CHALMERS

Analysis of spatial structure of epidermal nerve entry point patterns based on replicated data

Mari Myllymäki*, Ioanna G Panoutsopoulou** and Aila Särkkä***

*Department of Biomedical Engineering and Computational Science, Aalto University, P.O. Box 12200, FI-00076 Aalto, Finland

**Department of Dermatology, Mayo Mail Code 98, 420 Delaware Street S.E., University of Minnesota, Minneapolis, Minnesota 55455, U.S.A

***Department of Mathematical Sciences, Chalmers University of Technology, 41296 Gothenburg, Sweden; Department of Mathematical Sciences, University of Gothenburg, 41296 Gothenburg, Sweden

Correspondence to: M. Myllymäki. Tel.: +358 50 512 4490; e-mail: mari.myllymaki@aalto.fi

Keywords: Epidermal nerve fiber, mixed model, pooled K function, replicated pattern, spatial point pattern.

SUMMARY

Epidermal nerve fiber (ENF) density and morphology are used to diagnose small fiber involvement in diabetic, HIV, chemotherapy induced, and other neuropathies. ENF density and summed length of ENFs per epidermal surface area are reduced, and ENFs may appear clustered within the epidermis in subjects with small fiber neuropathy compared to healthy subjects. Therefore, it is important to understand the spatial behavior of ENFs in healthy and diseased subjects. This work investigates the spatial structure of ENF entry points, which are locations where the nerves enter the epidermis (the outmost living layer of the skin). The study is based on suction skin blister specimens from two body locations of 25 healthy subjects. The ENF entry points are regarded as a realization of a spatial point process and a second-order characteristic, namely Ripley's K function, is used to investigate the effect of covariates (e.g. gender) on the degree of clustering of ENF entry points. First, the effects of covariates are evaluated by means of pooled K

functions for groups and, second, the statistical significance of the effects and individual variation are characterized by a mixed model approach. Based on our results the spatial pattern of ENFs in samples taken from calf is affected by the covariates but not in samples taken from foot.

1 INTRODUCTION

Epidermal nerve fibers (ENFs) are unmyelinated nerve fibers that originate from the subepidermal neural plexus which is located in the dermis and penetrate the basement membrane to innervate the epidermis, which is the outmost living layer of the skin, see more details in Kennedy & Wendelschafer-Crabb (1993). ENFs can be visualized via light (Wang *et al.*, 1990) or confocal microscopy studies (Kennedy & Wendelschafer-Crabb, 1993). Their diagnostic value has been established through a series of studies. For example, Kennedy *et al.* (1996) report diminished numbers of ENFs per surface area in diabetic subjects, as well as reduced summed length of all ENFs per epidermal surface area, that is, reduced epidermal innervation.

Kennedy *et al.* (1999) report that nerve fiber loss due to small fiber neuropathy (for example diabetic neuropathy) results in a more clustered ENF

pattern across the epidermis in subjects with small fiber neuropathies than in non-diseased subjects. Waller *et al.* (2011) were able to quantify this observation based on analysis of ENF entry point data from suction-induced skin blister images (Kennedy *et al.*, 1999) from the thighs of one non-diseased patient, two patients with mild diabetic neuropathy, two with moderate diabetic neuropathy and two with severe diabetic neuropathy. The spatial pattern of the ENF entry points, i.e. locations where the ENFs penetrate the epidermis, was described by second-order methods for spatial point processes. Waller *et al.* (2011) conclude that the second-order summary statistic, Ripley's K function, could be used to show that ENF entry point patterns from subjects with moderate or severe diabetic neuropathy were clearly more clustered than the pattern from the non-diseased subject. However, since the study was based on limited amount of data, more data would be needed to confirm the pattern changes in diabetic subjects.

In order to better understand the spatial structure of ENFs within healthy subjects, we analyze data from skin blister specimens taken from the right foot and right calf of 25 healthy volunteers. This data was collected by the Kennedy laboratory (see Panoutsopoulou et al., 2009) during a study where they compared two different biopsy methods, the suction skin blister and the

punch skin biopsy, in order to visualize and quantify the ENFs. In our paper, the entry point patterns from the blister samples are regarded as realizations of spatial point processes and studied by the K function as in Waller *et al.* (2011). We are interested in whether the spatial pattern, especially the scale of clustering of entry points, varies in each body location of skin sample with age, gender and body mass index (BMI).

Since not many replicated point pattern data have been available, the tools for analysing replicated spatial point patterns, especially with covariates and hierarchy, are still under development. Most of the studies are based on pooled summary statistics as presented in Diggle *et al.* (1991), Baddeley *et al.* (1993) Diggle *et al.* (2000) and Schladitz *et al.* (2003). We divide the indivuals into subgroups according to body location, age, gender and BMI and estimate the K function separately for each subgroup. Our construction of pooled K functions is a slight modification of the approach presented by Diggle *et al.* (2000) and Schladitz *et al.* (2003). This visual analysis is followed by a linear mixed model approach where we try to characterize statistical significance of the effects of age, gender and BMI and, in addition, the magnitude of variation between subjects and within subjects.

Mixed models for replicated spatial point process data have been intro-

duced by Bell & Grunwald (2004) and Illian & Hendrichsen (2010) by incorporating random effects to pseudo-likelihood estimation of Gibbs point processes. Random effects have been used in point process models also e.g. in Hossain & Lawson (2009) and Rue *et al.* (2009), but in these works the effects operate for the first-order property (intensity) of non-replicated point patterns. Landau & Everall (2008) have proposed to model empirical K function values at a given distance by linear mixed models. As far as we know, the analysis we present in this paper is the first time mixed models have been used to model the entire K function. Instead of making assumptions of the point process model, we try to detect effects of covariates on the second-order spatial structure through the K function.

A linear mixed model approach has been used for ENFs already by Panoutsopoulou *et al.* (2009), who modeled log ENF density by a mixed linear model based on the foot and calf data. They concluded that the ENF density is lower among older subjects than among younger subjects, and that the density tends to be higher among women than among men. However, based on their study the ENF density does not depend significantly on the body location or BMI. We aim at finding factors that affect the second-order spatial structure of nerve entry points, and fit linear mixed models to the empirical

second-order K function.

Ultimately, we want to be able to recognize abnormal structure of ENFs. The linear mixed model analyses performed in this paper suggest that, particularly in calf, it is important to take the covariate effects into account when looking for signs of small fiber neuropathies. Since the covariates seem not to have effect on the second-order spatial structure of ENF entry points in foot, it might be preferable to take samples from foot instead of taking them from calf. However, since all the covariates of this study are easy to measure, they should be recorded in future studies as well. A further possible reason to favor foot is that the number of nerve entry points tends to be larger in samples taken from foot than in those taken from calf (Panoutsopoulou *et al.*, 2009). On the other hand, some other studies have shown that the ENF density decreases as we move distally on the body, see e.g. Lauria et al. (1999). Additional studies including small fiber neuropathy patients are needed to investigate which body location would best show the changes in spatial pattern due to neuropathy.

The rest of the paper is organized as follows. We start by introducing the data in Section 2. The spatial point processes and the second order properties of them as well as the basic theory of linear mixed models are

recalled in Section 3. These methods are then applied to the ENF data in Section 4, and the results are discussed in Section 5.

2 DATA

Two skin blister specimens were obtained from the right calf and from the right foot of 25 healthy adult volunteers using the suction skin blister method, see Panoutsopoulou *et al.* (2009). ENFs were immunostained, imaged confocally, and traced to determine entry point coordinates for each image. Three to six images (usually four) per each body location of each volunteer were obtained. These replicates were based on two blisters, from which (usually two) images with the surface area of approximately 330×432 microns, were analyzed.

We study the spatial pattern of the ENF entry points. Figure 1 shows two examples of such point patterns from the foot blister images of two individuals. The entry point pattern on the left has 41 entry points and the one on the right 21 entry points. The number of entry points varies quite a lot within and between individuals. The mean number (standard deviation) of entry points per sample (image) is 25.7 (14.6) for foot and 22.8 (11.5) for

calf. In the later analyses we exclude patterns with less than ten points, since the use of the spatial summary function seems not to be meaningful based on a very small number of points. After excluding the patterns with less than ten ENF entry points, the mean number of entry points is 27.5 (13.9) for foot and 25.1 (10.5) for calf.

Figure 1: about here.

3 METHODS

3.1 Second-order summary statistics

Spatial point processes describe a family of stochastic process models where events generated by the model have an associated (random) location in space. Illian *et al.* (2008), Diggle (2003), Cressie (1993, Chapter 8), and Waller & Gotway (2004, Chapter 5) provide details regarding theory and applications from many diverse fields, like astronomy, cellular biology, forestry and public health.

Our intention is to study the spatial pattern of the nerve entry points as a

realization of a stationary (translation invariant implying constant expected intensity across the study area) and isotropic (rotation invariant) point process. The analyses can be based on several summary statistics, for example the distribution of the distance from a randomly selected nerve entry point to its nearest neighboring entry point or the distribution of the distance from a randomly selected location to the nearest entry point, or a combination of these two (see e.g. Illian *et al.*, 2008). However, these methods examine behavior only at the "nearest neighbor" scale.

We have chosen to consider Ripley's K function (Ripley, 1977), which is a second order property of an observed point process. If λ is the intensity (mean number of entry points per area) of the point process, then $\lambda K(r)$ denotes the mean number of entry points within distance $r > 0$ from a typical entry point (which is not counted). When the point pattern is completely spatially random (CSR), $K(r)$ equals πr^2. Under regularity, $K(r)$ tends to be less than πr^2 and, under clustering, greater than πr^2. Since $K(r)$ is a function of all interevent distances it is possible for a given realization to have, for example, $K(r) > \pi r^2$, $r < r^*$, and $K(r) < \pi r^2$, $r > r^*$ for some distance r^*. Thus, r^* is associated with the spatial scale of clustering or regularity found in the data. Note though that the K function is a cumulative function of

distance as it measures the expected number of nerve entry points up to a certain distance.

Besag (1977) suggests a variance-stabilizing transformation of the K function,

$$L(r) = \sqrt{K(r)/\pi}. \tag{1}$$

The L function allows a more readily interpretable diagnostic tool, since we can plot r vs. $L(r)-r$ and compare the resulting curve to zero (expected value under CSR) in order to see whether the pattern differs from a completely spatially random pattern.

We estimate the K function using a standard estimator (see e.g. Cressie, 1993; Illian *et al.*, 2008) with the isotropic edge correction suggested by Ripley (1976). We further use $\widehat{L}(r) = \sqrt{\widehat{K}(r)/\pi}$ as an estimator for the L function. For estimation we utilize the R library spatstat (Baddeley & Turner, 2005).

3.2 Pooled summary statistic

3.2.1 *Pooled K function*

Often we observe only one point pattern from which we estimate the K (L) function. If repetitions are available, the K function can be estimated from

each repetition, and the individual K functions can be pooled together into one estimate which then represents the entire group, see Diggle *et al.* (1991), Baddeley *et al.* (1993), Diggle *et al.* (2000), and Schladitz *et al.* (2003). The pooled L function is estimated by transforming the pooled K function as in (1).

In our data, 3-6 repetitions are available per subject and body location. After the subject specific K (L) functions have been estimated from the replicates, the K function for each subject i and for an entire group can be estimated. The index set $\mathcal{G}$ contains the individuals $i \in \{1, ..., N\}$ that belong to a particular group that may for example consist of samples of all subjects taken from calf or samples of all females from foot. Therefore, we begin by estimating the K function for each replicate j, $j = 1, \ldots, m_i$, of each subject i, $i = 1, \ldots, N$, where N is the number of subjects and m_i is the number of replicates for subject i. Then the subject specific mean functions can be estimated as

$$\bar{K}_i(r) = \sum_{j=1}^{m_i} w_{ij} \widehat{K}_{ij}(r), \quad i = 1, \ldots, N, \tag{2}$$

where the replicate specific K_{ij} functions are weighted by the squared number of points n_{ij}^2 in the point pattern in question, i.e. $w_{ij} = n_{ij}^2 / \sum_{j=1}^{m_i} n_{ij}^2$ (Diggle *et al.*, 2000; Schladitz *et al.*, 2003). Following Schladitz *et al.* (2003), the

overall mean function for a group $\mathcal{G}$ of individuals becomes

$$\bar{K}_{\mathcal{G}}(r) = \frac{1}{n_{\mathcal{G},2}} \sum_{i=1}^{N} \mathbf{1}(i \in \mathcal{G}) n_i^2 \bar{K}_i(r), \tag{3}$$

where $n_{\mathcal{G},2} = \sum_{i=1}^{N} \mathbf{1}(i \in \mathcal{G}) n_i^2$, $n_i = \sum_{j=1}^{m_i} n_{ij}$ and $\mathbf{1}(i \in \mathcal{G})$ equals 1 if subject i belongs to the group $\mathcal{G}$ and 0 otherwise. We have decided to use the squared point-number-weighted group means, since particularly the ENF entry point patterns from different subjects can not be assumed to be realizations of point processes with the same intensity.

3.2.2 *Variance estimation*

In addition to the mean K function we need an estimate for its variance. One possible approach would be to use the sampling variance of the $\widehat{K}_{ij}$ functions at fixed r to obtain pointwise bands. Here, we create the pointwise bands based on a bootstrap estimate of the overall mean function $\bar{K}_{\mathcal{G}}(r)$ following the bootstrap procedure of Schladitz *et al.* (2003), see also Diggle *et al.* (1991, 2000). In this procedure, the residual K functions $\widehat{R}_{ij}(r) = n_{ij}[\widehat{K}_{ij}(r) - \bar{K}_i(r)]$ are resampled by drawing at random and with replacement, keeping number of samples per subject fixed. Thus, in a bootstrap sample, a new residual K function, $\widehat{R}^*_{ij}$, is attached to each replicate, and the bootstrapped K functions are obtained as $\widehat{K}^*_{ij}(r) = \bar{K}_{\mathcal{G}}(r) + n_{ij}^{-1}\widehat{R}^*_{ij}$ for all i and j. The function (3)

is calculated for each bootstrap sample, and the pointwice 2.5% and 97.5% quantiles from the B bootstrapped means are used as the band for the overall mean (3).

The resampling of the residual K functions assumes that the replication is the same across groups or sufficiently large within each group (see Diggle *et al.*, 1991). In our data, there are four replicates per location for most subjects, while a few have anywhere from two to six.

3.3 Linear mixed model

Typically, the K (L) function is used only in the preliminary analysis of the data mainly to see whether a point pattern differs from a completely spatially random pattern. If there is non-spatial covariate information available, it can be interesting to investigate also whether the form of the K function is affected by the covariates. Our study focuses on how to model the K function, or in fact the centered L function, $L(r) - r$, using the linear mixed models approach which is commonly used to model growth curves. Our "time variable" is distance r.

The starting point of a linear mixed model (LMM) is a linear model $\mathbf{E}[y] = \mathbf{X}\beta$, where the fixed effects are in β and $\mathbf{X}$ is a known matrix.

An LMM includes some fixed effects and in addition, some random effects. Therefore, the model can be written as

$$\mathbf{E}[y|u] = \mathbf{X}\beta + \mathbf{Z}\mathbf{u},$$

where $\mathbf{u}$ is a vector of random effects and $\mathbf{Z}$ is a known model matrix. As can be seen in the formula above the model is specified conditionally on the unobserved but realized values of $\mathbf{u}$. Fixed effects describe the behaviour of the entire population, and random effects are associated with the individual experimental unit (group) sampled from the population.

Typically, it is assumed that the within-group errors are independent and identically normally distributed, with mean zero and variance σ^2, and that they are independent of the random effects. Further, the random effects are assumed to be normally distributed, with mean zero and covariance matrix Φ (not dependent on the group) and that they are independent for different groups. See more about linear mixed models e.g. in McCulloch (2001).

4 ANALYSIS OF THE ENF PATTERNS

We study the nerve entry point patterns by using the second-order L function. First, the subjects are divided into subgroups based on body location, age,

gender and BMI, and the L function is estimated for each subgroup. Then, for each body location, we fit a linear mixed model to the centered L functions estimated from the data having age, gender and BMI as covariates in the model.

4.1 K-functions for subgroups

We start by estimating the overall mean L (or K) function (3) separately for the calf data and for the foot data. We have chosen to plot the mean K functions with bootstrap envelopes for each subgroup without performing any formal tests. The pooled centered L functions together with 95% bootstrap envelopes constructed from 1000 resamples are shown in Figure 2. Both the L function estimated from the calf data and the one estimated from the foot data indicate clustering of the ENF entry points. However, there are some differences between the calf and foot data: The L function based on the foot data reaches its maximum approximately at distance 20 microns, while the L function based on the calf data reaches its maximum at 30 microns. One should note though that the L functions estimated from the samples taken from the same body location of a subject may look quite different. The within subject variation can be inspected from Figure 3, where the gray

solid lines show the sample specific centered L functions (for $10 \leq r \leq 60$). For example, the L functions of the three samples from Subject 1103 are quite similar, whereas the four samples from Subject 1113 look quite different from each other. Large variation within Subject 1113 is likely caused by reasonably small number of nerve entry points in the samples taken from this subject. Note that in calculating the pooled K functions the individual K functions are weighted by the squared number of points.

Figure 2: about here.

Figure 3: about here.

We have split the calf and the foot data further into subgroups by age (Wang *et al.*, 1990), by gender, and by BMI. For the two body locations, we study the groups formed above ($n = 12$) and below ($n = 13$) the median age (50.5 years). The overall means in these subgroups are shown in Figure 4 (top). In both foot and calf data, the L functions of the two subgroups reach the maximum value approximately at the same distance, 30 microns for the

calf data and 20 microns for the foot data, indicating that the cluster radius is the same in these two age groups. However, since the L function of the older group lies above the L function of the younger group, one can conclude that the clusters of the nerve entry pattern of older people tend to have more points relative to the total number of points than the clusters in the nerve pattern of younger people.

Next we split the data according to BMI. Individuals with BMI < 25 ($n = 11$) belong to the low BMI class and individuals with BMI > 25 ($n = 14$) belong to the high BMI class. BMI does not seem to affect the cluster radius since the maximum in each subgroup (both in foot and calf data) is reached approximately at the same distance. However, for the calf data the clusters in the nerve entry point pattern among individuals with low BMI have more points relative to the total number of points than among those with high BMI, while in the foot data it is the other way around, see Figure 4 (middle).

Figure 4 (bottom) shows the L functions separately for women ($n = 15$) and for men ($n = 10$). The results are similar to the ones based on the division into low and high BMI. Indeed, in the data women tend to have slightly lower BMI than men (the mean BMI for women is 24.6, and for men 27.5) and only two out of 11 are men in the low BMI group. The high BMI

group, on the other hand, consists of eight men and six women.

We further continue by splitting the data both by age and by BMI, which gives four subgroups for each body location. The number of individuals in the groups are 6, 7, 5 and 7. We plotted the mean centered L functions of the different groups calculated for the calf and the foot data (figure not shown). In the calf data the group of older people with low BMI has a more clustered (more points per cluster relative to the total number of points) entry point pattern than the other groups. The other three groups behave very similarly. In the foot data, on the other hand, the two groups with younger people have less clustered nerve entry point patterns than the two older groups, whereas the differences between the low and high BMI groups within these age groups are slightly smaller.

We can conclude that the spatial pattern of nerve entry points appears to be different between calf and foot. Furthermore, there is some indication that the pattern may vary with age, and possibly also with gender and BMI. These observations should be considered with care since the bootstrap procedure does not account for unspecified sources of between-subject variation, see discussion in Diggle *et al.* (1991). One way to accommodate for these unspecified sources of between-subject variation is to assume a parametric

random effects model for the K functions.

Figure 4: about here.

4.2 Modelling second-order structure by linear mixed models

We analyzed above the spatial structure of the nerve entry points by using the second-order L function. Through visual observation we have noticed that the spatial pattern may depend on the body location and other covariates such as age, BMI and gender. We further study the second-order spatial structure by mixed models, and investigate how the covariates affect the form of the centered L function. The L function is modeled using linear mixed models usually used to model growth curves. Our "time variable" is distance r, which is initially chosen to take values $r = 10, 15, ..., 60$ (microns). Figure 2 shows that the centered L function levels down around at distance 60 microns, and therefore $r = 60$ is chosen as the maximum value of r considered here. Our future goal is to be able to compare ENF patterns of healthy subjects with patterns of subjects with neuropathy. The hypothesis is that the

diseased ENF patterns are more clustered than the healthy patterns. Therefore, the behaviour at short distances is not of primary interest here; we are more interested in the scale and amplitude of clustering at longer distances. Therefore, we ignore the possible effects of covariates on the inhibition between nerve trunks and choose the minimum value for r as 10. Inspection of the distribution of $L(r) - r$ estimates for different values of r reveals that for $r = 10, 15, ..., 60$ the distribution is quite symmetric.

We first fit a full fixed model including r, age, gender, BMI, body location and all interactions between them. Subject as well as the body location within subject were added as random effects to allow random variation but to keep the model reasonably simple. Distance r was regarded as an ordered factor with levels $10, 15, \ldots, 60$. We started with the full model and excluded the non-significant terms ($p > 0.05$) from the model stepwise. The resulting model included body location, age, r and the interaction between r and body location. (The marginal effect of body location was kept in the model since its interaction with r was highly significant.) From the summary of the fitted model we can investigate the orthogonal polynomial contrasts and conclude that only the linear, quadratic, third and maybe the fourth power matter. (Note though that this may depend on the denseness of r values.)

We then fit a model with only the first four powers of r considering r a continuous variable. We proceeded with the model including body location, age, r and interaction between r and body location, where the dependent variable was modeled as a polynomial with respect to the distance variable. Since r was now considered continuous, we took a denser set of 26 r values: $r = 10, 12, 14, \ldots, 60$. We ended up with the same fixed effects as above.

Based on the experimenting, we can conclude that the shape of the L function is different for the calf and for the foot data since the interaction between r and body location is strong. This difference is also visible in Figure 2. In the following, our main purpose is to find out which covariates affect the shape and the level of the L function in each body location.

4.2.1 *Foot*

The joint analysis for foot and calf data above showed that it is enough to include r in the model as a fourth order polynomial. First we fit the fourth order polynomials for each of the subjects separately, and even for each sample within subjects separately. Figure 5 shows boxplots of the fitted regression coefficients. The results show that the variation within a subject is much larger than the variation between the subjects indicating that we

should include sample specific but not necessarily subject specific random effects in the model. We will investigate both scenarios.

Figure 5: about here.

Our model is

$$L_{ijk} - r_k \;\; = \;\; \mathbf{x}_{ik}\beta + \mathbf{z}\mathbf{u}_j + \epsilon_{ijk}$$

for subjects $i = 1, \ldots, N$, repetitions $j = 1, \ldots, m_i$ within subject i and r_k-values, $k = 1, \ldots, 26$. Here ϵ_{ijk} is assumed to be an unstructured error term or, more precisely, ϵ_{ijk}s are independent zero-mean Gaussian variables with variance $\sigma^2_{ijk} = \sigma^2 \cdot 1/n^2_{ij}$, where n_{ij} is as above and σ^2 constant. The dependence of $L_{ijk} - r_k$ from the replicate j for different r_k is taken care of by having distance r both in the fixed effect term and in the random effects $\mathbf{u}_j$ (or $\mathbf{u}_{ij}$) to be specified below. The fixed part of the model we start with includes interaction of r^i, $i = 1, 2, 3, 4$, with age, gender, BMI and with joint effect of gender and BMI as well as all the marginal effects. Then, one term at the time is dropped out at the 5% significance level. The interaction effects of the covariates and r are treated as a group (i.e. the significance of Age $\cdot\, r$, Age $\cdot\, r^2$, Age $\cdot\, r^3$ and Age $\cdot\, r^4$ is assessed simultaneously) and the constant

and marginal effects in the presence of interaction effects are kept in the model throughout. Our resulting fixed effect model is $\mathbf{x}_{ik} = (1, r_k, r_k^2, r_k^3, r_k^4)$. So, in fact, none of the covariate effects are included. Preliminary analysis with pooled L functions (Figure 4, right) indicates that the level of $L(r) - r$ may be affected by age, BMI and gender. However, these effects are not statistically significant according to the estimated model. When we inspect the full model without the (non-significant) interaction effects of gender and BMI (with r), we also observe that none of the marginal covariate effects are statistically significant.

We first used sample specific random effects for the intercept and all powers of r, but we further studied whether some of these could be left out from the model (Akaike information criterion, AIC, and Bayesian information criterion, BIC, were used to compare the models, both gave the same result). As a result the random effect for r^3 was dropped out. (The fixed effect conclusions where the same whether or not this random effect was in the model.) More precisely, the chosen estimated model is

$$\begin{aligned} L_{ijk} - r_k &= 3.3_{(1.5)} + u_{0j} + (1.7_{(0.2)} + u_{1j}) r_k + (-0.08_{(0.01)} + u_{2j}) r_k^2 \\ &\quad + 0.001_{(1 \cdot 10^{-4})} r_k^3 + (-8 \cdot 10^{-6}_{(1 \cdot 10^{-6})} + u_{4j}) r_k^4 + \epsilon_{ijk}, \end{aligned} \tag{4}$$

where the estimates of β are plugged in and their standard errors are given in

the parantheses under the estimates. It is assumed that $\mathbf{u}_j = (u_{j0}, u_{j1}, u_{j2}, u_{j4}) \sim N(0, \Phi)$, where Φ is a diagonal matrix. The standard deviations (95% confidence limits) of $u_{ji}, i = 0, 1, 2, 4$, on the diagonal of Φ, are estimated as $10.9\,(9.3, 12.9)$, $0.8\,(0.6, 0.9)$, $1.7 \cdot 10^{-2}\,(1.4 \cdot 10^{-2}, 2.1 \cdot 10^{-2})$, $2.1 \cdot 10^{-6}\,(1.7 \cdot 10^{-6}, 2.5 \cdot 10^{-6})$, respectively. The estimation was done by maximizing the restricted log-likelihood using R library nlme (Pinheiro & Bates, 2000).

We then added the subject specific random effects for the same terms as we have the sample specific random effects. However, this led to no improvement of the model according to AIC/BIC. The subject specific random effect estimates were very small compared to the sample-level random effects. The model with so many random effects may also be overparametrized.

Finally, we investigate some residual plots for the model (4). The standardized residuals are centered around zero and have approximately the same variance, see Figure 6 (top left). The figure of the standardized residuals versus $1/n_{ij}^2$ also indicates that the chosen heteroscedastic structure is reasonable. (We also tested for the error variance to be constant or $\sigma_{ijk}^2 = \sigma^2 \cdot 1/n_{ij}$, but the model with $\sigma_{ijk}^2 = \sigma^2 \cdot 1/n_{ij}^2$ was superior (AIC/BIC).) Further, the fitted values are in close agreement with the observed values of the centered L function even though there are a few outliers (at 0.05 significance level). The

normal plot of the standardized residuals (see Figure 6 bottom left) shows that the distribution of the within sample errors is quite close to normal. It has slightly heavier tails and pointier peak than expected under normal distribution, but the tails are distributed quite symmetrically apart from a few outliers. Therefore, changing the error distribution to some other than normal distribution should not affect the estimates of the fixed effects too much (Pinheiro & Bates, 2000). The normality of the random effects holds well, but we observe that some of the random terms, intercept, r, r^2, and r^4, are correlated. The model might be further improved by allowing correlation between r and r^2, which we did. This indeed improves the model (according to AIC/BIC), but the conclusions remain unchanged.

Figure 6: about here.

In conclusion, the shape of the second-order summary statistic can be described by a fourth order polynomial of the distance r for $10 \leq r \leq 60$. The level of clustering (i.e. height of the curve or the number of points per cluster) and the scale of clustering (i.e. the shape of the curve or the cluster radius) vary between the samples taken from an individual, which has been

adjusted by adding the within subject effect as a random effect in the model. Figure 3 shows comparison between the observed centered L functions and the predicted ones obtained by the model. We consider the (random effects) estimates quite good, but still some correlation remains in the residuals.

4.2.2 *Calf*

As for the foot data, we start with individual fits of the model for each subject and sample and find out that the variation within subject is clearly larger than the variation between subjects. Therefore, we start with the same fixed effect model as for the foot data and end up with the model

$$\begin{aligned} L_{ijk} - r_k \;=\; & -16.2_{(12.8)} + 69.1_{(19.5)}\,\mathrm{Gender}_i + 0.3_{(0.2)}\,\mathrm{Age} - 0.02_{(0.4)}\,\mathrm{BMI}_i \\ & -2.2_{(0.7)}\,\mathrm{Gender}_i \cdot \mathrm{BMI}_i + u_{0j} \\ & +(1.7_{(0.2)} - 0.9_{(0.7)}\,\mathrm{Gender}_i + u_{1j})r_k \\ & +(-0.04_{(0.01)} + 0.03_{(0.02)}\,\mathrm{Gender}_i + u_{2j})r_k^2 \\ & +(2 \cdot 10^{-4}_{2 \cdot 10^{-4}} - 5 \cdot 10^{-4}_{(4 \cdot 10^{-4})}\,\mathrm{Gender}_i)r_k^3 \\ & +(9 \cdot 10^{-7}_{2 \cdot 10^{-6}} + 3 \cdot 10^{-6}_{3 \cdot 10^{-6}}\,\mathrm{Gender}_i + u_{4j})r_k^4 \\ & +\epsilon_{ijk}. \end{aligned} \tag{5}$$

Terms related to gender are for male subjects, females are used as a control group. That is, at the significance level 0.05, we could not exclude the marginal effect of age and the joint effect of gender and BMI on the level of $L(r) - r$ and also not the joint effect of gender with r. The random effects are the same as for the foot data, namely sample specific intercept, r, r^2 and r^4 and their standard deviations (95% confidence limits) are estimated as $10.8\,(9.1, 12.9), 0.6\,(0.5, 0.8), 1.2 \cdot 10^{-2}\,(0.9 \cdot 10^{-2}, 1.6 \cdot 10^{-2}), 1.5 \cdot 10^{-6}(1.1 \cdot 10^{-6}, 1.9 \cdot 10^{-6})$, respectively.

We proceed with similar examination of the fitted model as for the foot data. We added the subject specific random effects, but again this led to no improvement of the model according to AIC and BIC. Then we studied the residuals and random effects. The standardized residuals are centered around zero and their variance is approximately constant (Figure 1 top right), but the left tail of the distribution is slightly heavier than in the case of the foot data (Figure 6 bottom right). We further added the block diagonal covariance matrix for the random effects releasing the correlation between r and r^2 to be estimated. This slightly improved the model (smaller AIC/BIC). Furthermore, strictly speaking, gender does not seem to have joint effect with r anymore ($p = 0.08$ in the joint Wald test for Gender$\cdot r$, Gender$\cdot r^2$, Gender$\cdot r^3$

and Gender $\cdot r^4$). All the marginal effects of the model (5) remain significant.

To conclude, age, gender and BMI seem to affect the level of the centered L function on samples taken from calf: Based on our data, among men the clustering is more pronounced with low BMI than with high BMI. However, there are two male subjects with BMI < 25 and their patterns seem most clustered among men. These two individuals affect the result for men and, consequently, also the found joint effect of gender and BMI. Thus, more data would be needed to confirm the observation. Higher age seems to indicate more pronounced clustering. Variances of random effects of the foot and calf data are of the same magnitude.

5 DISCUSSION

We have studied the ENF entry pattern in samples taken from healthy subjects using second-order K (L) function for spatial point processes and some covariate information. We have first divided the samples into subgroups by the body location, calf and foot, and then within each body location by age, gender, BMI and finally age and BMI. The L function was estimated separately for each subgroup. The results show that the spatial pattern of ENF

entry points is always clustered (with some small scale inhibition). The cluster radius in the foot data is shorter than in the calf data indicating that the ENF entry point clusters in the foot are tighter and the clusters in the calf are more spread out. This may be an interesting observation, since in early stages of small fiber neuropathy the ENF density and distribution may be normal on the calf but abnormal on the foot (which is more distally located on the body). Furthermore, age, gender and BMI also slightly affect the scale and level of clustering, but exactly how seems to depend on the body location.

After the visual inspection of the estimated (pooled) L functions, we modeled the centered L functions by linear mixed growth models separately for each body location. Age, gender and BMI were taken into the model as covariates, and the within subject variation as random effect. According to the estimated model, in the samples taken from foot, the covariates do not seem to have much effect on the function. In the samples taken from calf, on the other hand, there is some indication that age, gender and BMI all have some effect on the spatial structure of the nerve entry points. The results based on the mixed models differ from the visual inspections of the K functions. One should note that age and BMI are considered continuous

variables in the mixed model analysis while they each have only two values (young-old and low-high, respectively) in the visual inspection. Furthermore, unspecified sources of between-subject variation are not taken into account in the visual inspection using bootstrap.

When modeling K function with mixed models in Section 4.2 we have made some choices and simplifications. In our final models, we have left out the subject specific random effects since they were found out to be small compared to sample specific random effects. Further, we have assumed that all the sample specific random effects origin from the same normal distribution, even though it might be more reasonable to allow the within-subject variation to vary from subject to subject. Particularly, Subject 1116 seems to have most extraordinary variation in K function compared to the others, see Figure 5. We left out this subject and re-fitted the model both for calf and for foot. For foot, the results remained the same (no significant covariate effects at the 0.05 significance level). For calf, strictly speaking, the marginal effect of age (earlier significant in the model, $p = 0.03$) was now only borderline significant ($p = 0.10$). The estimated random effect variances were approximately the same with and without Subject 1116. The main reason for the changes in the effects is probably that Subject 1116 is the oldest of

all the subjects (age 62).

The analysis of the entry point patterns of healthy subjects shows that the within subject variation even within a body location is very large (particularly for some subjects), whereas the between subject variation among the healthy volunteers is rather small (after accounting for covariates). Due to the large within-subject variation, it is important also in future studies to take several samples in order to obtain reliable results of the ENF pattern of a subject. Even a better solution would be to take a much larger sample than the ones taken here that would include more ENF entry points. Since two images comprise only a small fraction of the total area of each blister, larger images could be obtained at least in theory, but this would require much more effort.

We have chosen to model the cumulative K function. However, for detailed inspection of the scale of clustering/regularity at a particular distance, one should use the corresponding non-cumulative function (i.e. pair correlation function, see e.g. Illian *et al.*, 2008) instead. We have chosen the cumulative K function because estimation of non-cumulative functions is not as standardized as estimation of cumulative ones: to use non-cumulative functions in statistical testing is usually not recommended, following the common

practice in classical statistics (Illian *et al.*, 2008). Furthermore, the cumulative K function can be related to growth curves, which are often modeled by mixed models.

We have assumed that the entry point patterns are realizations of stationary point processes, and used the (homogeneous) K function to describe the second-order properties of the patterns. As pointed out by Waller *et al.* (2011), the underlying physiology may affect the locations of the ENF entry points causing heterogeneities due to the locations of the dermal papilla and the inhomogeneous K function (Baddeley *et al.*, 2000) could be used, if some heterogeneities were present. The images in this study are relatively small as in Waller *et al.* (2011) and do not reveal any obvious heterogeneities. Therefore, the stationary assumption seems reasonable.

The suggested approach is meant for modeling the dependence of second-order structure of repeated point pattern data on covariates. The preliminary investigations with pooled L functions together with deeper mixed model analysis for L curves may serve as a starting point for analysing these kind of data. In the ENF data, in addition to ENF entry points, locations of end points of nerve fibers would provide us with information on the spatial pattern of ENFs. A similar approach as presented here, could be used to

analyze the pattern of the end points of fibers. The end point patterns consist of many more points than the corresponding entry point patterns, and therefore, the spatial point pattern approach could be even more suitable for the analysis of end points than for the analysis of entry points. Further, similar analyses could be done for subjects with small fiber neuropathy. The health status could be added as a covariate in the linear mixed model in order to see whether the spatial structure is different between healthy and diseased subjects. Such spatial analysis of ENFs may help us to detect and diagnose small fiber neuropathies in early stages even when the ENF density is within the normal range. Note, however, that the mixed model may need to be revised for specific problem at hand, and therefore we are currently investigating also some more flexible alternative models. Our final goal is the modelling of the whole ENF structure of both healthy patients and patients with some small fiber neuropathy.

Acknowledgements

The authors thank William R. Kennedy and Gwen Wendelschafer-Crabb, University of Minnesota, for blister immunostaining, quantification and mor-

phometry as well as for the valuable comments regarding this paper. Further, the authors thank Lance Waller and Gavino Puggioni for their comments on an earlier version of this paper and two anonymous referees for comments which led to further improvement of the paper. The research has been funded by the Academy of Finland (Project number 250860), and by the Swedish Research Council and the Swedish Foundation for Strategic Research. In addition, the project was supported by the American Legion Brain Sciences Chair, and the RW Goltz Professorship in Dermatology.

References

Baddeley, A.J., Moyeed, R.A., Howard, C.V. & Boyde A. (1993). Analysis of a Three-Dimensional Point Pattern with Replication. *Appl. Stat.* **42**, 641–668.

Baddeley A.J., Møller J. & Waagepetersen, R. (2000). Non- and semiparametric estimation of interaction in inhomogeneous point patterns. *Stat. Neerl.* **54**, 329–350.

Baddeley, A. & Turner, R. (2005). Spatstat: an R package for analyzing spatial point patterns. *J. Stat. Softw.* **12**,1–42.

Bell, M.L. & Grunwald, G.K. (2004). Mixed models for the analysis of replicated spatial point patterns. *Biostatistics* **5**,633–648.

Besag, J.E. (1977). Comment on 'Modelling spatial patterns' by B.D. Ripley. *J. Roy. Stat. Soc. B Met.* **39**, 193–195.

Cressie, N.A.C. (1993). *Statistics for Spatial Data*. 2nd edition. New York: Wiley.

Diggle, P.J., Lange, N. & Beneš, F.M. (1991). Analysis of variance for replicated spatial point patterns in clinical neuroanatomy. *J. Am. Stat. Assoc.* **86**, 618–625.

Diggle, P.J., Mateu, J. & Clough, H.E. (2000). A comparison between parametric and non-parametric approaches to the analysis of replicated spatial point patterns. *Adv. Appl. Probab.* **32**, 331–343.

Diggle P.J. (2003). *Statistical Analysis of Spatial Point Patterns*. London: Academic Press.

Hossain, M.M. & Lawson, A.B. (2009). Approximative methods in Bayesian point process spatial models. *Comput. Stat. Data An.* **53**, 2831–2842.

Illian, J., Penttinen, A., Stoyan, H. & Stoyan, D. (2008). *Statistical Analysis and Modelling of Spatial Point Patterns*. Chichester: Wiley.

Illian, J. & Hendrichsen, D.K. (2010). Gibbs point process models with mixed effects. *Environmetrics* **21**, 341–353.

Kennedy, W.R. & Wendelschafer-Crabb, G. (1993). The innervation of human epidermis. *J. Neurol. Sci.* **115**, 184–190.

Kennedy, W.R., Wendelschafer-Crabb, G. & Johnson, T. (1996). Quantitation of epidermal nerves in diabetic neuropathy. *Neurology* **47**, 1042–1448.

Kennedy, W.R., Nolano, M., Wendelschafer-Crabb, G., Johnson, T.L. & Tamura, E. (1999). A skin blister method to study epidermal nerves in peripheral nerve disease. *Muscle Nerve* **22**, 360–371.

Landau, S. & Everall, I.O. (2008). Nonparametric bootstrap for K-functions arising from mixed-effects models with applications in neuropathology. *Stat. Sinica* **18**, 1375–1393.

Lauria, G., Holland, N., Hauer, P., Cornblath, D.R., Griffin, J.W. & McArthura, J.C. (1999). Epidermal innervation: changes with aging, topographic location, and in sensory neuropathy. *J. Neurol. Sci.* **164**, 172–178.

McCulloch, C.E. (2001). *Generalized, linear and mixed models*. New York: Johan Wiley & Sons.

Panoutsopoulou, I., Wendelschafer-Crabb, G., Hodges, J.S. & Kennedy, W.R. (2009). Skin blister and skin biopsy for quantifying epidermal nerve fibers: a comparative study. *Neurology* **72**, 1205–1210.

Pinheiro, J.C. & Bates, D.M. (2000) *Mixed-effects models in S and S-PLUS*. New York: Springer.

Ripley, B.D. (1976). The second-order analysis of stationary point processes. *J. Appl. Prob.* **13**, 255–266.

Ripley, B.D. (1977). Modelling spatial patterns. *J. Roy. Stat. Soc. B Met.* **39**, 172–192.

Rue, H., Martino, S. & Chopin, N. (2009). Approximate Bayesian Inference for Latent Gaussian Models Using Integrated Nested Laplace Approximations (with discussion). *J. Roy. Stat. Soc. B Met.* **71**, 319–392.

Schladitz, K., Särkkä, A., Pavenstädt, I., Haferkamp, O. & Mattfeldt, T. (2003). Statistical analysis of intramembranous particles using freeze fracture specimens. *J. Microsc.*, **211**, 137–153.

Waller, L.A. & Gotway, C.A. (2004). *Applied Spatial Statistics for Public Health Data*. Hoboken, NJ: Wiley.

Waller, L.A, Särkkä, A., Olsbo, V., Myllymäki, M., Panoutsopoulou, I.G., Kennedy, W.R. & Wendelschafer-Crabb, G. (2011). Second-order spatial analysis of epidermal nerve fibers. *Stat. Med.* **30**: 2827–2841.

Wang, L., Hilliges, M., Jernberg, T., Wiegleb-Edström, D. & Johansson, O. (1990). Protein gene product 9.5-immunoreactive nerve fibers and cells in human skin. *Cell Tissue Res.* **261**, 25–33.

Figure captions.

Fig. 1. Two point patterns of nerve entry points taken from the right foot of Subject 1103 (left) and Subject 1113 (right).

Fig. 2. Overall mean L-functions (thick solid lines) with r-wise 95% bootstrap envelopes (dashed lines) for calf (black) and for foot (gray).

Fig. 3. Comparison of the observed (gray solid lines) and predicted centered L functions (black dashed lines) for the foot data. The dotted line shows the fixed effect prediction of the linear mixed model. The ranges for r (on the x-axis) and $L(r) - r$ (on the y-axis) are $(10, 60)$ and $(-20, 50)$, respectively.

Fig. 4. Overall mean centered L functions (thick solid lines) in subgroups given by age (on the top), BMI (in the middle) and gender (on the bottom) with r-wise 95% bootstrap envelopes (dashed lines) for calf (left) and foot (right).

Fig. 5. Boxplots of the regression coefficients from separate fits of the fourth order polynomials to each subject and each sample within subject for the foot data.

Fig. 6. Residual plots of the mixed models of the foot data (left) and for the calf data (right). On the top: Plot of the standardized residuals versus $1/n_{ij}^2$. On the bottom: Normal probability plot of the within-group standardized

residuals.