Invertebrate Biology

Invertebrate Biology 130(4): 344–350.

DOI: 10.1111/j.1744-7410.2011.00238.x

# Genome-wide search for leech antiplatelet proteins in the non-blood-feeding leech *Helobdella robusta* (Rhyncobdellida: Glossiphoniidae) reveals evidence of secreted anticoagulants

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**Abstract.** The genome of the non-blood-feeding glossiphoniid leech *Helobdella robusta* was screened for leech antiplatelet protein (LAPP), an anticoagulant that specifically inhibits collagen-stimulated platelet aggregation. Previously identified LAPP sequences from *Haementeria officinalis* were used as queries against the predicted genes in the genome, employing a variety of BLAST protocols. Matches were reciprocally BLASTed against GenBank databases as a cross-validation of the predicted annotations of the genes. A total of eight loci, positioned as a tandem array, were recovered with significantly low e-values; these showed high sequence similarity (32.49% average sequence similarity of shared amino acid positions) to the known anticoagulants. Moreover, six of these possessed a predicted signal-peptide toward the N-terminus, indicating their secretion by the leech. All eight loci, together with known LAPP sequences from *Ha. officinalis*, as well as several sequences from publicly available expressed sequence tag libraries of *Ha. depressa* and *He. robusta*, were aligned and subjected to phylogenetic analysis. The resulting tree showed a monophyletic clade consisting of the *He. robusta* loci, which was sister to a clade comprised of *Haementeria*-derived sequences. To corroborate the evolution of the anticoagulants with the evolution of leeches more generally, the topology of the LAPP-tree was compared to that of a previously published leech phylogeny; these showed compatible topologies with respect to the included genera. These results corroborate recent phylogenetic work, which suggests that this non-blood-feeding leech has a hematophagous ancestry.

*Additional key words:* genomics, hematophagy, liquidosomatophagy, phylogeny, *Helobdella robusta*, anticoagulants, LAPP

Leeches (Hirudinida) have evolved suites of anticoagulant salivary proteins that are injected into the feeding site to facilitate blood feeding. They are used by leeches not only to prevent blood from coagulating around the incision wound of the prey, but also to maintain the blood in a suitable state during the long periods of digestion by the leech (Salzet 2001). One of these salivary proteins is leech antiplatelet protein (LAPP), which was first isolated from the glossiphoniid leech *Haementeria officinalis* De Filippi 1849, and characterized as an inhibitor of collagen-stimulated platelet aggregation (Connolly et al. 1992). A normal thrombus formation, following injury to vascular walls, is mediated by von Willebrand factor (vWf) via its conformational change of proaggregatory collagen (Ruggeri 1997) and an irreversible binding to the surface glycoprotein complex GP Ib/IX/V (Obert et al. 1999; Watson et al. 2000). This leads to activation of the platelet and subsequent secretion of its granular contents, which form aggregates stimulating both thrombosis and the further activation of platelets (Connolly et al. 1992; Carter et al. 1998). Like saratin, which is a LAPP homolog first isolated from the European medicinal leech *Hirudo medicinalis*

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Linnaeus 1758 (Cruz et al. 2001), LAPP residues bind to subendothelial collagen, thus inhibiting the vWf-mediated activation of the platelets. LAPP and saratin have been isolated from a variety of blood feeding leeches from different taxonomic families (Connolly et al. 1992; Barnes et al. 2001; Min et al. 2010) including *Haementeria depressa* Ringuelet 1972 and *Ha. officinalis* (Glossiphoniidae), *Hi. medicinalis* (Hirudinidae), and *Macrobdella decora* (Say 1824) (Macrobdellidae).

*Helobdella robusta* Shankland et al. 1992 is a non-blood-feeding freshwater leech in the family Glossiphoniidae. Members of this species feed primarily on the haemolymphal fluids of freshwater snails, a strategy known as liquidosomatophagy. However, if members of this lineage had a recent hematophagous past, remnants of that ancestry may still be encoded in their genomes. Owing largely to the transparent bodies of these organisms, but also to the fact that their egg-cases (cocoons) are rather large and easily maintained in a laboratory setting, *He. robusta* has rapidly become central to evolutionary developmental studies of annelids (e.g., Shain 2009). In 2007, the DOE Joint Genome Institute released results from a full-genome sequencing effort of *He. robusta*. This genome enables investigations of the presence of anticoagulants in this non-blood-feeding leech. Herein, we focus on investigating the presence of LAPP in *He. robusta*, the putative secretion of the protein, and on corroborating the evolution of the anticoagulant in the context of the evolution of the leech species that possess it.

## Methods

### Characterization of putative salivary peptides

The full genome of *Helobdella robusta* is available on the Joint Genome Institute (JGI) portal website (http://genome.jgi-psf.org/Helro1/Helro1.home.html). It consists of 2,354,463 reads in 1993 scaffolds, for a total of 235.4 Mbp.

Amino acid sequences of previously characterized LAPP and saratin from blood-feeding leeches were employed as queries to matches against the *He. robusta* genome. Queries were conducted using the tBLASTn and BLASTp algorithms on the JGI portal website with an e-value cut-off of $1E^{-2}$, without filtering low complexity regions, and using a gapped alignment with a BLOSUM62 scoring matrix. Complementary to this, word searches were performed for both anticoagulants among the gene annotations already accomplished for the *He. robusta* genome. All matching regions were retained as nucleotide sequence records and localized on the JGI genome browser so as to correlate with gene predictions at each locus. Where more than one gene prediction model identified multiple identical loci, only one was retained to avoid redundancy. For all anticoagulant loci recovered in the *He. robusta* genome, both the nucleotide sequences (with introns removed) and their translated amino acid sequences were individually compared against the non-redundant (nr) GenBank nucleotide and protein sequence databases (using tBLASTx and BLASTp, respectively) as a definitive cross-validation of the predicted annotation (reciprocal BLAST or "bi-directional best hit" approach; Fang et al. 2010). To localize expressed putative orthologs, candidate anticoagulants from the *He. robusta* genome were each compared using the tBLASTx and BLASTn algorithms to available expressed sequence tag (EST) libraries (in GenBank, and in a stand-alone *Hirudo medicinalis* EST library at http://genomes.ucsd.edu/leechmaster/database). In addition, to recover potential phylogenetic outgroups, LAPP from *Haementeria officinalis* and saratin from *Hi. medicinalis* were queried against the entire GenBank nr nucleotide and protein databases, and the GenBank EST database, as well as the entire genome of the polychaete *Capitella teleta* Blake et al. 2009 on the JGI website, using BLASTn and BLASTx algorithms.

Signal peptides at the N-terminus were predicted using the SignalP 3.0 (Bendtsen et al. 2004) server at the Center for Biological Sequence Analysis website (http://www.cbs.dtu.dk/services/SignalP/) employing both neural networks and hidden Markov models for prediction. To identify and characterize conservation levels within the full genome-derived sequences, these were aligned with known anticoagulants using RevTrans 1.4 (Wernersson & Pedersen 2003) in accordance with their inferred amino acid states. The alignment used Clustal W 1.83 (Thompson et al. 1994) as implemented in RevTrans and used the Standard Genetic Code translation table. The amino acid alignment was visualized using Jalview ver. 2 (Waterhouse et al. 2009), where percent similarity was calculated by hand.

### Phylogenetic analysis

Alignment of nucleotide sequences used for the phylogenetic analysis was accomplished with RevTrans 1.4 in accordance with their inferred amino acid states. Aligned sequences were subjected to phylogenetic analysis under the parsimony criterion using TNT (Goloboff et al. 2008). A heuristic search was performed using the traditional search option

with 100 random addition sequences and employing TBR branch swapping. Support values for the nodes were retrieved through standard bootstrap re-sampling with 1000 iterations each consisting of ten random addition sequence replicates and with the same settings as above. For branch length comparisons, the matrix and resulting TNT trees were imported into PAUP* ver. 2.0b10 (Swofford 2002) and branch lengths were calculated. The tree was rooted using saratin from *Hi. medicinalis*.

## Results

### Leech antiplatelet protein loci

A total of eight loci matching LAPP from *Haementeria officinalis* were found in the *Helobdella robusta* genome (Table 1). When reciprocally BLASTed against the GenBank protein database, all of these loci matched leech antiplatelet proteins better than anything else; six of these were matched at e-values $<1E^{-5}$, whereas two of them only hit with marginal e-values (BLASTp scores of 0.004 and 0.005). All eight of these putative LAPP-loci co-localized in a tandem array in the *He. robusta* genome. Significant matches for the eight full-genome-derived loci also were found in EST libraries that are available for leeches (Table 1). No matches were found in the *Hirudo robusta* genome for saratin, and no putative LAPP orthologs were found in the *Hirudo medicinalis* EST library.

### Protein conservation and secretion

The amino acid alignment included nine loci; all eight of the full genome-derived loci from *He. robusta*, and LAPP from *Ha. officinalis*. The average similarity of shared amino acid positions was 32.49%. The six disulfide-bond-forming cysteines, indicative of antiplatelet proteins (Min et al. 2010), showed full conservation across the eight full-genome-derived sequences. In addition, Signal P predicted the presence of signal peptide regions in all but two sequences from the *He. robusta* genome (positions 10–27; Fig. 1).

### Phylogeny

The BLASTx and BLASTn analyses using LAPP from *Ha. officinalis* and saratin from *Hi. medicinalis* against the entire GenBank nr database returned no hits, suggesting that no orthologous proteins are present outside of the leech taxa used in the present study. Thus, no outgroups could be used for the phylogenetic analysis performed herein. The LAPP data set included 23 nucleotide sequences: eight loci derived from the full genome of *He. robusta*, ten *He. robusta* EST sequences, three LAPP-loci from *Ha. officinalis* and *Ha. depressa* EST libraries, and two saratin loci from *Hi. medicinalis* and *M. decora*. A total of 549 aligned sites were analyzed, 279 of which were parsimony informative. The heuristic search returned a single most parsimonious tree with

**Table 1.** Top matches in the GenBank nr nucleotide and protein databases, as well as EST libraries, using *Helobdella robusta* loci as queries. The *He. robusta* loci were identified through a tblastn search using a known anticoagulant sequence as query (GenBank Accn. M81489).

| *He. robusta* locus | tblastx GenBank nr. | blastp GenBank nr. | tblastx *Helobdella robusta* EST library (GenBank) | tblastx *Haementeria depressa* EST library (GenBank) |
|---|---|---|---|---|
| e_gw1.2.270.1 | M81489 LAPP ($1E^{-12}$) | Q01747 antiplatelet protein ($1E^{-13}$) | EY344275 ($2E^{-64}$) | CN807637 LAPP ($2E^{-16}$) |
| fgenesh4_pg.C_scaffold_2000933 | - | Q01747 antiplatelet protein ($5E^{-3}$) | EY344275 ($2E^{-64}$) | CN807637 LAPP ($2E^{-7}$) |
| e_gw1.2.291.1 | - | 1I8N_A LAPP ($2E^{-7}$) | EY349090 ($2E^{-56}$) | CN807637 LAPP ($1E^{-5}$) |
| fgenesh4_pg.C_scaffold_2000928 | - | Q01747 antiplatelet protein ($2E^{-5}$) | EY361718 ($4E^{-61}$) | CN807637 LAPP ($5E^{-6}$) |
| estExt_Genewise1.C_21054 | - | Q01747 antiplatelet protein ($2E^{-7}$) | EY349090 ($1E^{-75}$) | CN807637 LAPP ($8E^{-5}$) |
| fgenesh4_pg.C_scaffold_2000934 | - | Q01747 antiplatelet protein ($3E^{-5}$) | EY341220 ($3E^{-59}$) | CN807637 LAPP ($3E^{-5}$) |
| fgenesh4_pg.C_scaffold_2000930 | M81489 LAPP ($4E^{-3}$) | Q01747 antiplatelet protein ($5E^{-7}$) | EY392612 ($5E^{-56}$) | CN807637 LAPP ($2E^{-6}$) |
| fgenesh4_pg.C_scaffold_2000932 | - | Q01747 antiplatelet protein ($4E^{-3}$) | EY378603 ($6E^{-59}$) | CN807637 LAPP ($4E^{-5}$) |

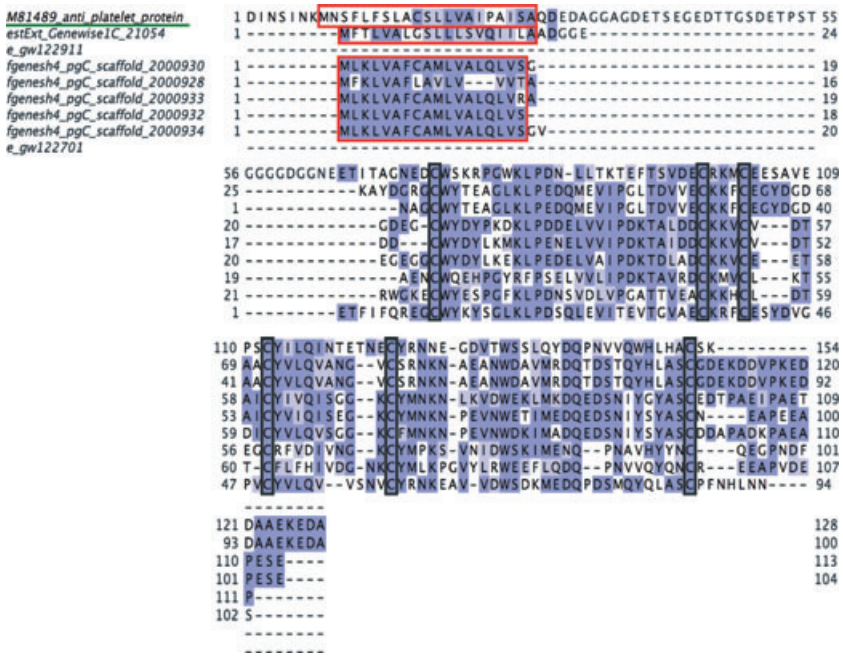


**Fig. 1.** Alignment of inferred amino acid sequences for *Helobdella robusta* leech antiplatelet protein loci together with the known anticoagulant from *Haementeria officinalis*. Red boxes indicate predicted signal peptides, black boxes indicate fully conserved cysteines and green underlining denotes the taxon with a known sequence of the anticoagulant. Shading intensity corresponds to BLOSUM62 conservation.

1068 steps (Fig. 2a). The tree revealed a cluster grouping LAPP from *Ha. officinalis* (the annotated sequence M81489) with two EST loci from *Ha. depressa* (CN807637, CN807641). Sister to that group is a monophyletic cluster of all *He. robusta* loci with full-genome-derived and EST-derived loci interspersed. Several of the loci derived from the full genome are almost identical to the corresponding *He. robusta* EST sequences. In turn, the *Helobdella*/*Haementeria* group was recovered as sister to the *Hi. medicinalis*/*M. decora* group. To corroborate the phylogenetic hypothesis derived from the *Helobdella* and *Haementeria* LAPP dataset, the topology of the LAPP tree was compared to that of a previously published phylogenetic hypothesis of leeches (Fig. 2b; Min et al. 2010). The topology of the LAPP tree, concerning the relationships between LAPP orthologs of *Haementeria* and *Helobdella* versus the saratin orthologs, mirrors the topology of the more data-rich phylogeny of leeches.

## Discussion

### Hematophagous ancestry

The similarity of predicted genes in the *Helobdella robusta* genome with a known leech salivary gland-secreted anticoagulant (LAPP) is powerful corroboration of recent phylogenetic work, which suggests that this non-blood-feeding leech has a hematophagous ancestry (Siddall et al. 1995, 1996; Trontelj et al. 1999). Because Glossiphoniidae is typically recovered toward the base of the phylogeny of leeches in independent studies (Siddall et al. 1998, 2001; Apakupakul et al. 1999; but see Trontelj et al. 1999), this result shows that possession of anticoagulants is not restricted to taxa in the more derived parts of the phylogenetic tree. That is, our finding belies the long-held notion that hematophagy is a recently derived trait (Mann 1962; Sawyer 1986; see Siddall et al. 1996).

The present study represents the first evidence of an anticoagulant in a non-blood-feeding glossiphoniid leech. Interestingly, Kim et al. (1996) isolated and biochemically characterized the serine protease inhibitor Guamerin II from the macrophagous hirudinid (*sensu* Phillips & Siddall 2009) *Whitmania edentula* (Whitman 1886). Furthermore, Hovingh & Linker (1999) identified a hyaluronoglucuronidase in the macrophagous erpobdellids *Erpobdella obscura* (Verrill 1872) and *E. punctata* (Leidy 1870). Taken together, these findings corroborate independent losses of blood feeding throughout the evolutionary history of leeches as hypothesized by Siddall et al. (1995, 1996) and Trontelj et al. (1999).

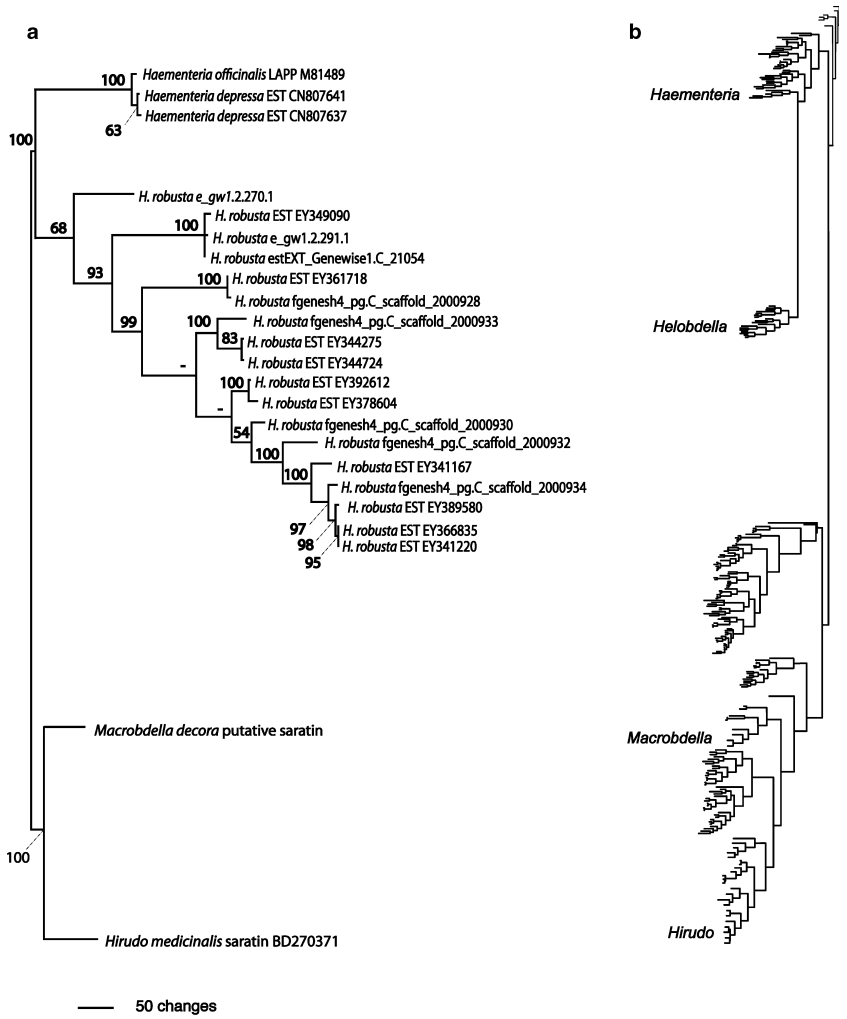


**Fig. 2.** Phylogenetic hypotheses concerning leech antiplatelet protein (LAPP) loci and leech genera. **a.** Single most parsimonious tree recovered from the heuristic search using the LAPP data set (L=1068 steps; CI=0.658; RI=0.784). Bootstrap values >50% are shown above each node. **b.** Phylogenetic hypothesis of leeches modified from Min et al. (2010) with the genera *Haementeria, Helobdella, Macrobdella*, and *Hirudo* (all discussed in the text) highlighted. Trees A and B are in agreement concerning the aforementioned genera.

## The role of LAPP and its genomic positioning in *Helobdella robusta*

Because sequences almost identical to LAPP were found in *He. robusta* EST libraries, anticoagulant-like proteins seem to be expressed in this leech. Furthermore, several of the putative vWf-blocking proteins contain a signal-peptide-encoding region toward the N-terminus, indicating their secretion by the leech (Fig. 1). However, the functional role of these secreted anticoagulant proteins in *He. robusta* is not yet clear.

Anticoagulation factors and lytic proteases have previously been isolated from oligochaetous clitellates (Mihara et al. 1991; Jeon et al. 1995; Popovic et al. 1998). Some of these are specifically able to lyse collagen and laminin (Alberts et al. 1994; Jeon et al. 1995). The paraphyletic status of Clitellata with several oligochaetous clitellates diverging earlier than Hirudinida (Rousset et al. 2007), coupled with the apparent patchy expression pattern of anticoagulants and (paralogous) collagen proteases across this group, raises some questions concerning the evolutionary history of the proteins. One hypothesis would be the retention of these proteins throughout Clitellata, enabling the change in feeding strategy from macrophagy in several clitellates (including oligochaetes) to liquidosomatophagy in, e.g., *He. robusta*. Like a macrophagous lifestyle, liquidosomatophagy would require the breaking down of subendothelial collagen, with anticoagulation properties as a secondary effect. In turn, this would

suggest that the LAPP-like proteins already present in the genomes of related proboscis-bearing (rhyncobdellid) species may be used for dual purposes: as a means for keeping blood flowing in and around the incision wound, and as a collagen protease. This breaking down of collagen is not as obvious a need for jaw-bearing (arhynchobdellid) hematophagous species, which restrict their incisions to the skin surface, and this may be why the expression of collagen-binding/lysing proteins is not obvious in these species (e.g., *Hirudo medicinalis*, as mentioned above).

Interestingly, all the eight predicted genes matching LAPP are positioned as tandem repeats in the *He. robusta* genome. This may be due to linked functionality between the loci. For example, where no independent promoter region exists between the loci, the RNA polymerase may simultaneously transcribe them all in a single pass. This would enable rapid, high-copy translation of a variety of LAPPs; given the diversity of collagen (Eyre 1980), it is tempting to speculate that this tandem array of LAPPs would be capable of simultaneously targeting an array of different collagen types. Notably, platelet glycoproteins such as Ib and IIIa exhibit a variable number of tandem repeats (Carter et al. 1998). Although the platelets targeted by LAPP and saratin are of different kinds than glycoprotein Ib and IIIa, at this stage we cannot rule out that the agonists also have a structural connection to the platelets. Koh & Kini (2009) demonstrated that the structure of both Kazal-type proteinase inhibitors and antistasin-like inhibitors include tandem repeat domains. Unfortunately, that study does not include antiplatelet proteins. X-ray crystallography and/or nuclear magnetic resonance studies would likely shed light on any interactions both between the domains in each LAPP and between these consecutive loci.

### Phylogeny

There are three possible major topological outcomes of the phylogenetic analysis conducted in the present study. Specifically, (i) LAPP from the two species of *Haementeria* could be identified as sister to saratin, (ii) LAPP from *He. robusta* could be identified as sister to saratin, and (iii) the *Haementeria* and *He. robusta* orthologs could be identified as sister to each other. The hypothesis presented herein shows the latter topology, and this is congruent with the phylogenetic hypothesis of leeches presented by Min et al. (2010). In other words, the topology of the phylogenetic tree of leeches is in agreement with the tree derived from the LAPP-loci in terms of the concerned genera.

## Conclusions

Through a combination of similarity analyses (BLASTn, BLASTp, tBLASTn, and tBLASTx) and phylogenetic analysis, we have shown that the genome of the non-blood-feeding glossiphoniid leech *He. robusta* possesses putative orthologs of a known anticoagulant, LAPP. In light of previous phylogenetic hypotheses recovering *Helobdella* at the base of the leech tree, this finding suggests that the presence of anticoagulants is plesiomorphic in Hirudinida. The eight LAPP-like loci found in *He. robusta* are represented as a tandem array, a phenomenon that already characterizes several other anticoagulation factors, and one that may bring significant benefits to the transcription efficiency of these specific DNA regions.

**Acknowledgments.** We thank the Joint Genome Institute for making the *Helobdella robusta* genome publicly available online. The sequence data were produced by the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/) in collaboration with the user community. We are grateful to Anna Phillips, Alejandro Oceguera-Figueroa, and two anonymous reviewers whose comments on an early draft of the manuscript led to a much-improved version. This contribution was funded by the Swedish-American foundation fellowship and a Helge Ax:son Johnsons foundation stipend to SK, and NSF grant DEB-0640463 to MES. INS is supported in part by DHHS/NIH/NLM grant R01LM009725.

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