation found here between the electrophoretic HDL% and the HDL-C measured using a colorimetric method on an automated spectrophotometer, which is still faster and cheaper than the electrophoretic technique although does not provide information about the other lipoproteins, suggest that this method can be widely employed for the monitoring of lipid and lipoprotein profiles also in large animals.

230 The differences recorded in lipidograms of calves, showing lower HDL% and VLDL% and higher
231 LDL% compared to adult cows, are likely dependent on a more active energy metabolism in adult
232 cows, due to the intense lipomobilization associated with early lactation (Contreras et al., 2010).
233 Interestingly, the differences between calves and adult cows reflect the age-related changes already
234 reported for PON-1 activity (Herosimczyk et al., 2013). In the cited study, the immaturity of liver of
235 newborns was also suggested among the possible causes of the differences recorded between calves
236 and adult cows. The same hypothesis may be suggested here; liver is recognized as a pivotal organ
237 for lipoprotein metabolism, thus the reduced function of newborns compared with adults may be
238 responsible for the different lipoprotein profile in calves observed in the present study. This
239 hypothesis has already been suggested in a previous study (Turk et al., 2010).
240 The significantly lower levels of PON-1 and HDL and the higher level of VLDL in calves affected
241 by inflammatory conditions confirms that also in calves HDL decreases early during inflammation,
242 as previously suggested (Yamamoto et al., 2000) and in agreement with what reported in other
243 species (Carpentier and Scruel, 2002; Khovidhunkit et al., 2004). This decrease may be detected
244 using both the methods employed in this study for measuring HDLs. However, in sick calves
245 HDL% correlates better than HDL-C with PON-1 activity, a marker of oxidative metabolism
246 associated with inflammation and therefore it may be preferable to the colorimetric measurement of
247 HDL to investigate oxidative phenomena in calves with inflammation.
248 The correlation between PON-1 and HDL has been once again confirmed in the present study. This
249 is not surprising, since PON-1 is known to be an HDL-associated enzyme esterase which appears to
250 contribute to the antioxidant and anti-atherosclerotic capabilities of HDL-C (Aviram et al., 2013).
251 Results of the present study, in fact, showed that PON-1 activity was positively correlated with
252 concentration of HDL-c in both sick and healthy calves. This suggests, as already reported in people
253 and in cows (Florentin et al., 2008; Ileri-Büyükoğlu et al., 2009), that a strict association between
254 lipid metabolism and oxidative phenomena associated with inflammation exists. A limitation of this
255 study is that the other molecules with oxidant or anti-oxidant properties have not been measured,

and therefore no conclusive information on the actual presence of oxidative stress in calves with and without inflammation can be drawn from this study. However, the results of this study should encourage further studies on oxidative stress, since the association between changes of PON-1 and HDLs, either in the comparison between age groups or in the comparison between healthy and sick animals strongly suggest the strict association between oxidation, lipid metabolism and inflammation.

The results reported here showed that, in healthy calves, the best correlation between these two parameters is detected when HDL is measured colorimetrically instead of using electrophoretic percentage of HDL. This is probably due to the fact that fractions obtained with the lipidograms should be then transformed in absolute values. This is only possible when the total concentration of lipoproteins is known. Unfortunately is not easy to measure the total concentration of plasma lipoproteins, since the most reliable methods available are not applicable in routine practice. On the other hand, the measurement of HDL using the colorimetric method represents a more reliable method since this value is obtained after the other lipoproteins have been precipitated.

5. Conclusions

In conclusion, this study evidenced that the lipidogram of calves differs from that of adults. During inflammation, decreases of HDLs may be detected either using lipoprotein electrophoresis or the colorimetric measurement of HDL, confirming the association between inflammation and lipid metabolism suggested also by the correlation between PON-1 and HDL values in diseased calves.

Conflict of interest statement

The Authors do not have any conflict of interest potentially influencing the results of this study

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Table 1 Reference intervals calculated in healthy neonatal calves for lipoprotein electrophoretic fractions and HDL measured by a colorimetric method

Analyte	N	Mean	SD	Median	Min	Max	RI	Lower Ref Lim 90% CI	Upper Ref Lim 90% CI	Dist	Method
HDL-C (mg/dL)	38	41.5	11.2	43	20	61	17.4-64.1	11.4-24.6	59.0-68.2	G	R
HDL (%)	38	77.6	8.6	78.6	58.3	93.9	59.8-95.4	56.3-63.9	91.6-99.2	G	R
VLDL (%)	38	2.6	2.5	2.1	0.4	11.1	0.3-9.7(S)	0.3-0.5	6.8-13.3	G	R
LDL (%)	38	19.7	7.4	19.5	4.9	34.1	4.9-35.3	2.1-8.4	31.9-38.6	G	R

(S) = suspected outliers were present; G, Gaussian; R, robust;

RI: Reference interval; Ref Lim: Reference Limit; Dist: Distribution

Figure legends

Fig. 1: Examples of densitometric analyses of lipidogram from a clinically healthy cow (A: HDL = 91.8%, VLDL = 4.2%, LDL = 4.0 %), from a clinically healthy calf (B: HDL = 80.3%, VLDL = 3.6%, LDL = 16.1 %;), a calf with a respiratory disease (C: HDL = 77.5%, VLDL = 4.4%, LDL = 18.1 %) and a calf with neonatal diarrhoea (D: HDL = 73.4%, VLDL = 17.5%,LDL = 9.1 %). From the left to the right, peaks represent HDL (black arrow), VLDL (light grey arrow) and LDL (dark grey arrow).

Fig. 2: Differences between clinically healthy and sick calves regarding colorimetric HDL, electrophoretic HDL%, VLDL% and LDL% and PON-1 activity. The boxes indicate the I–III interquartile range (IQR), the horizontal line indicates the median, whiskers extend to further observation within the I quartile minus 1.5*IQR or to further observation within the III quartile plus 1.5*IQR. Near outliers are indicate by the symbol '+' and far outliers by the red asterisk . The black bolded asterisks indicate significant differences between groups: *= $P < 0.05$; **= $P < 0.01$; ***= $P < 0.001$.

Figure 1

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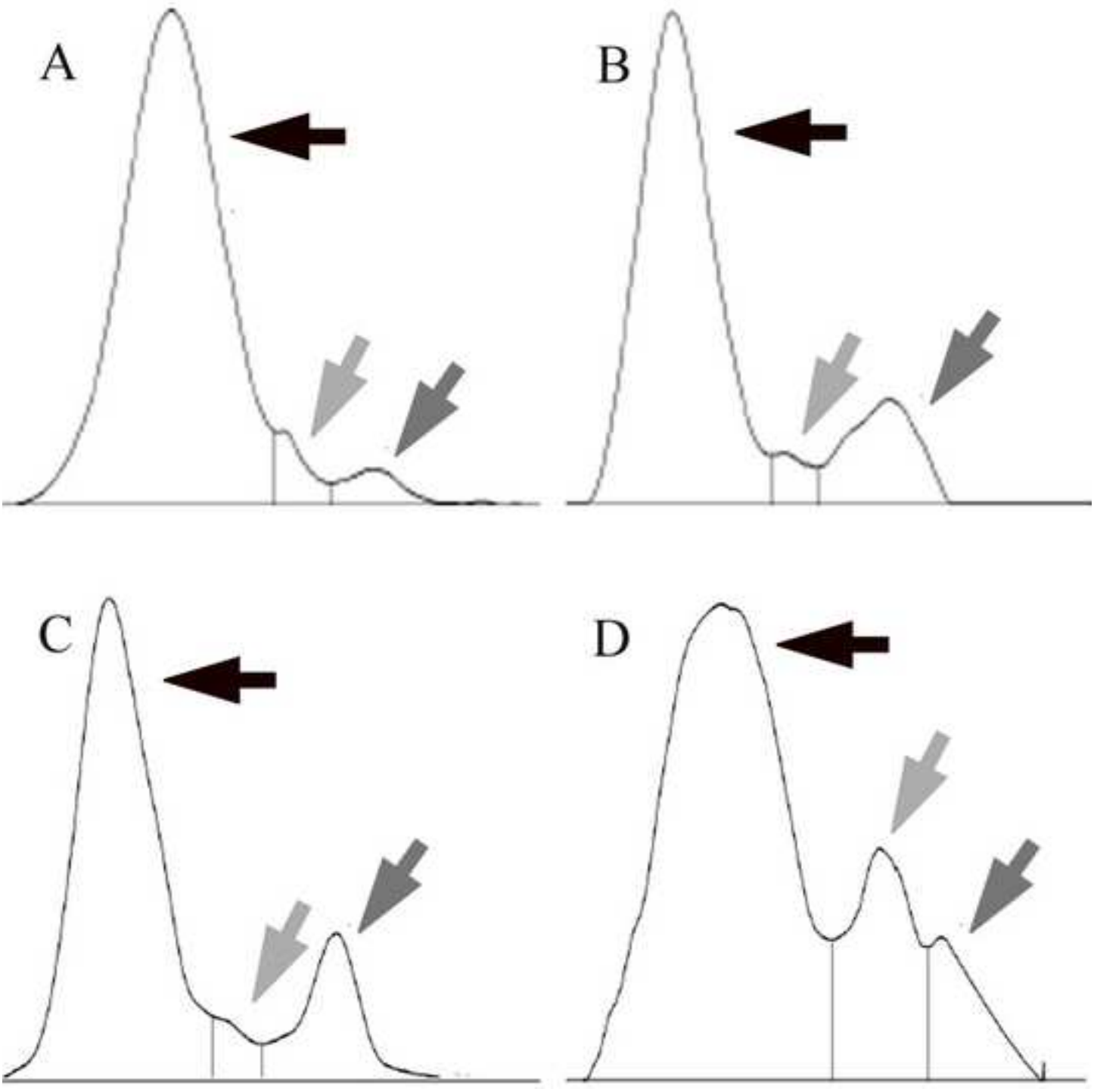

Figure 2

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