Rheumatology 2001;40:48–54

Evidence of association and linkage disequilibrium between a novel polymorphism in the transforming growth factor β1 gene and hip bone mineral density: a study of female twins*

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Abstract

Objective. Bone mineral density (BMD) in later life is a major determinant of osteoporotic fracture risk and has been shown to be under strong genetic influence. Transforming growth factor β1 (TGF-β1) is an important regulatory cytokine, is found in high concentrations in the bone matrix, and is a plausible candidate for the genetic regulation of BMD.

Methods. This study investigated whether a novel polymorphism within the *TGF-β1* gene is associated with BMD in a large normal female population of 1706 dizygotic (DZ) twins (age range 18–76 yr).

Results. A T→C polymorphism was identified in intron 5, the C allele having a frequency of 0.25. Subjects homozygous for the presence of the *TGF-β1* C allele had a 4% reduction in femoral neck BMD compared with the other two genotype groups ($P < 0.025$). No effect was seen at the lumbar spine or ultradistal radius, or with calcaneal ultrasound measurements. Results were unaffected after adjustment for potential confounders. These findings were predominantly seen in pre-menopausal subjects, suggesting that this locus has an effect on the attainment of peak BMD. In pre-menopausal women, subjects who were homozygous for the C allele had a 5-fold excess risk of having osteoporosis at the femoral neck compared with the other genotype groups. A within-pair analysis using the sibling transmission disequilibrium test confirmed these findings in pre-menopausal women and supported the candidacy of the *TGF-β1* locus in the genetic regulation of hip BMD.

Conclusions. These results indicate that allelic variation at the *TGF-β1* gene contributes to the development of osteoporosis at the hip. The study also highlights the power of candidate gene analysis in twins, in whom loci having modest effects on disease risk can be identified.

KEY WORDS: Genetics, Osteoporosis, Alleles, Sibling TDT, Growth factor.

Osteoporosis is a common age-related condition and is characterized by reduced bone mineral density (BMD), deterioration in skeletal microarchitecture and an increased risk of fragility fracture [1]. BMD is the strongest predictor of fracture [2], and a large number of twin and family studies have suggested a strong genetic influence on this trait, up to 85% of the population variance in BMD being attributable to genetic factors [3–5].

Submitted 29 February 2000; revised version accepted 24 July 2000.

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*Presented as an oral presentation at the 2nd Joint Meeting of the ASBMR and IBMS, 1–6 December, 1998.

Over the last few years a number of genes have been implicated as having a potential role in the determination of BMD and the risk of osteoporotic fracture [6]. Most of these studies have been association-based in either patient groups or general population cohorts, and have examined polymorphisms within candidate genes of interest. More recently, genome-wide searches have been performed in sib-pairs, positive linkage results identifying chromosomal regions that may harbour novel quantitative trait loci [7]. Although association analyses are generally more powerful than linkage analyses in the identification of loci having modest effects on complex traits [8], results may be affected by population stratification or admixture [9].

To overcome some of these problems, the transmission disequilibrium test (TDT) has been developed [10] and has recently been modified for the analysis of sibling-only data and of quantitative traits [11, 12].

In this analysis, the transforming growth factor β1 gene (*TGF-β1*) was examined as a potential candidate gene for the determination of osteoporosis in non-identical, dizygous (DZ) twins. The aim of the study was to examine the regulatory and coding regions of the *TGF-β1* gene for novel genetic variation and to assess the relationship between any novel polymorphisms and bone mass at multiple skeletal sites. By using twin pairs, it was possible to perform association analysis across the twin pairs, with subsequent TDT analysis within the DZ pairs to confirm any initial positive findings.

Methods

Subjects

The subjects studied were Caucasian female DZ twins (age range 18–76 yr), recruited after national media campaigns. All subjects were healthy and did not suffer from diseases specifically affecting bone, and were broadly representative of the normal UK population, as described previously [13]. The twins completed a nurse-administered questionnaire detailing medical, obstetric and gynaecological histories, full drug histories, dietary assessment and smoking status. The zygosity of the twins was determined by questionnaire and in doubtful cases this was confirmed by multiplex DNA fingerprinting. Post-menopausal status was defined as the absence of menstruation for at least 12 months and was confirmed by measurement of serum sex hormone levels.

Measurements

BMD was measured at the lumbar spine (L1–4), non-dominant hip (femoral neck, total hip), and non-dominant ultradistal radius using dual-energy X-ray absorptiometry on a Hologic QDR-2000 (Hologic, Waltham, Massachusetts, USA). Reproducibility, as assessed by the coefficient of variation from duplicate measures in healthy volunteers, was between 0.8 and 1.6% at the skeletal sites measured. Subjects were classified as having osteoporosis according to the World Health Organization diagnostic criteria if their BMD measurement was 2.5 standard deviations (S.D.) below the mean peak young adult value (i.e. a *T*-score less than -2.5)

Calcaneal ultrasound was measured using a McCue Cuba Clinical scanner. This produced two output variables: broadband ultrasound attenuation (BUA) and velocity of sound (VOS). Reproducibility, as assessed by the coefficient of variation in duplicate measures on 30 subjects, was 2.5% (BUA) and 0.44% (VOS).

Identification of polymorphisms

DNA was prepared for each subject from peripheral blood leucocytes using a standard phenol extraction method. Common single-nucleotide polymorphisms (SNPs) in the *TGF-β1* gene were detected by sequence analysis of 24 unrelated DZ individuals and comparison was made with the published *TGF-β1* sequence (accession no. Y00112) [14, 15]. This strategy was chosen in order to identify polymorphisms with an allele frequency of at least 0.1 within the study group. Oligonucleotide primer pairs were designed to cover the *TGF-β1* coding regions, and the promoter and 5′-untranslated region up to position –1363. After amplification of the DNA by the polymerase chain reaction (PCR), the products were purified, sequenced using dRhodamine Terminator cycle sequencing, and analysed on an ABI 377 DNA sequencer (Perkin Elmer Applied Biosystems, Foster City, California, USA).

Restriction enzyme digestion

Polymorphism screening within the DZ group was performed using PCR–restriction fragment length polymorphism (RFLP)-based methods. DNA samples from subjects with known genotype were included as positive controls. Two PCR–RFLP assays were used in this study.

BstUI PCR–RFLP analysis of the intron 5 SNP

PCR amplification was performed using the oligonucleotide primers 5′-ATGGTGGTAGCCCCTCCCT-3′ and 5′-GCATCTCGTAGCCCGGTGG-3′. Reactions were performed in 25 μl, with the following composition: 0.3 μM primers, 0.2 mM dNTPs, 1 mM $MgCl_2$, 1$\times$Taqgold buffer, 1.25 units Taqgold (Perkin Elmer Applied Biosystems) and 50 ng genomic DNA. Thermocycling was performed on an MJ Research (Waltham, MA, USA) DNA Engine Tetrad PTC-225 thermal cycler using the following conditions: 95°C for 4 min, 35 cycles of 94°C for 15 s, 60°C for 15 s, 72°C for 30 s, followed by final extension at 72°C for 10 min. The 230 base-pair (bp) PCR product was digested with 3 units of *Bst*UI (New England Biolabs, Hitchin, Herts, UK) at 60°C for 2 h, producing polymorphic fragments of 202 and 28 bp. Products were analysed by agarose gel electrophoresis, with size determination after transillumination under ultraviolet light. Alleles were coded as follows: A_1 = absence of restriction site; A_2 = presence of site.

StuI PCR–RFLP analysis of the codon 255 SNP

PCR was performed as described above, except that the oligonucleotide primers used were 5′-ACTGCTCCTGTGACAGCAG-3′ and 5′-ATCCAGGCTACAAGGCTCAC-3′ and the annealing temperature was reduced from 60 to 55°C. Digestion of the 354 bp PCR product was performed with 3 units of *Stu*I (New England Biolabs) at 37°C for 2 h.

Statistical analysis

All analyses testing for the effects of the *TGF-β1* intron 5 polymorphism were done within a structural equation modelling framework using Mx software [16]. Parameters were estimated by normal-theory maximum

likelihood, in which the models were fitted to the raw data [16, 17].

Test for association

A full model was specified, which estimated means (and their 95% confidence intervals) for each of the possible genotypes (*A1A1*, *A1A2* and *A2A2*) and the residual sib-pair variance–covariance matrix, thereby taking account of the relatedness between the sibs. The effect of the *TGF-β1* intron5 polymorphism was assessed by comparing the full model, in which the means were estimated separately for each genotype (i.e. $\mu A1A1 \neq \mu A1A2 \neq \mu A2A2$), with the reduced model, in which the means were constrained to be equal across genotypes (i.e. $\mu A1A1 = \mu A1A2 = \mu A2A2$). This gives a χ^2 test with 2 degrees of freedom (d.f.), since the difference between minus twice the log-likelihood ($-2\ln L$) for a reduced model and that of the full model is approximately distributed as χ^2, with d.f. equal to the difference in the number of parameters estimated in the full and reduced models. The effect of potential confounding variables was accommodated in the model by incorporating the effect of age and menopausal status as a linear regression on the trait value. Comparison of the residual variances in the full and reduced models also enabled us to estimate the percentage of the variation in the trait that was explained by the *TGF-β1* intron 5 locus. The analytical approach described above is similar to one used and described previously [18].

Test for sibship transmission disequilibrium

To test whether any significant associations might have been due to population stratification or admixture, significant results were followed up with a sibship TDT (s-TDT) for quantitative traits. We adapted the TDT described for the situation of sib-pairs only [19], which provides a test for linkage in the presence of association. This test is essentially the same as that described by Fulker *et al*. [12] and explores the fact that population admixture can only result in differences between sib-pairs, not within sib-pairs. Testing whether within-sib-pair differences in the trait can be explained by the locus therefore makes the s-TDT insensitive to the effects of population admixture.

For the s-TDT, the locus effect on the trait was modelled using parameterization in which a score a is assigned to *A1A1* subjects, d to *A1A2* subjects, and $-a$ to *A2A2* subjects (Table 1) [20]. The last two columns of Table 1 list the deviations from the sib-pair means that are used by the s-TDT to model the within-sib-pair differences due to the locus. It must be noted that only sib-pairs discordant for their genotype are informative for the s-TDT, which can be seen in Table 1. The effect of confounders was again incorporated in the model by including the effects of age and menopausal status as a linear regression on the trait value. For the s-TDT, the effect of the locus was tested by comparing the full model, in which the parameters a and d were estimated, with the reduced model, in which the effect of both a and d was set to zero, which gives a χ^2 test with 2 d.f.

Results

Sequence analysis of 24 unrelated individuals identified two novel polymorphisms in the *TGF-β1* gene in addition to six previously identified polymorphisms within the 5′ and coding regions [21]. The two novel polymorphisms included a C→A substitution at codon 255 and a T→C substitution in intron 5 (20 bp upstream of exon 6). The first substitution in codon 255 represented a synonymous codon change (CCG to AGG) and had no effect on the amino acid sequence at this point (arginine). Subsequent testing of this polymorphism using a *Stu*I PCR–RFLP showed, however, that this allelic variant was very rare, as it was identified in only one of the 400 subjects tested. The polymorphic T→C substitution in intron 5 introduces a *Bst*UI site (recognition sequence 5′-CG→CG-3′), and subsequent screening for this within the whole DZ cohort using the *Bst*UI PCR–RFLP showed the allele frequencies to be 0.75(T) and 0.25(C), the genotype distributions being in Hardy–Weinberg equilibrium ($\chi^2 = 1.21$, $P > 0.05$).

In total, phenotypic data were available on 1706 DZ subjects (853 pairs). Intron 5 genotype results were available for 1501 (88%) of the DZ subjects. Reasons for the absence of genetic results included inadequate DNA extraction and failure of the PCR–RFLP assay. Because no significant differences were observed between subjects with and without *TGF-β1* genotype results, no attempt was made to repeat the genotype analysis on this subset. Characteristics of the study population are shown in Table 2. There were no statistically significant differences in height, weight, smoking status or hormone replacement therapy (HRT) use between the

Table 1. Genotypic values, locus contributions to the sib-pair means and deviation from the sib-pair means for all six possible combinations of sib-pairs if genotyped for a biallelic locus

	Sib-pair		Genotypic value		Sib-pair mean	Deviation from sib-pair mean	
	Sib 1	Sib 2	Sib 1	Sib 2	(Sib 1 + Sib 2)/2	Sib 1	Sib 2
1	*A1A1*	*A1A1*	$+a$	$+a$	$+a$	0	0
2	*A1A1*	*A1A2*	$+a$	d	$a/2 + d/2$	$a/2 - d/2$	$-a/2 + d/2$
3	*A1A1*	*A2A2*	$+a$	$-a$	0	$+a$	$-a$
4	*A1A2*	*A1A2*	d	d	d	0	0
5	*A1A2*	*A2A2*	d	$-a$	$-a/2 + d/2$	$a/2 + d/2$	$-a/2 - d/2$
6	*A2A2*	*A2A2*	$-a$	$-a$	$-a$	0	0

TABLE 2. Mean ($\pm$ S.D.) characteristics of female DZ twin subjects according to intron 5 genotype

Variable	DZ total ($n=1706$)	DZ subjects according to *TGF-β1* intron 5 genotype		
		A1A1 ($n=824$)	*A1A2* ($n=587$)	*A2A2* ($n=90$)
Age (yr)	47.8 (11.2)	47.8 (10.9)	47.8 (11.8)	49.5 (9.7)
Height (cm)	162 (6)	162 (6)	162 (6)	162 (6)
Weight (kg)	65.6 (12.3)	65.8 (12.2)	65.0 (12.1)	65.4 (12.6)
Post-menopausal subjects (%)	51.9	50.5	54.2	56.7
Subjects ever smoked (%)	47.9	45.2	49.4	50.7
Subjects ever used HRT (%)	30.3	28.9	31.8	30.7

TABLE 3. Maximum likelihood-based means (95% confidence intervals) of BMD at lumbar spine, hip and forearm, and calcaneal ultrasound measurements in DZ subjects according to their *TGF-β1* intron 5 genotype

Variable	*TGF-β1* intron 5 genotype			Test for locus effect (χ^2)
	A1A1 ($n=824$)	*A1A2* ($n=587$)	*A2A2* ($n=90$)	
Lumbar spine BMD (g/cm^2)	0.999 (0.987–1.010)	1.004 (0.991–1.017)	1.008 (0.978–1.038)	0.60
Femoral neck BMD (g/cm^2)	0.814 (0.804–0.825)	0.803 (0.792–0.814)	0.777 (0.750–0.803)	7.95*
Total hip BMD (g/cm^2)	0.923 (0.913–0.933)	0.919 (0.907–0.930)	0.898 (0.872–0.925)	3.01
Radial BMD (g/cm^2)	0.454 (0.449–0.459)	0.455 (0.449–0.461)	0.449 (0.435–0.463)	0.57
VOS (m/s)	1659 (1655–1663)	1659 (1655–1664)	1660 (1650–1670)	0.04
BUA (dB/MHz/cm)	77.4 (76.0–78.8)	77.0 (75.4–78.7)	77.3 (73.2–81.3)	0.11

*$P=0.019$.

three *TGF-β1* intron 5 genotype groups. There were, however, small differences in the ages and the proportion of post-menopausal women between these groups, although these were not statistically significant (Table 2).

The *TGF-β1* intron 5 genotype *A2A2* was associated with hip BMD when compared with the other genotypes within the whole study population, with a 4% reduction in femoral neck BMD ($P < 0.025$) and a 2.4% reduction in total hip BMD, although the latter did not reach significance (Table 3). The effect on femoral neck BMD remained significant after adjustment for menopausal status and age, with a χ^2 value of 6.53 ($P=0.038$). The hip BMD data were normally distributed around each genotype. No genotypic association was seen at the lumbar spine, ultradistal radius or with the calcaneal ultrasound parameters of BUA and VOS. The proportion of the population variance in femoral neck BMD explained by the intron 5 *TGF-β1* polymorphism was estimated to be 0.43%.

To ensure sure that these results did not merely reflect the higher proportion of post-menopausal women in the *A2A2* group, we conducted a stratified analysis of twin pairs who were either both pre-menopausal or post-menopausal. The upper part of Table 4 (Association test) shows that the effect of the polymorphism was especially apparent in pre-menopausal twins, in whom it explained 0.87% of the population variance. However, the effect did not reach statistical significance in post-menopausal twins, in whom it explained only 0.67% of the trait variance. Adjustment for age did not change these results.

Overall, the prevalence of clinically defined osteoporosis (*T*-score less than -2.5) at the femoral neck in the genotype group *A2A2* was 15.5% compared with 9.9% in each of the *A1A1* and *A1A2* groups. The *A2A2* genotype group tended to an increased risk of having osteoporosis at the femoral neck compared with the other two genotypes, with an odds ratio (95% confidence interval) of 1.69 (0.94, 3.04) ($P=0.08$). Similarly, this relationship was most apparent in pre-menopausal women, in whom the *A2A2* genotype group had a greater than 5-fold increased risk, with an odds ratio of 5.38 (1.96, 14.89). In the post-menopausal twin pairs there was no increase in risk, with an odds ratio of 1.09 (0.52, 2.56).

The s-TDT was subsequently used to test whether the association between the *TGF-β1* intron 5 locus and femoral neck BMD was real or whether it might have been due to population stratification. The results are

shown in the lower part of Table 4 (s-TDT). In spite of the reduced power resulting from the loss of non-informative sib-pairs, who constituted more than 50% of the total sample, the effect of the locus remained highly significant and was not affected by adjustment for age and menopausal status. The stratified analysis also confirmed the difference in the effect of the locus between pre- and post-menopausal individuals.

Discussion

In this study we have identified a T→C polymorphism in intron 5 of the human *TGF-β1* gene. The data demonstrate both association and linkage disequilibrium between this polymorphism and hip BMD in a large group of unselected, normal female DZ twins. The C allele was present at a frequency of 0.25 in the twin population, and there appeared to be a recessive pattern of risk associated with this allelic variant. Hip BMD was up to 4% lower in women who were homozygous for the allele compared with women who were either heterozygous or homozygous for the commoner allele. Overall, women who were homozygous for the polymorphism tended to have an increased risk of having osteoporosis at the femoral neck in comparison with the other two genotypes. Stratification of results by menopausal status demonstrated that the significant findings were predominantly observed in the pre-menopausal subjects.

The observation that these findings were confined to the hip (and predominantly at the femoral neck) rather than being seen at the spine and distal radius suggests a site-specific association. The absence of any association of genotype with bone ultrasound measures also suggests that the *TGF-β1* intron 5 polymorphism has a predominant effect on BMD rather than on bone quality or structure. Modelling of data in twins has suggested that there are both common and specific genetic factors acting on bone at different skeletal sites and on different aspects of bone quality [22]. There are also data from animal models of osteoporosis suggesting that the effect of genes may be site-specific [23]. The finding that the relationship between the intron 5 genotype and BMD was strongest in pre-menopausal women suggests an influence of this genotype on the attainment of peak BMD, and is of interest as the action of *TGF-β1* on bone is mediated via oestrogen [24, 25].

The *TGF-β1* gene has also been associated with the risk of osteoporosis in other studies. A recent study has also identified a further novel polymorphism in intron 4 of the *TGF-β1* gene (a C deletion 8 bp upstream of exon 5) [26]. This polymorphism was rare (technically it is a mutation), being present in only 10 of 161 osteoporotic patients with spinal fracture (allele frequency 0.03) and two of 131 controls (allele frequency 0.008). Although there was no overall significant association between this polymorphism and BMD, subgroup analysis in patients with both spinal fracture and low BMD (defined as Z-score less than -1) did show an association with carriage of the polymorphic allele and BMD at the spine only. In 287 Japanese post-menopausal women, a T→C polymorphism at codon 10 was associated with spinal BMD and risk of vertebral fracture [27]. In addition, an association between circulating levels of serum *TGF-β1* was also observed. The -509 promoter polymorphism has also been shown to be associated with serum *TGF-β1* levels [18], although the serum levels did not appear to correlate with BMD [28].

These data illustrate the candidacy of the *TGF-β1* locus, although they highlight the difficulties of interpreting results when allelic variants are examined in isolation. It is becoming apparent from other disease areas that full characterization and testing of all single-nucleotide polymorphisms in genes may be required to detect disease-related alleles [29, 30]. Earlier work has also identified strong linkage disequilibrium across the *TGF-β1* gene [21], thereby suggesting that the construction and examination of haplotypes would be possible with additional polymorphism information. Unfortunately, in this study we had full coding sequence data available on only 24 subjects (i.e. 48 chromosomes), and this was too small for the estimation of linkage disequilibrium or for the construction of haplotypes.

The study has potential limitations in addition to those previously addressed. The proportion of population variance in BMD attributable to the *TGF-β1* polymorphism appears small ($< 1\%$), probably reflecting the fact that the risk associated with this locus was apparent only in the rare homozygous CC genotype

TABLE 4. Test results for the effect of the *TGF-β1* intron 5 genotype on femoral neck BMD

		Unadjusted		Adjusted[b]	
	n[a]	$\chi^2_{(2)}$	P	$\chi^2_{(2)}$	P
Association test					
All twins	729	7.95	0.019	6.53	0.038
Pre-menopausal twins	297	8.89	0.012	9.14	0.010
Post-menopausal twins	332	4.06	0.131	4.15	0.125
s-TDT					
All twins	237	7.41	0.025	8.49	0.014
Pre-menopausal twins	86	9.38	0.009	9.40	0.009
Post-menopausal twins	109	3.57	0.168	3.52	0.172

[a]Number of twin pairs with complete genotypic and phenotypic data.
[b]Adjusted for age and menopausal status in all twins (for age only within the pre- or post-menopausal groups).

group. This, however, highlights the power of association studies in large numbers of subjects to detect loci having modest effects on trait values. In addition, loci having only small effects on BMD may still have important influences on the risk of fracture, as illustrated by recent findings about the type I collagen 1α gene and osteoporosis [31]. In this study, the polymorphic *s* allele was associated with a 2-fold increased risk of fracture despite explaining only 0.3 and 0.4% of the population variance in BMD at the hip and spine respectively. The functional significance of our findings with an intronic polymorphism on TGF-β1 activity is also uncertain, although as it is located 20 bp upstream of exon 6 it could have some influence on the active component of the TGF-β1 protein. It has been reported that up to 15% of human diseases are caused by point-sequence variation in splice regions, resulting in either exon-skipping or cryptic splicing [32], and although the intron 5 polymorphism is not located in a splice donor or acceptor site it could affect a branch point. The intronic polymorphic sequence change may also affect messenger RNA stability. If the intron 5 polymorphism is not functional, then the results from the s-TDT would suggest that the *TGF-β1* polymorphism is actually in linkage disequilibrium with a novel disease locus mapping to this chromosomal region. Given the strong linkage disequilibrium across the *TGF-β1* gene, this suggests that other sequence variation within the gene should be examined. In addition, other loci mapping to within 1–2 cM (centimorgans) of the *TGF-β1* on chromosome 19q13 should also be examined. The use of twins and the applicability of the findings to the wider population is also sometimes questioned. However, the DZ twins in this study had baseline characteristics similar to those of women from the UK population, and we believe that our results will be applicable to unrelated individuals. DZ twins also offer advantages for the genetic analysis of complex, age-related traits such as osteoporosis, particularly the matching for age and shared environment. Statistical analysis both across and within DZ pairs also offers advantages in the power to detect loci having modest effects on disease risk [33].

In summary, this study has identified additional and novel sequence variation in the *TGF-β1* gene. A T$\rightarrow$C polymorphism in intron 5 of the *TGF-β1* gene has shown an association with femoral neck BMD in a large group of normal DZ female twins. Subjects who were homozygous for the polymorphic allele had reduced BMD and an increased risk of osteoporosis at the hip. The demonstration of linkage disequilibrium through the use of the s-TDT within the twins also confirms the candidacy of this locus as having significant effects on the genetic determination of hip BMD. The effects attributable to this genotype were seen predominantly in pre-menopausal rather than post-menopausal women, suggesting that this locus may have some influence either on the attainment of peak BMD or on differential rates of post-menopausal bone loss. Further studies are required to confirm these findings and to determine the effect of this locus on the risk of future fractures. The study also highlights the power of candidate gene analysis in twins, in whom loci having modest effects on disease risk can be identified.

Acknowledgements

The Twin & Genetic Epidemiology Research Unit was helped by grants from the Arthritis Research Campaign, the Chronic Disease Research Foundation, Gemini Genomics plc, the Medical Research Council, the Special Trustees of St Thomas' Hospital and the Wellcome Trust. We should like to specifically acknowledge and thank all the staff at the Twin & Genetic Epidemiology Research Unit for patient recruitment and clinical assessment, and also the twin volunteers, without whom this study would not have been possible.

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