Received: 14 February 2017 | Revised: 7 June 2017 | Accepted: 11 June 2017
DOI: 10.1002/ajp.22683

RESEARCH ARTICLE

WILEY AMERICAN JOURNAL OF PRIMATOLOGY

Significant differentiation in the apolipoprotein(a)/lipoprotein(a) trait between chimpanzees from Western and Central Africa

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Funding information
Medical University of Innsbruck Research Project Fund, Grant number: P5030-011-011

Elevated Lipoprotein(a) (Lp(a)) plasma concentrations are a risk factor for cardiovascular disease in humans, largely controlled by the *LPA* gene encoding apolipoprotein(a) (apo(a)). Lp(a) is composed of low-density lipoprotein (LDL) and apo(a) and restricted to *Catarrhini*. A variable number of kringle IV (KIV) domains in *LPA* lead to a size polymorphism of apo(a) that is inversely correlated with Lp(a) concentrations. Smaller apo(a) isoforms and higher Lp(a) levels in central chimpanzees (*Pan troglodytes troglodytes* [PTT]) compared to humans from Europe had been reported. We studied apo(a) isoforms and Lp(a) concentrations in 75 western (*Pan troglodytes verus* [PTV]) and 112 central chimpanzees, and 12 bonobos (*Pan paniscus* [PPA]), all wild born and living in sanctuaries in Sierra Leone, Republic of the Congo, and DR Congo, respectively, and 116 humans from Gabon. Lp(a) levels were severalfold higher in western than in central chimpanzees (181.0 ± 6.7 mg/dl vs. 56.5 ± 4.3 mg/dl), whereas bonobos showed intermediate levels (134.8 ± 33.4 mg/dl). Apo(a) isoform sizes differed significantly between subspecies (means 20.9 ± 2.2, 22.9 ± 4.4, and 23.8 ± 3.8 KIV repeats in PTV, PTT, and PPA, respectively). However, far higher isoform-associated Lp(a) concentrations for all isoform sizes in western chimpanzees offered the main explanation for the higher overall Lp(a) levels in this

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subspecies. Human Lp(a) concentrations (mean 47.9 ± 2.8 mg/dl) were similar to those in central chimpanzees despite larger isoforms (mean 27.1 ± 4.9 KIV). Lp(a) and LDL, apoB-100, and total cholesterol levels only correlated in PTV. This remarkable differentiation between chimpanzees from different African habitats and the trait's similarity in humans and chimpanzees from Central Africa poses the question of a possible impact of an environmental factor that has shaped the genetic architecture of *LPA*. Overall, studies on the cholesterol-containing particles of Lp(a) and LDL in chimpanzees should consider differentiation between subspecies.

KEYWORDS
blood lipids, cholesterol, copy number variation, genetics, *Pan troglodytes*

1 | INTRODUCTION

Humans and their closest evolutionary relatives, the great apes, differ in susceptibility to develop atherosclerosis that can manifest as coronary artery disease. Elevated Lipoprotein(a) (Lp(a)) plasma concentrations are the strongest common genetically determined risk factor for cardiovascular disease (CVD) in humans (reviewed in Erqou et al., 2010), yet little is known about Lp(a) in chimpanzees. Comparative studies of Lp(a) in chimpanzees could add further insight into the pathophysiological mechanisms of human Lp(a), and possibly help to reveal the trait's still mysterious physiological function.

Lp(a) is a hepatic plasma lipoprotein consisting of a low-density lipoprotein (LDL) -like moiety that is covalently bound to apolipoprotein(a) (apo(a)), the specific protein that is encoded by the *LPA* gene. *LPA* (*Homo sapiens*: ENSG00000198670; *Pan troglodytes verus* [PTV]: ENSPTRG00000018770) is only found in old world monkeys and apes. It has evolved about 40 million years ago from a duplication of the neighboring plasminogen gene (*PLG*) (McLean et al., 1987). *LPA* has lost the kringle I, II, and III structures of *PLG*, while the kringle IV (KIV) has multiplied and diverged into then different types (KIV1–10), distinguished by their amino acid composition in humans (McLean et al., 1987). The KV and the protease domain of *PLG* are maintained, but the latter seems to have lost plasmin activity (McLean et al., 1987). The basic domain structure of *LPA* is shared between humans and chimpanzees (Chenivesse, Huby, Wickins, Chapman, & Thillet, 1998; Leibundgut et al., 2013).

LPA displays a size polymorphism due to a copy number variation (CNV) of its kringle IV-2 domain, with 1 to ~40 KIV-2 repeats per allele in humans. This KIV-2 CNV results in apo(a) isoforms of variable sizes. Both *LPA* alleles are expressed codominantly, but apo(a) size correlates inversely with Lp(a) plasma concentrations in humans (Sandholzer et al., 1991), chimpanzees (Doucet, Huby, Chapman, & Thillet, 1994), and other non-human primates (Azrolan, Gavish, & Breslow, 1991; Enkhmaa et al., 2015; Williams-Blangero & Rainwater, 1991). A causal mechanism underlies this correlation as the posttranslational retention time and rate of pre-secretory degradation of apo(a) depends on isoform size (Brunner et al., 1996; White, Hixson, Rainwater, & Lanford, 1994). However, other sequence variation within *LPA* alleles also influences individual Lp(a) levels, which may vary between individuals even for same-sized apo(a) isoforms (reviewed in Schmidt, Noureen, Kronenberg, & Utermann, 2016).

In all human populations studied so far, Lp(a) levels are highly heritable and mainly controlled by the *LPA* gene and range from <0.1 mg/dl to >200 mg/dl between healthy individuals. Mean levels between populations differ by a factor of two to three, with Sub-Saharan Africans displaying the highest concentrations (Sandholzer et al., 1991). In chimpanzees, Lp(a) levels were higher than in the human populations they were compared with (Doucet et al., 1994; Ronke et al., 2015). However, such comparisons only included European populations. Also, small apo(a) isoforms, that is, by convention with up to 22 KIV repeats (=the nine non-repetitive KIV plus 13 KIV-2), were found to be more abundant in non-human primates, including a small sample of central chimpanzees (*Pan troglodytes troglodytes* [PTT]) (Doucet et al., 1994; Enkhmaa et al., 2015). A small apo(a)/high Lp(a) profile has been shown to confer increased CVD risk in case of human *LPA* (Erqou et al., 2010).

A recent study on lineage-specific changes in 33 blood biomarkers also reported high, but quite diverse Lp(a) concentrations for different subspecies samples of wild-born chimpanzees (Ronke et al., 2015). The median Lp(a) level in western chimpanzees (PTV) was approximately four times higher than in central chimpanzees (PTT), and two and a half times higher than in bonobos (*Pan paniscus* [PPA]). However, that study did not investigate whether these differences in Lp(a) levels were related to variation of KIV-2 CNV sizes and hence to genetic differentiation at the *LPA* locus between subspecies, and neither had Lp(a) and other lipid biomarkers been analyzed for their possible correlation. Our study aimed to fill these gaps, and therefore we now assessed apo(a) isoform sizes and their association with Lp(a) concentrations in those three subspecies samples. We compare these results with those of humans from Gabon.

TABLE 1 Basic characteristics and biomarkers in three samples of chimpanzees, and humans from Gabon

(Sub-) species	*Pan troglodytes verus* (PTV)	*Pan troglodytes troglodytes* (PTT)	*Pan paniscus* (PPA)	*Homo sapiens* (HSA)
Country	Sierra Leone	Republic of the Congo	DR Congo	Gabon
Number of individuals	75[a]	112[b]	12	116
Age (years)				
Median (min–max)	7 (1.5–23)	9.75 (1–42)	5.5 (4–20)	33 (6–62)
Weight (kg)				
Median (min–max)	28.5 (9.9–58.5)	40 (10–72)	17 (7.8–45)	
Sex				
Females/males	43/32	49/63	6/6	64/52
Lp(a) (mg/dl)				
Median (min–max)	181.0 (61.0–303.7)	42.8 (0.9–279.3)	107.5 (3.5–371.8)	43.2 (1.0–155.2)
Mean ± SE	180.8 ± 6.7	56.5 ± 4.3	134.8 ± 33.4	47.9 ± 2.8
Skewness	−0.13	1.85	0.82	0.97
TC (mmol/L)				
Median (min–max)	5.07 (3.67–6.80)	4.62 (2.65–7.20)	5.74 (4.60–8.77)	
Mean ± SE	5.12 ± 0.09	4.74 ± 0.08	6.11 ± 0.38	
LDL-C (mmol/L)				
Median (min–max)	2.66 (1.61–4.11)	2.43 (0.73–4.83)	2.95 (2.01–6.06)	
Mean ± SE	2.64 ± 0.06	2.45 ± 0.06	3.24 ± 0.35	
HDL-C (mmol/L)				
Median (min–max)	1.67 (0.85–2.71)	1.56 (0.67–3.40)	1.87 (0.89–2.45)	
Mean ± SE	1.67 ± 0.05	1.61 ± 0.04	1.78 ± 0.13	
ApoB (g/L)				
Median (min–max)	0.70 (0.40–0.95)	0.59 (0.19–1.00)	0.82 (0.52–1.34)	
Mean ± SE	0.68 ± 0.01	0.58 ± 0.01	0.86 ± 0.08	
TG (mmol/L)				
Median (min–max)	1.13 (0.61–1.95)	1.04 (0.45–3.77)	1.14 (0.64–2.32)	
Mean ± SE	1.16 ± 0.04	1.10 ± 0.04	1.18 ± 0.12	
CRP (mg/L)				
Median (min–max)	0.53 (0.00–53.87)	2.05 (0.00–94.52)	9.18 (0.63–90.63)	
Mean ± SE	2.98 ± 1.01	6.57 ± 1.23	21.75 ± 8.41	

Results of the six pairwise tests on the distribution and the median of the total Lp(a) plasma concentrations each (by independent samples Mann–Whitney *U* test and median test, respectively) between groups are explained in the main text. Bonferroni adjusted threshold for *p*-values is 0.008. TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; apoB: apolipoprotein B-100; TG: triglycerides; CRP: C-reactive protein; min–max: minimum and maximum; SE: standard error (=standard deviation/$\sqrt{n}$). For one PTV and two PTT, no data for other lipid parameters than Lp(a) were available.

[a]Only for Lp(a), for all other $N = 74$.

[b]For TC, LDL-C, HDL, and TG $N = 110$. Data on weight were missing for one bonobo and one western chimpanzee each, and for 10 central chimpanzees.

2 | METHODS

2.1 | Samples

Sera from 112 PTT, 75 PTV, and 12 PPA were used for this study. Basic data on sex, age, and weight are given in Table 1. The chimpanzees were wild-born and live in Tchimpounga Sanctuary, Pointe Noire, Republic of the Congo (PTT), Tacugama Sanctuary, Freetown, Sierra Leone (PTV), and Lola Ya Bonobo Sanctuary, Kinshasa, Democratic Republic of the Congo (PPA). After overnight fasting, blood samples were collected under anesthesia from the femoral vein (for details see Ronke et al., 2015). Serum was separated before samples were transferred on dry ice to the laboratory at the university hospital in Leipzig, Germany. In 2011, an aliquot was transferred on dry ice to the Medical University of Innsbruck, Austria, for analyses of Lp(a) levels and apo(a) isoforms. Our laboratory in Innsbruck only analyzed a

subset of the samples described in the recent study by Ronke et al. (2015) Here, we report the results of all samples we have analyzed with the methods described in this manuscript.

For comparison, data from 116 unrelated individuals of a human population of Gabonese Bantu living in the rural rainforest area south of Lambaréné in the province of Moyen-Ogooué, Gabon, were included. This population was described in detail in a previous study (Schmidt, Kraft, Parson, & Utermann, 2006).

2.2 | Humane care guidelines and ethics

This research adhered to the American Society principles for the ethical treatment of primates. The chimpanzees were not subjected to any experimental procedures. Blood samples were left-over aliquots collected by veterinarians in 2007 and 2009 carrying out planned annual routine health checks. Authorization was obtained from the respective Ministries of Environment as well as by the Ministère de la Recherche Scientifique (DRC) to "Les Amis des Bonobos du Congo", and the Ministère de l'Enseignement Supérieur et de la Recherche Scientifique from the Republic of the Congo. The international transport of samples was authorized (CITES numbers: Uganda E-3520/05, Kenya E-1259/05, DRC E-0908/07, Republic of the Congo E-1274/07). The proposal that included this research (233297, TWOPAN) was approved by the European Commission. Human samples from Gabon were anonymized and informed consent was obtained from all participants during the sampling process in 1998/99, and the study on *LPA* had been approved by the ethics committees of the Medical Faculty of the University of Tuebingen, Germany (reference 08/98), of the Albert-Schweitzer-Hospital, Lambaréné, Gabon, and of the Medical University of Innsbruck, Austria (reference UN4485, 305/4.1).

2.3 | Lp(a) measurements

Lp(a) plasma concentrations were measured with an enzyme-linked immunosorbent assay (ELISA) as described elsewhere (Kronenberg et al., 2000). The monoclonal 1A2 antibody against apo(a) used as the blotting antibody in the Western blots and as the detection antibody in the ELISA targets the epitope SRTPEYYPNAGL on the KIV-2 domain of humans (Dieplinger et al., 1995). This epitope is also recorded in the KIV-2 orthologous region of PTV. For PTT, no reference sequence for the complete KIV-2 orthologs is released, and for PPA, no sequence data exist beyond KIV-1. The antibody does not cross-react with plasminogen in humans, and the crucial binding epitope YYPN is not encoded in *PLG* in either humans or published chimpanzee sequences (PTV and PTT). However, as in humans, it is also present in chimpanzee apo(a) KIV types 3, 4, and 6–8. A peptide search of the UniProt database for *P. troglodytes* (based on the PTV reference sequence) for the YYPN motif did result in 33 matches outside *LPA*, but none of these in plasma proteins and all also recorded in the respective human orthologs. Compared to assays relying on a non-repetitive epitope in apo(a), assays using antibodies that recognize multiple epitopes in apo(a) have been reported to underestimate Lp(a) concentrations associated with small apo(a) isoforms (18 KIV) by about 10%, and to overestimate those of large isoforms (30 KIV) by about 35% (Marcovina, Albers, Gabel, Koschinsky, & Gaur, 1995).

2.4 | Measurements of apo(a) isoform size and apo(a) isoform-associated Lp(a) levels

Apo(a) isoforms were determined for 198 of the 199 samples. For one PTT, no sample was left after Lp(a) quantification. Apo(a) isoforms were detected on Western blots after SDS gel electrophoresis with the described 1A2 antibody. A human apo(a) isoform size standard composed of isoforms with 13, 19, 23, 27, and 35 KIV repeats was applied on six out of the 36 lanes on each gel. The primary antibody was visualized with a horseradish peroxidase-conjugated goat-anti-mouse IgG antibody. Two exposures on photographic film of the chemiluminescent detection were made. All blots were then evaluated by two investigators (K.S. and A.N.). In order to increase the detection of low expressed isoforms, any sample that showed only one band in the standard application of 30 ng Lp(a) was run on a second gel with a larger amount of sample (120 ng) loaded. Only for four samples, this resulted in the detection of a second isoform. The method was shown to detect apo(a) isoforms associated with Lp(a) levels below 0.5 mg/dl (Schmidt et al., 2006); however, samples where one isoform is associated with a 50 times lower Lp(a) concentration than the predominant isoform are likely to escape detection due to oversaturation of the blot with background signals. For samples with two clearly spaced apo(a) isoforms, the proportional signal intensity of the bands was used to calculate apo(a) isoform-associated Lp(a) levels as described (Kronenberg et al., 2000). In the human samples, KIV-2 CNV genotyping by pulsed-field electrophoresis (PFGE) allowed to distinguish size homozygotes and carriers of one non-expressed or null allele. Still, for cross-species comparison of isoform-associated Lp(a) levels, single band phenotypes were excluded for all groups.

2.5 | Measurement of other biomarkers

For the chimpanzees, levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), apolipoprotein B-100 (apoB), triglycerides (TG), and C-reactive protein (CRP) were available from measurements as previously described in detail (Ronke et al., 2015).

2.6 | Statistics

Lp(a) concentrations and apo(a) isoform size were compared pairwise between groups with non-parametric tests (independent samples median and Mann–Whitney *U* tests). Contributions of the apo(a) size polymorphism to the variance of Lp(a) levels were calculated from Pearson's correlation coefficients of square root-transformed apo(a) isoform-associated Lp(a) levels, which best fulfilled assumptions for linear regression analysis. Statistical analyses were conducted with SPSS 22. Where indicated, significance is evaluated after Bonferroni-correction for multiple testing. The observed apo(a) isoform frequency distributions were tested population-wise for Hardy–Weinberg

equilibrium (HWE) with Arlequin 3.5.2.2 (Excoffier & Lischer, 2010). In the absence of genotype data, it was assumed that the observed phenotypes equaled those of the genotype, that is, single-banded phenotypes were entered as size homozygotes.

2.7 | Sequence comparisons

Genomic sequences were compared between (sub-)species using the *LPA* references sequence for humans (HSA; ENSG00000198670, GRCh38.p3) and PTV (ENSPTRG00000018770; CHIMP2.1.4.), the corresponding scaffold sequence from PPA (NCBI Reference Sequence: NW_014013999.1), and the published partial sequence for PTT from cloned samples from Gabon (Chenivesse et al., 1998; Huby et al., 2001). Positioning of bases and amino acids follows the human sequence with six KIV-2 copies. To comply with HGVS standards nomenclature, bases were counted from the ATG start codon. This deviates from alternate labeling of positions in the promoter (Huby et al., 2001) or a cloned apo(a) cDNA (McLean et al., 1987).

3 | RESULTS

3.1 | Lipoprotein(a) plasma concentrations in the three samples of chimpanzees

With 181 mg/dl, mean and median Lp(a) levels were three and four times higher in PTV than in PTT, respectively, with the much smaller sample of PPA lying in between these two samples of chimpanzees (Table 1). In all three samples, individuals with very high Lp(a) concentrations (>100 mg/dl) were observed, but these were rare in PTT while in PTV, all individuals had Lp(a) levels higher than 60 mg/dl (Figure 1). Sixty percent of PTT and 40% of PPA had lower Lp(a) concentrations than any PTV, respectively. Individuals with an Lp(a) concentration below 10 mg/dl were seen in PPA and especially in PTT, and for PTT, the frequency distribution of Lp(a) levels was skewed toward lower concentrations. However, considering multiple testing, significance was only reached for the differences in distributions and medians between PTT and PTV ($p < 0.001$) (Table 1). Distribution and median of total Lp(a) plasma concentrations in the 116 Gabonese Bantu were very similar to those of PTT ($p = 0.361$ and $p = 0.845$) (Table 1 and Figure 1), but significantly different to those in PTV ($p < 0.001$).

3.2 | Apo(a) isoforms

On Western blots, two bands indicating the presence of apo(a) isoforms of different size were observed in 76%, 82.6%, and 58.3%, of the samples from PTV, PTT, and PPA, respectively. Correspondingly, frequencies of single-band phenotypes were 24%, 17.4%, and 41.7%. The latter can either reflect homozygosity for apo(a) isoforms of the same size, or indicate the presence of one non-expressed allele, respectively an isoform associated with very low Lp(a) concentration not passing the detection threshold. If single-band phenotypes were indeed size homozygotes, a moderate deviation from HWE would result. In all three samples, the observed heterozygosity was lower than the expected one (0.760 versus 0.855, $p = 0.027$ for PTV; 0.811 versus 0.932, $p = 0.013$ for PTT; 0.583 versus 0.873, $p = 0.156$ for PPA).

Considering only double-band phenotypes, the distribution of isoform sizes was significantly different between PTV and both other samples. Mean isoform size was slightly smaller in PTV, and small apo(a) isoforms with up to 22 KIV repeats were considerably more abundant in PTV (77.2%) than in PTT (52.8%) (Figure 2 and Table 2). Isoforms size range was narrower in PTV than PTT or PPA, with the largest isoform detected in PTV at 28 KIV repeats, and in PTT 15.6% of all isoforms being larger than that. Large isoforms with 28 KIV and 36 KIV were also found in PPA. With 10–37 KIV repeats, the apo(a) isoform size range of Gabonese Bantu, as observed on Western blots, slightly exceeded that seen in any of the three samples of chimpanzees, but was more similar to PTT and PPA than to PTV (Figure 2). However, mean and median apo(a) isoform size (27.1 and 28 KIV repeats) in Gabonese were markedly larger than in any chimpanzee subspecies, and all pairwise comparisons showed a highly significant difference (Table 2).

3.3 | Apo(a) isoform-associated Lp(a) concentrations

We could estimate apo(a) isoform-associated Lp(a) levels for 98 isoforms (65.3% of all alleles) from PTV, 172 isoforms (70.5%) from PTT, and 12 isoforms (50%) from PPA. In all three samples, isoform-associated Lp(a) concentrations varied considerably for same sized isoforms with similar variances (Figure 3). However, isoform-associated Lp(a) levels were decidedly higher in PTV than in PTT throughout the apo(a) size range, especially for very small apo(a) isoforms. The inverse correlation of Lp(a) levels with apo(a) isoform size was weakest in PTT (Spearman's rho for PTV: −0.636, PTT: −0.459, PPA: −0.735, all at least $p < 0.006$). Apo(a) size explained 37.1% of the variance in isoform-associated Lp(a) levels in PTV, but only 21.3% in PTT, and 52.9% in the very small set of PPA (all at least $p < 0.007$). A clear drop in average Lp(a) concentrations for large apo(a) isoforms in PTT can be seen in the graphical display of the distribution of apo(a) isoform-associated Lp(a) levels (Figure 3), while the inverse correlation appears very weak over a wide range of small to intermediate isoform sizes. Considering only the size range up to 28 KIV repeats, for which we observed isoforms in all three chimpanzee samples, the differences between them were more pronounced. Here, apo(a) size explained only 9.5% in PTT ($N = 144$ isoforms) compared to the 37.1% in PTV ($p < 0.001$ for both), and 43.8% for PPA ($N = 11$ isoforms, $p = 0.027$).

In the 84 samples of Gabonese Bantu suitable for this comparison (see section 2), apo(a) isoform size correlated strongly with isoform-associated Lp(a) levels (Spearman's rho = −0.664, $p < 0.001$) and explained 39% of the variation in Lp(a) concentrations ($p < 0.001$). However, as in PTT, for isoform sizes up to 28 KIV, this correlation was much weaker, explaining only 11.7% ($p < 0.001$). Still, besides isoform size, species is an additional significant contributor to the variance in isoform associated Lp(a) levels (univariate analysis of variance, $p < 0.001$).

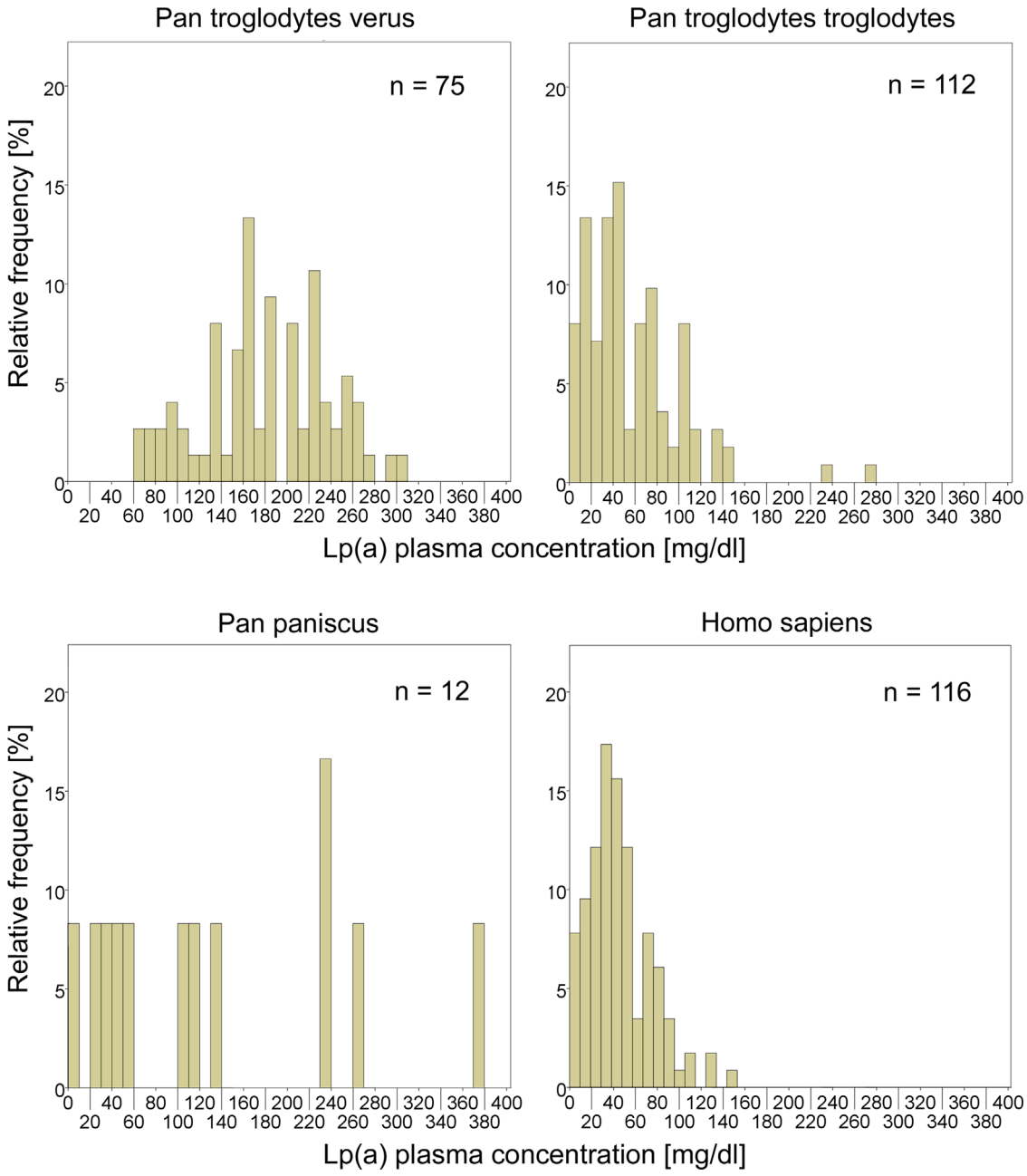

FIGURE 1 Distribution of lipoprotein(a) plasma concentrations. Shown is the frequency distribution of Lp(a) plasma concentration in mg/dl as measured by the 1A2 ELISA in the different (sub-)species of chimpanzees and in humans from Gabon

3.4 | Potential bias from null alleles and low-expressed alleles

Without information on the actual KIV-2 CNV genotypes, single-band phenotypes pose a problem for the analyses of HWE and isoform-associated Lp(a) levels. In humans, *LPA* alleles without detectable isoforms in plasma occur across the whole size range of isoforms. In the Gabonese, 11 (4.7%) of such null alleles were observed among 232 *LPA* alleles analyzed by PFGE and Western blots (Figure 2).

In theory, all single-band phenotypes could carry one null allele, thus maximal frequencies of null alleles were 12% and 8.7% in PTV and PTT, respectively (we excluded PPA from this analysis due to small sample size). However, size homozygotes for apo(a) isoforms are expected under HWE as well. If the populations were indeed in HWE and only null alleles were accountable for the observed excess of homozygosity in the simplified HWE model, then 9.5% and 6.8% of alleles were expected to be actual null alleles in PTV and PTT, respectively. Consequently, 0.9% and 0.5% of samples could be expected to be homozygous for null alleles in these subspecies. This would equal an expected observation of null homozygotes in less than one sample each. Thus the lack of observation of samples with undetectable Lp(a) does not argue against the presence of null alleles in any of the subspecies, and null alleles could indeed explain the calculated deviation from HWE.

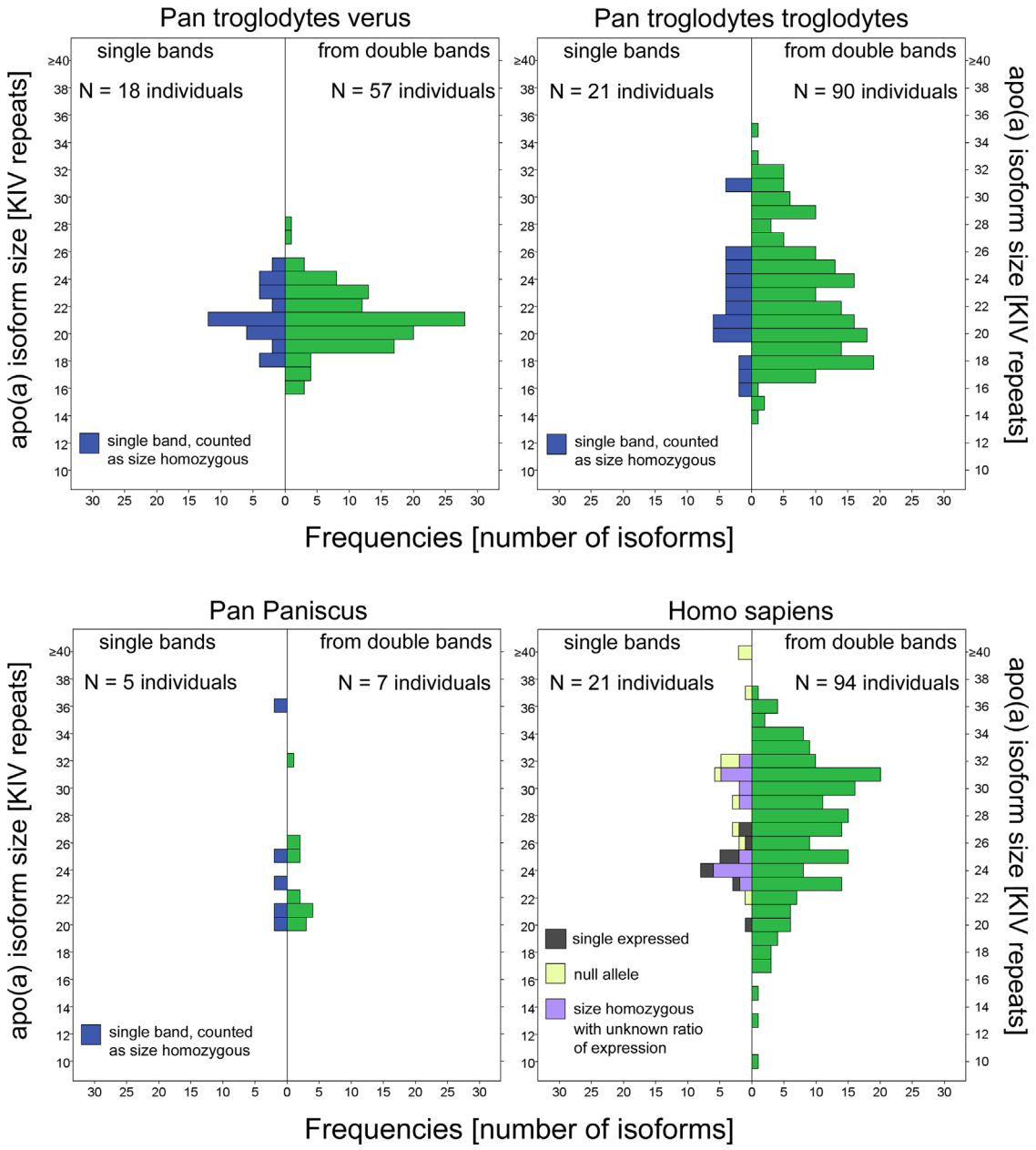

FIGURE 2 Frequency of apo(a) isoforms. The count of apo(a) isoforms as detected on Western blots is given for samples with two isoforms of different size (right side, in green) and those with only one band (left side). In this figure, single-band phenotypes are depicted as size homozygotes in chimpanzees. In humans, KIV-2 CNV genotypes allowed differentiating between single-band phenotypes caused by size homozygosity and those deriving from the presence of one allele without corresponding apo(a) isoform in plasma ("null allele")

34.7% of PTV and 22.5% of PTT were excluded from the analysis of isoform-associated Lp(a) concentrations because they exhibited single-band phenotypes or two isoforms of neighboring size. If many isoforms with very high Lp(a) levels from PPT and with very low Lp(a) levels from PTV were among these single-band samples, the two subspecies might be more similar than seen in the subset of heterozygotes with densitometric data described above. To investigate for such an effect, we assumed that in samples without densitometric data, both isoforms were expressed equally and carried half of the total Lp(a) concentration. When plotted against the measured apo(a) isoform-associated Lp(a) concentrations from the other samples with actual densitometric data, isoform-associated Lp(a) levels appear similarly scattered for both sets of samples (Supplemental Figure S1).

3.5 | Comparison of *LPA*/apo(a) sequences between (sub-)species

Within the minimal promoter region spanning in *LPA* from nucleotide positions −239 to −1, none of the referenced chimpanzee sequences differed from each other at positions −144, −143, −134 which had been described as being essential for a fivefold higher transcriptional

TABLE 2 Apo(a) isoforms in the three samples of chimpanzees and in humans from Gabon

	Apo(a) isoform distribution									
	Double-band phenotype					Single-band phenotype				
	Number of individuals	Mean ± SD	Median	Min–max	Skewness	Number of individuals	Mean ± SD	Median	Min–max	Skewness
PTV	57	20.9 ± 2.2	21	16–28	0.290	18	21.2 ± 2.0	21	18–25	0.211
PTT	90	22.9 ± 4.4	22	14–35	0.502	21	22.7 ± 3.9	22	16–31	0.567
PPA	7	23.8 ± 3.4	21.5	20–32	1.535	5	25 ± 6.1	23	20–36	1.767
HSA	94[a]	27.1 ± 4.9	28	10–37	−0.503	21[b]	26.6 ± 3.4	25	20–32	0.291

SE: standard deviation; pairwise tests (p value for: independent samples median test, Mann–Whitney U test; only tested for double band phenotypes) all $p \leq 0.001$ except PTV versus PPA $p = 0.349$; $p = 0.026$ and PTT versus PPA: $p = 0.580$; $p = 0.675$ and PPA versus HSA: $p = 0.004$; $p = 0.001$. Frequencies and distributions of apo(a) isoform for samples with two and one band seen on immunoblots.

[a]For one Gabonese, only a smeary signal was detected on the Western blot, and for one PTT, plasma was lacking for Western Blotting.

[b]PFGE showed that 11 of these 42 alleles are actually null alleles and only 10 individuals are size homozygotes, the null alleles are excluded from the values given here.

activity of the chimpanzee promoter from PTT compared to humans (Huby et al., 2001) (Figure 4a). PTV and PTT differ from each other at five further nucleotide positions (−1,250, −1,118, −703, −469, and, by a synonymous substitution, +30G), and here two (−1,118 and −469) are shared between PTV and humans. The PPA promoter shows several more bases which appear to be specific for this subspecies.

Within the available coding sequence from KIV-3 to the protease domain, the amino acid compositions of PTV and PTT differ at 33 positions in the same way from humans. In further two amino acids, PTT differs from both PTV and humans, and in another amino acid, PTV from PTT and humans (Figure 4b). For the region covered in the PTV sequence between the KIV-1 and the KIV-3 orthologs, which by the relative position within the gene corresponds to the KIV-2 CNV in human *LPA*, three different amino acid sequences are encoded by the four domains included (Figure 4c). Only the first two copies are identical. Other than that, the copies all diverge in six to seven amino acids from each other, with these differences being dispersed over 10 of the domain's 114 amino acids. In comparison, the corresponding KIV-3 domain also deviates at six to eight amino acids from those KIVs. For the human reference sequence, no variation is reported between any of its six KIV-2 copies. No sequence data for this region are available for PPA, and for PTT, only the last unit has been partially included in cloned cDNA (Chenivesse et al., 1998). This partial sequence of 30 amino acids is identical to its ortholog in humans but differs from all four corresponding sequences in PTV in at least one position.

3.6 | Impact of factors other than *LPA*

While our hypothesis was that *LPA* was responsible for differences in Lp(a) levels between chimpanzee subspecies, we also explored our data for other parameters. No significant difference in either Lp(a) plasma concentrations or apo(a) isoform distributions or medians were detected between sexes in any of the subspecies ($p > 0.05$ for all comparisons). No biomarker covering hepatic and kidney function implied renal disease or impaired hepatic function (for details see Ronke et al., 2015). However, C-reactive protein was significantly higher in PTT and PPA (Table 1).

In all chimpanzee subspecies, other lipid parameters were in the range of normolipidemic human subjects (Table 1). Pairwise comparisons between subspecies yielded the following significant differences (30 tests, thus threshold $p < 0.0017$): PTT versus PTV for apoB (median and distributions), and PTT versus PPA for apoB and TC (distributions).

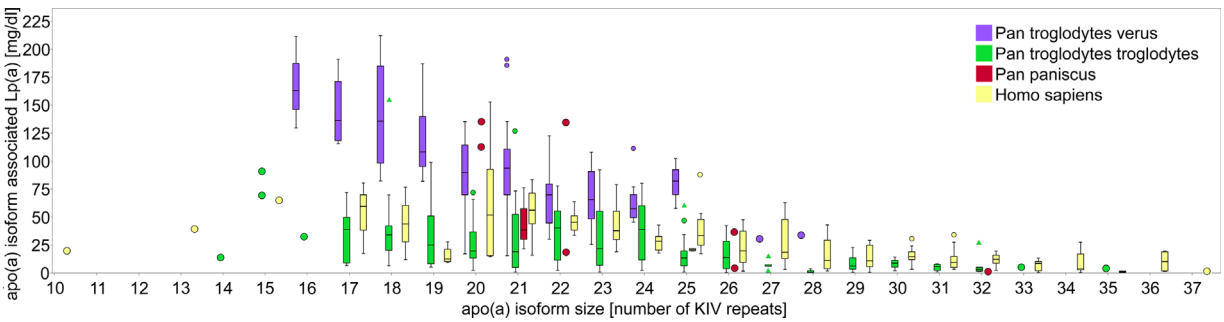

FIGURE 3 Apo(a) isoform-associated Lp(a) concentrations measured in heterozygous samples. Boxes give medians and the 25 and 75 percentiles of densitometrically measured isoform-associated Lp(a) concentrations, whiskers show minimum and maximum values that are not statistically outlying. For apo(a) size categories with only one or two observations per (sub-)species, data points are entered as large circles. Smaller circles show outliers, and triangles extremes. Single-banded phenotypes were excluded for all (sub-)species

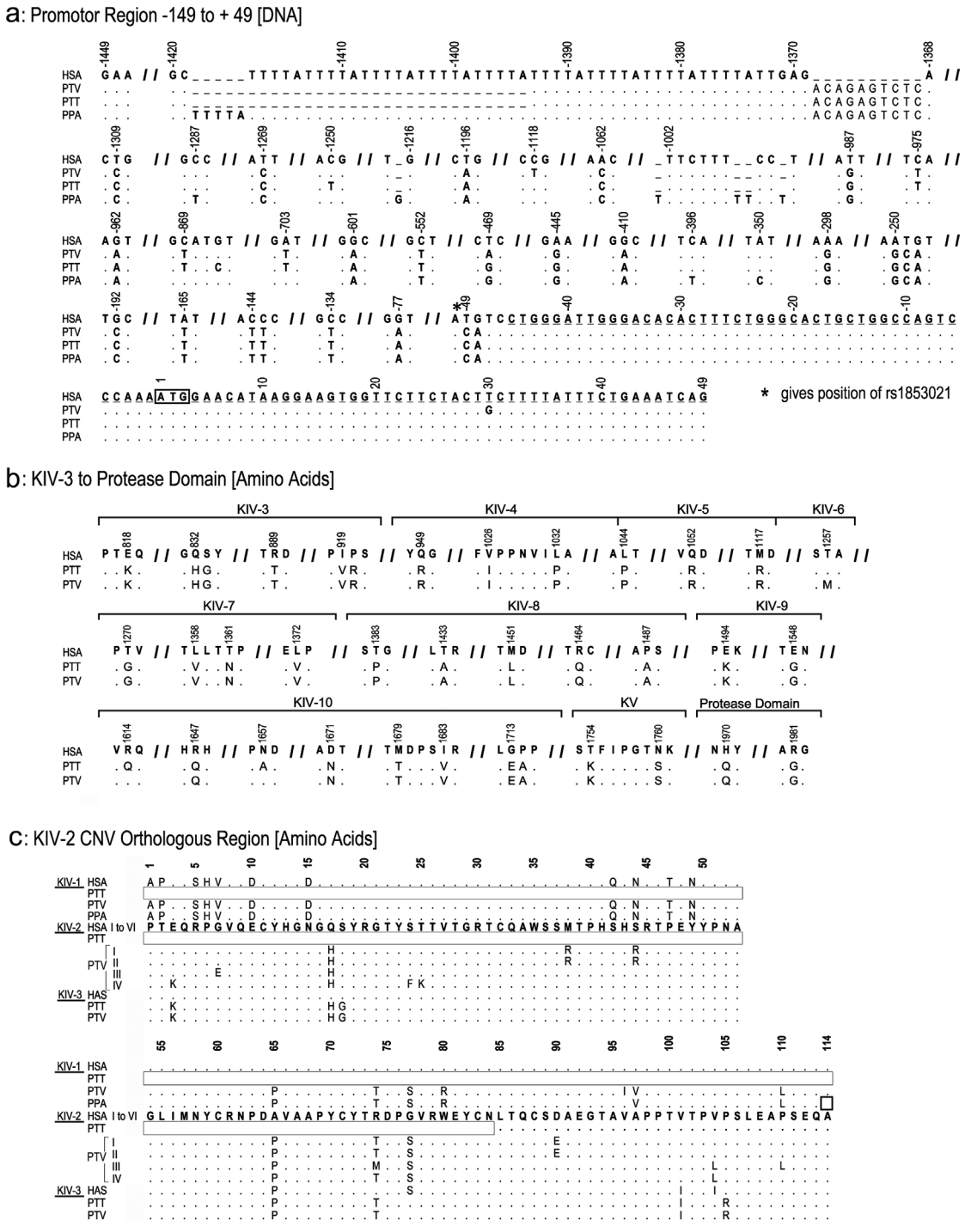

FIGURE 4 Comparison of *LPA* sequences from chimpanzees and humans. Available DNA respectively amino acid sequences from chimpanzees are compared with the human reference sequence. Labeling of positions is according to the start codon (black solid box, panel a). Diverging bases/amino acids ± at least one position are depicted. (a) Promoter region. Note that the human reference sequence contains the variant allele of dbSNP rs1853021, which introduces an additional start codon reducing proper transcription due to an in-frame stop-codon, and that its number of TTTTA repeats at −1,372 to −1,418 is one higher than the most common allele in humans. (b) No sequence for PPA is available for the region of KIV-3 to the protease domain. (c) The KIV-2 domains are encoded by two exons (160 and 182 bp). In humans, but not in PTV, KIV-1 exon 2 and KIV-3 exon 1 are homologous to the corresponding KIV-2 exons, and all KIV-2 copies (I–VI) share the same amino acid sequence

TABLE 3 Correlation between lipid parameters within the two samples of common chimpanzees

		Lipid parameter 1						
		Lp(a)	LDL-C	ApoB	TC	HDL-C	TG	
Lipid parameter 2	Lp(a)		0.39	0.38	0.227*			
	LDL-C			0.88	0.87	0.34**		
	ApoB		0.83		0.71			
	TC		0.86	0.70		0.70		
	HDL-C		0.38		0.67			
	TG		−0.26***			−0.32		In PTV
							In PTT	

Spearman's rank correlation coefficients rho for different lipid parameters of individuals (given as "lipid parameter 1" and "lipid parameter 2"), analyzed separately for subspecies. Upper/right panel: *Pan troglodytes verus* (PTV); lower/left panel: *Pan troglodytes troglodytes* (PTT). 2 × 15 tests were conducted, Bonferroni threshold for significance is $p < 0.0017$. Results are only shown for rho with $p \leq 0.05$. $p < 0.001$ except $^{*}p = 0.052$, $^{**}p = 0.003$, $^{***}p = 0.005$. Lp(a): lipoprotein(a); LDL-C: low-density lipoprotein cholesterol; apoB: apolipoprotein B-100; TC: total cholesterol; HDL-C: high-density lipoprotein cholesterol; TG: triglycerides.

Several further differences missed significance only due to Bonferroni correction, among them those of PTT versus PTV as well as PPA for the distribution of LDL ($p = 0.027$ and 0.020, respectively) and PTT versus PTV for TC (distribution and median; $p = 0.002$ and 0.017, respectively) (Supplemental Table S1). While correlations between LDL-C, TC, apoB, and HDL were all nearly identical in PTV and PTV, only in PTV a significant correlation of intermediate strength was found for Lp(a) levels with LDL-C, TC, and apoB plasma concentrations (Table 3). However, our measurements of LDL-C did not differentiate between LDL-C deriving from Lp(a) and LDL particles. Based on data on the lipid composition of Lp(a) from Gabonese PTT (Doucet et al., 1994), we have calculated "corrected LDL-C" (cLDL-C) by subtracting 40% of the Lp(a) mass from the total LDL-C (converted from mmol/L to mg/dl using the human conversion factor of 38.6) for each individual and compared the results for PTT and PTV (Figure 5). In PTV, a larger proportion of LDL-associated cholesterol appears to be carried by Lp(a) than by LDL particles.

4 | DISCUSSION

Our study demonstrates considerable differentiation between chimpanzee subspecies in the apo(a)/Lp(a) trait. It also shows that the previous observation of higher Lp(a) concentrations in chimpanzees than in humans cannot be generalized, as contrary to the comparison of a small sample of ($N = 29$) PTT from Gabon with humans from Europe (Doucet et al., 1994), humans from Gabon and PTT from the Republic of Congo have virtually identical Lp(a) levels and distributions. However, among the three subspecies of chimpanzees, PTV from West Africa display a far more pronounced high Lp(a) level/small apo(a) isoform profile, setting this subspecies apart from both humans and chimpanzees from Central Africa. The bonobos (PPA) show, even in our small sample, broad variation in both Lp(a) levels and apo(a) isoform sizes, for both covering the total range observed in the chimpanzees. It is noteworthy that our sample of PTT displays similar mean Lp(a) concentrations as that previous sample of PTT from Gabon measured by Doucet et al. (1994) with a different assay (56.5 mg/dl vs. 61 mg/dl). While comparisons between Lp(a) assays are known to be problematic, and furthermore our assay is slightly isoforms size dependent as the number of epitopes varies accordingly (see section 2), the latter effect could not explain the threefold higher mean Lp(a) concentration in PTV compared to PTT. Taken together, we are confident that our results are indeed reflecting a massive differentiation in the apo(a)/Lp(a) trait between subspecies.

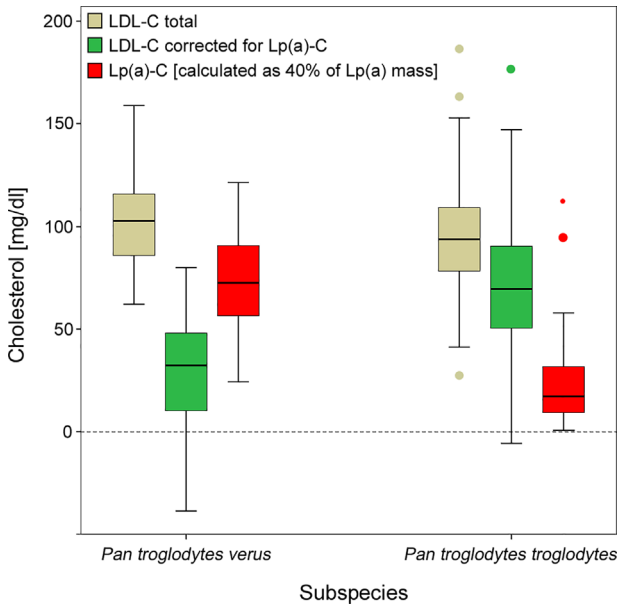

FIGURE 5 Total LDL cholesterol and calculated Lp(a) cholesterol. Shown are the plasma concentrations of total LDL-C and corrected, that is, non-Lp(a) associated LDL-C, as well as Lp(a)-C, that is, cholesterol assigned to Lp(a), for the two *Pan troglodytes* subspecies. LDL-C was converted from mmol/L to mg/dl applying the human conversion factor of 38.6. Lp(a)-C was calculated assuming 40% of the measured Lp(a) mass is cholesterol. LDL-C was then calculated by subtracting Lp(a)-C from total LDL-C in each individual. Boxes give medians and the 25 and 75 percentiles, whiskers show minimum and maximum values that are not statistically outlying. Larger dots show outliers, smaller dots extremes

4.1 | General lipid profile

While all measured concentrations of lipid parameters were higher in PTV than PTT, it is remarkable that mean LDL-C and mean TC levels were similar in both subspecies, with a difference of only 7.5 mg/dl and 15 mg/dl between the samples, respectively, compared to the mean Lp(a) concentration being 124.3 mg/dl higher in PTV than in PTT. Lp(a) is composed of one molecule of apo(a) and one LDL-particle and the latter is included in LDL measurements. On the other hand, our Lp(a) values are actually based on the measurement of apo(a). For PTT, the fraction of free apo(a), that is, apo(a) circulating in plasma not attached to an LDL-like particle, has been reported to be low (Doucet, Wickings, Chapman, & Thillet, 1998), and the lipid composition of Lp(a) to be similar to that of humans (Doucet et al., 1994), with 40% of particle mass being cholesterol. If these two assumptions were applied to all of our samples, this would result in far higher proportions of the total and LDL cholesterol circulating as Lp(a) in PTV than in PTT. In principle, such a shift of the LDL/Lp(a) particle ratio is in line with published results from density gradient ultracentrifugation of plasma from PTT with comparatively high Lp(a) levels (Doucet et al., 1998). However, it remains possible that the LDL-like moiety of Lp(a) is carrying less than 40% cholesterol or that the concentration of free apo(a) is high in some individuals. Furthermore, variation in LDL densities has also been reported for *P. troglodytes* of undisclosed subspecies (Chapman, Forgez, La-grange, Goldstein, & Mills, 1984). Nonetheless, the fact that we see a significant positive correlation of Lp(a) concentrations with LDL-C, TC, and apoB-100 in PTV but not in PTT also indicates that in PTV, the contribution of Lp(a) to cholesterol and apoB is high, while in PTT, this is not the case. So far, PTT are better studied in respect of the biophysical properties of Lp(a) (Doucet et al, 1994, 1998). Our results highlight the importance to include information on chimpanzee subspecies in similar studies. Altogether, the massive differences in Lp(a) values between PTT and PTV appear to represent true differentiation between subspecies in Lp(a) phenotypes. These differences might be caused by genetic differentiation or by environmental effects.

4.2 | Non-genetic factors

Non-genetic factors known to impact Lp(a) levels in humans seem an unlikely cause for the impressive differences in Lp(a) concentrations between chimpanzee subspecies, assuming that the same factors played a role in chimpanzees. Biomarkers neither indicated a bias by renal disease, known to increase Lp(a) levels (Kronenberg et al., 2000), nor by hepatic failure, reducing apo(a) synthesis (Geiss, Ritter, Richter, Schwandt, & Zachoval, 1996). While it is debated to which extent apo(a)/Lp(a) reacts as an acute phase protein (Anuurad et al., 2008) and CRP was significantly different between all three samples of chimpanzees, CRP levels did not indicate a bias by acute inflammation. Some data had suggested an impact of diet on Lp(a) concentrations in monkeys (Rainwater, Kammerer, & VandeBerg, 1999), but diet was also similar for all our samples (Ronke et al., 2015). This notwithstanding, a highly significant difference ($p < 0.0001$) in Lp(a) levels between wild-born PTV in Sierra Leone ($N = 121$, including our 112 samples), and PTV held in captivity in Germany ($N = 19$) was reported (Ronke et al., 2015). However, all the latter samples had been measured by a different assay (immuno-turbidimetric Tina-quant Lp[a], Roche, Diagnostics Deutschland GmbH, Mannheim, Germany) that in direct comparison was shown to underestimated the very high Lp(a) concentrations in PTV from Sierra Leone.

4.3 | Genetic factors affecting Lp(a) levels

While a high heritability of Lp(a) levels has been found in all human populations and could always be allocated primarily to the *LPA* locus, no such studies exist for chimpanzees. However, Lp(a) plasma levels seem to be highly heritable in baboons (Rainwater, Manis, & VandeBerg, 1989). Furthermore, the causal effect of the KIV-2 CNV size on Lp(a) levels had also been established in baboon cell lines (White et al., 1994). Thus, it appears likely that the general inverse correlation of apo(a) size and Lp(a) levels that we described in all three chimpanzee samples is genetically determined by the size of the KIV-2 CNV, with the narrower size spectrum of apo(a) isoforms and the higher frequency of small isoforms in PTV than in PTT and PPA being of genetic origin. We are confident that the observed phenotypic distributions of apo(a) isoform sizes reflect the actual genetic distribution of KIV(-2) CNV alleles as any potential bias by very low expressed isoforms appears unlikely when transferred to sample numbers. Still, apo(a) isoform-associated Lp(a) concentrations show clearly that the difference in Lp(a) levels between subspecies are not explained by the higher frequency of small apo(a) isoforms in PTV alone, but that in PTV these are also associated with far higher Lp(a) plasma concentrations than in PTT.

Thus, it remains to be determined what causes such deviation in isoform-associated Lp(a) concentrations, additionally to the differences in the strength of the inverse correlation between apo(a) size and Lp(a) levels, and what might explain the apparently different apo(a) size spectra. Actually, the same questions exist regarding apo(a)/Lp(a) in human populations from different continents (Sandholzer et al., 1991).

4.4 | Overall genetic differentiation between chimpanzees

In general, genetic differentiation between chimpanzee (sub-) species is at least 15-fold higher ($F_{ST} = 0.68$ and 0.49 for PPA versus PTV and PTT, respectively, and $F_{ST} = 0.29$ between PTT and PTV) (Fischer, Pollack, Thalmann, Nickel, & Paabo, 2006) than between human Sub-Saharan African populations ($F_{ST} = 0.019$) (Gurdasani et al., 2015) or at least double that seen between the most distant human continental groups ($F_{ST} = 0.15$) (Fischer et al., 2006). This can be explained by divergence times (Hey, 2010) and

by limited, unidirectional gene flow between chimpanzee subspecies (Wegmann & Excoffier, 2010). PTT are genetically most diverse with the largest effective population size (Prado-Martinez et al., 2013); nucleotide diversity within PTT ($\pi = 0.19\%$) is considerably higher than in PPA or PTV ($\pi = 0.10\%$ and 0.08%, respectively) (Fischer et al., 2006). The smaller effective population size in PTV might also result in less variability of KIV-2 CNV and thus apo(a) sizes, and less genetic diversity concerning single-nucleotide polymorphisms (SNPs) in *LPA* could explain the stronger inverse correlation between apo(a) isoform size and Lp(a) levels in PTV, as in addition to the KIV-2 CNV, SNPs in *LPA* are known to affect Lp(a) levels by different mechanisms (reviewed in Schmidt et al., 2016). However, we observe a similar variance of isoform associated Lp(a) concentrations across subspecies for the same apo(a) sizes, but in PTV the mean values are far higher and decrease steadily with increasing apo(a) size, while in PTT means hardly follow an inverse correlation over the small and intermediate apo(a) size range—another characteristic they share with the human population from Gabon, for whom the strong genetic control of Lp(a) levels by the *LPA* gene has been confirmed by a family study (Schmidt et al., 2006). Thus, not only total genetic diversity in terms of additional SNPs in *LPA* but also their association with KIV-2 CNV size had to be considered. Currently, no such data exist for chimpanzees.

The available sequence data did not reveal major differentiation between subspecies for apo(a) domains that were related to Lp(a) assembly in humans (Koschinsky & Marcovina, 2004) or for sites with previously identified functional effects within the promoter region (Huby et al., 2001) which could explain the widely different Lp(a) concentrations. Variation in a more distant enhancer region (Huby et al., 2003) could not be compared as sequence data for PTT do not extend that far. Still, except for the promoter region in PTT with $N = 50$ (Huby et al., 2001), sequence data for either subspecies are currently restricted to that of single or very few individuals. As we also observed PTT with very high and PTV with comparatively low Lp(a) plasma levels, the sparse genetic data might derive from individuals that are not typical for their subspecies.

A promising target for further studies might reside within the KIV-2 CNV itself. Considerable differentiation between the four KIV copies composing the KIV-2 CNV orthologous region is already reported in the reference sequence of one single PTV. In contrast, the partial sequence described for PTT is identical to the human KIV-2 but deviates from all its orthologs in PTV. We have recently described new sequence variation in the KIV-2 copies of humans, especially in Africans, some with a likely impact on Lp(a) concentrations (Noureen, Fresser, Utermann, & Schmidt, 2015). This genomic region is still difficult to investigate due to the sequence homology of the KIV-2 copies (in humans), but the encoded domains composing a large part of the apo(a) protein could also be of functional significance (Kapetanopoulos et al., 2002) and also contribute to the differences in Lp(a) levels between chimpanzee subspecies.

4.5 | Functional considerations

In humans, high Lp(a) levels with small apo(a) isoforms have been identified as key parameters for the atherothrombotic risk attributed to Lp(a), especially for coronary heart disease (CHD), with a relative risk of about two (reviewed in Erqou et al., 2010). Still, CHD appears to be rare among chimpanzees, even under a more sedentary lifestyle and at high age as seen in captivity (reviewed in Varki et al., 2009). Human-specific binding sites for oxidized phospholipids (OxPL) on the KIV-10 domain have been suggested to contribute to the pro-atherogenic capacity of Lp(a) (Leibundgut et al., 2013), and the available sequences point toward no differentiation at these sites between chimpanzees subspecies. In that study, OxPL could not been detected on apo(a) or in Lp(a) when using a monoclonal anti-human OxPL antibody, and it was concluded that chimpanzee apo(a)/Lp(a) might lack OxPL altogether and thus has less atherogenic potential (Leibundgut et al., 2013). However, no information on subspecies (PTT or PTV) is available for that study.

As we show, previous conclusions on higher Lp(a) levels in chimpanzees than in human subjects (Doucet et al., 1994) cannot be generalized. Though the Gabonese carried significantly more large apo(a) isoforms than PTT, their Lp(a) concentrations closely matched. Still, across species apo(a) isoform-associated Lp(a) levels were similar in neighboring rural Gabon and Congo compared to the geographically distant PTV from Sierra Leone. Thus, it is tempting to speculate that the trait could have evolved similarly under selective pressure by the same environmental factor, possibly by a pathogen that can cross species barriers (Becquart et al., 2010; Krief et al., 2010; Leroy et al., 2004). Identifying sequence variants in *LPA* that are shared between humans and chimpanzees from the same geographic region but differ between chimpanzee subspecies might therefore shed light on functionally important domains of apo(a), and also provide more insight into *LPA* domains affecting Lp(a) levels by way of transcription, translation, Lp(a) assembly efficacy, or its still unrevealed catabolism.

ACKNOWLEDGMENTS

The excellent technical work of L. Fineder and A. Stöckl (Lp(a) ELISA and Western blotting) is greatly appreciated. The study was funded by Research Project Fund P5030-011-011 “Clinical Genetics” of the Medical University of Innsbruck to G.U.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Noureen A, Ronke C, Khalifa M, et al. Significant differentiation in the apolipoprotein(a)/lipoprotein(a) trait between chimpanzees from Western and Central Africa. *Am J Primatol*. 2017;79:e22683. https://doi.org/10.1002/ajp.22683