Early Treatment of Diabetic Retinopathy Scale (ETDRS)

Retinopathy status will be graded in standard 7-field stereoscopic color photographs using the Early Treatment of Diabetic Retinopathy Scale (ETDRS) modification of the Airlie House Classification scheme which assesses the level of retinopathy for each eye (1, 2). This grading system has been used extensively in clinical and epidemiological studies of diabetic retinopathy, including the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS), to assess baseline status of retinopathy and progression of disease (3-10). The applicable levels of an abbreviated version of the ETDRS scale to be used in the study are summarized below:

<table>
<thead>
<tr>
<th>Level</th>
<th>Severity</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>No retinopathy</td>
<td>Diabetic retinopathy absent</td>
</tr>
<tr>
<td>20</td>
<td>Very mild NPDR</td>
<td>MA only</td>
</tr>
<tr>
<td>35</td>
<td>Mild NPDR</td>
<td>MA plus hard exudates, cotton wool spots, and/or mild retinal hemorrhages</td>
</tr>
<tr>
<td>43*</td>
<td>Moderate NPDR</td>
<td>MA plus mild IRMA or moderate retinal hemorrhages</td>
</tr>
<tr>
<td>47</td>
<td>Moderate NPDR</td>
<td>More extensive IRMA, severe retinal hemorrhages, or venous beading in one quadrant</td>
</tr>
<tr>
<td>53</td>
<td>Severe NPDR</td>
<td>Severe retinal hemorrhages in four quadrants, or venous beading in at least two quadrants, or moderately severe IRMA in at least one quadrant</td>
</tr>
</tbody>
</table>

Chart derived from Diabetes Complications and Control Trial Final Version of the ETDRS Scale of Diabetic Retinopathy Severity for individual eyes (6).

NPDR: nonproliferative diabetic retinopathy
MA: microaneurysms
IRMA: intraretinal microvascular abnormalities

*Patients with levels of 43 or greater will be excluded from Group 1.

Because Early CAN diabetic subjects are limited to individuals with mild NPDR, a further division of Level 20 into two subgroups will be made. The division of Level 20 will be based on the number of microaneurysms counted in the retinal photographs: the subgroups will be 20a, with \( \geq 1 \) and <3 microaneurysms (these subjects will be ineligible for the study), and 20b, with \( \geq 3 \) microaneurysms (eligible for inclusion). The rationale for using microaneurysm counts to further subdivide Level 20 is based on observations that the number of microaneurysms at baseline correlates with severity of retinopathy (5, 7, 11, 12) and is a significant predictor of risk of progression to more severe stages of retinopathy, independent of glycemic status or type of diabetes (5, 7, 11). The division of Level 20 into those individuals with 1 or 2 microaneurysms and those with 3 or more microaneurysms is derived from the relative risk of progression. Data from a population-based, 10-year follow-up of individuals with early diabetic retinopathy suggest that a threshold effect is present at this level, since eyes with 3 or more microaneurysms in retinal photographs at baseline have a 2.3-fold relative risk of progression to proliferative diabetic retinopathy (and demonstrate augmented SS reactivity and early CAN), compared to those with fewer than 3 microaneurysms at a 10-year follow-up (11)
The ETDRS modification of the Airlie House classification scheme of diabetic retinopathy proposed for use in this study has been validated as a means of measuring progression of disease in prospective studies (3-6, 8, 10).

Subjects will be characterized according to retinopathy status and assigned to one of two major groups. Diabetic subjects with early CAN will comprise subjects with mild NPDR (Levels 20b-35). This classification is consistent with the classification of subjects in our Preliminary Studies in which subjects were divided by the absence of microaneurysms and the presence of >20 microaneurysms. Ophthalmologic exclusion criteria will include lack of consent to retinal photographs, features of ETDRS Level 43 (i.e. moderate NPDR) or greater on retinal photographs, evidence of prior retinal photocoagulation procedures or any ocular complication that may affect accurate readings of the retinal photographs or may suggest another cause of their retinopathy. Diabetic control subjects will comprise subjects without NPDR (level 10).

References

NEPHROPATHY
MANUAL OF OPERATIONS

URINARY ALBUMIN ASSESSMENT

Diabetic subjects will be evaluated for the presence of microalbuminuria using recommendations from the American Diabetes Association. 80% of subjects with type 1 diabetes who develop sustained microalbuminuria will increase the rate of urinary albumin excretion by 10-20% per year (ADA) to a stage of overt nephropathy over a period of 10-15 years. All patients will undergo three 24 h collections of urine with creatinine taken at intervals of one week. For the purposes of this study, all diabetic subjects will be excluded if any qualifying urine collection meets the criteria for diabetic nephropathy. Subjects will be instructed not to exercise for 24 h prior to the urine collection period, and the presence of fever, marked hyperglycemia, hypertension (>140/90), pyuria and hematuria will result in the postponement or exclusion of the urine collection.

<table>
<thead>
<tr>
<th>Category</th>
<th>24-h collection (mg/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No nephropathy</td>
<td>&lt;30</td>
</tr>
<tr>
<td>Microalbuminuria</td>
<td>30-299</td>
</tr>
<tr>
<td>Clinical albuminuria</td>
<td>≥300</td>
</tr>
</tbody>
</table>

References

PERIPHERAL NEUROPATHY
THE MICHIGAN DIABETIC NEUROPATHY SCORE (MDNS)

This provides a quantitative neurological assessment of sensation, strength and reflexes in the extremities, with emphasis on the hands and feet. This instrument is currently in use in the continuation study of the DCCT, the NIH funded Epidemiology of Diabetes and Complications Trial. We have validated this tool (1) and administered the MDNS to over 8,000 patients (2). Briefly, the MDNS consists of a quantitative clinical neurological examination. Vibratory threshold perception pain and light touch are assessed with a 128 Hz tuning fork, a pin and a 10-gram filament, respectively. Previous work suggests that monofilaments, as a measure of touch perception, can indicate the pressure threshold that confers protection against plantar ulceration (3). In the current protocol, the 10-gram filament is applied to the dorsum of the great toe and the patient is asked to respond yes if he or she feels the filament. Eight correct responses out of 10 applications are considered normal (score of 0); 1 to 7 correct responses indicates reduced sensation (score of 1) and no correct answers translates as absent sensation (score of 2). Tendon reflexes are scored as 0 for normal, 1 for abnormal, and 2 for absent responses. Muscle strength is scored as 0 for normal, 1 for mild-moderate and 2 for severe weakness, while complete loss of strength is scored as 3. For example, sensation that is present but reduced, reflexes that are present only with reinforcement, and mild-moderate but not severe weakness are each scored as “1.” Absent reflexes, absent sensation and severe weakness are scored as “2.” A score of ≥2 is abnormal.

THE MICHIGAN SENSORY NEUROPATHY INVENTORY (MSNI)

The MSNI is a simple tool to assess symptoms. Patients are asked to answer 15 questions. This questionnaire, like the MDNS, has been extensively field tested and validated. A positive response on 4 or more questions correlates with the presence of quantifiable neuropathy (2).

History
The history questionnaire is self-administered by the patient. Responses are added to obtain the total score. Responses of “yes” to items 1-3, 5-6, 8-9, 11-12, 14-15 are each counted as one point. A “no” response on items 7 and 13 counts as 1 point. Item #4 is a measure of impaired circulation and item #10 is a measure of general aesthenia and are not included in scoring. To decrease the potential for bias, all scoring information has been eliminated from the patient version.

Physical Assessment
For all assessments, the foot should be warm (>30°C).

Foot Inspection: The feet are inspected for evidence of excessively dry skin, callous formation, fissures, frank ulceration or deformities. Deformities include flat feet, hammer toes, overlapping toes, halux valgus, joint subluxation, prominent metatarsal heads, medial convexity (Charcot foot) and amputation.

Vibration Sensation: Vibration sensation should be performed with the great toe unsupported. Vibration sensation will be tested bilaterally using a 128 Hz tuning fork placed over the dorsum of the great toe on the boney prominence of the DIP joint. Patients, whose eyes are closed, will be asked to indicate when they can no longer sense the vibration from the vibrating tuning fork.
In general, the examiner should be able to feel vibration from the hand-held tuning fork for 5 seconds longer on his distal forefinger than a normal subject can at the great toe (e.g. examiner’s DIP joint of the first finger versus patient’s toe). If the examiner feels vibration for 10 or more seconds on his or her finger, then vibration is considered decreased. A trial should be given when the tuning fork is not vibrating to be certain that the patient is responding to vibration and not pressure or some other clue. Vibration is scored as 1) present if the examiner senses the vibration on his or her finger for < 10 seconds, 2) reduced if sensed for ≥ 10 or 3) absent (no vibration detection.)

**Muscle Stretch Reflexes:** The ankle reflexes will be examined using an appropriate reflex hammer (e.g. Trommer or Queen square). The ankle reflexes should be elicited in the sitting position with the foot dependent and the patient relaxed. For the reflex, the foot should be passively positioned and the foot dorsiflexed slightly to obtain optimal stretch of the muscle. The Achilles tendon should be percussed directly. If the reflex is obtained, it is graded as present. If the reflex is absent, the patient is asked to perform the Jendrassic maneuver (i.e., hooking the fingers together and pulling). Reflexes elicited with the Jendrassic maneuver alone are designated “present with reinforcement.” If the reflex is absent, even in the face of the Jendrassic maneuver, the reflex is considered absent.

**Monofilament Testing:** For this examination, it is important that the patient’s foot be supported (i.e., allow the sole of the foot to rest on a flat, warm surface). The filament should initially be prestressed (4-6 perpendicular applications to the dorsum of the examiner’s first finger). The filament is then applied to the dorsum of the great toe midway between the nail fold and the DIP joint. Do not hold the toe directly. The filament is applied perpendicularly and briefly, (<1 second) with an even pressure. When the filament bends, the force of 10 grams has been applied. The patient, whose eyes are closed, is asked to respond yes if he/she feels the filament. Eight correct responses out of 10 applications is considered normal: one to seven correct responses indicates reduced sensation and no correct answers translates into absent sensation.

**References**


MICHIGAN NEUROPATHY SCREENING INSTRUMENT
(Scoring Version)

History (To be completed by the person with diabetes)

Please take a few minutes to answer the following questions about the feeling in your legs and feet. Check yes or no based on how you usually feel. Thank you.

1. Are your legs and/or feet numb? □ 1 Yes □ 0 No
2. Do you ever have any burning pain in your legs and/or feet? □ 1 Yes □ 0 No
3. Are your feet too sensitive to touch? □ 1 Yes □ 0 No
4. Do you get muscle cramps in your legs and/or feet? □ 0 Yes □ 0 No
5. Do you ever have any prickling feelings in your legs or feet? □ 1 Yes □ 0 No
6. Does it hurt when the bed covers touch your skin? □ 1 Yes □ 0 No
7. When you get into the tub or shower, are you able to tell the hot water from the cold water? □ 0 Yes □ 1 No
8. Have you ever had an open sore on your foot? □ 1 Yes □ 0 No
9. Has your doctor ever told you that you have diabetic neuropathy? □ 1 Yes □ 0 No
10. Do you feel weak all over most of the time? □ 0 Yes □ 0 No
11. Are your symptoms worse at night? □ 1 Yes □ 0 No
12. Do your legs hurt when you walk? □ 1 Yes □ 0 No
13. Are you able to sense your feet when you walk? □ 0 Yes □ 1 No
14. Is the skin on your feet so dry that it cracks open? □ 1 Yes □ 0 No
15. Have you ever had an amputation? □ 1 Yes □ 0 No

Total: ______
(13 maximum)
MICHIGAN NEUROPATHY SCREENING INSTRUMENT
(Patient Version)

History (To be completed by the person with diabetes)

Please take a few minutes to answer the following questions about the feeling in your legs and feet. Check yes or no based on how you usually feel. Thank you.

1. Are your legs and/or feet numb? □ Yes □ No
2. Do you ever have any burning pain in your legs and/or feet? □ Yes □ No
3. Are your feet too sensitive to touch? □ Yes □ No
4. Do you get muscle cramps in your legs and/or feet? □ Yes □ No
5. Do you ever have any prickling feelings in your legs or feet? □ Yes □ No
6. Does it hurt when the bed covers touch your skin? □ Yes □ No
7. When you get into the tub or shower, are you able to tell the hot water from the cold water? □ Yes □ No
8. Have you ever had an open sore on your foot? □ Yes □ No
9. Has your doctor ever told you that you have diabetic neuropathy? □ Yes □ No
10. Do you feel weak all over most of the time? □ Yes □ No
11. Are your symptoms worse at night? □ Yes □ No
12. Do your legs hurt when you walk? □ Yes □ No
13. Are you able to sense your feet when you walk? □ Yes □ No
14. Is the skin on your feet so dry that it cracks open? □ Yes □ No
15. Have you ever had an amputation? □ Yes □ No

Total: ______________
## MICHIGAN NEUROPATHY SCREENING INSTRUMENT

### B. Physical Assessment

(To be completed by health professional)

#### 1. Appearance of Feet

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<thead>
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<th></th>
<th>Right</th>
<th></th>
<th>Left</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>No</td>
<td>Normal</td>
</tr>
<tr>
<td>a. Normal</td>
<td>□ 0 Yes</td>
<td>□ 1 No</td>
<td>□ 0 Yes</td>
</tr>
<tr>
<td>b. If no, check all that apply:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Deformities</td>
<td>□</td>
<td>Deformities</td>
<td>□</td>
</tr>
<tr>
<td>Dry skin, callus</td>
<td>□</td>
<td>Dry skin, callus</td>
<td>□</td>
</tr>
<tr>
<td>Infection</td>
<td>□</td>
<td>Infection</td>
<td>□</td>
</tr>
<tr>
<td>Fissure</td>
<td>□</td>
<td>Fissure</td>
<td>□</td>
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<tr>
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### 2. Ulceration

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<tbody>
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<td>Absent</td>
<td>Present</td>
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<tr>
<td>2. Ulceration</td>
<td>□ 0</td>
<td>□ 1</td>
<td>□ 0</td>
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</table>

### 3. Ankle Reflexes

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<tr>
<th></th>
<th>Right</th>
<th></th>
<th>Left</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Present</td>
<td>Present/</td>
<td>Absent</td>
</tr>
<tr>
<td>Ankle Reflexes</td>
<td>□ 0</td>
<td>□ 0.5</td>
<td>□ 1</td>
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</tbody>
</table>

### 4. Vibration perception at great toe

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th>Left</th>
</tr>
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<tr>
<td></td>
<td>Present</td>
<td>Decreased</td>
<td>Absent</td>
</tr>
<tr>
<td>Vibration</td>
<td>□ 0</td>
<td>□ 0.5</td>
<td>□ 1</td>
</tr>
</tbody>
</table>

### 5. Monofilament

<table>
<thead>
<tr>
<th></th>
<th>Right</th>
<th></th>
<th>Left</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Reduced</td>
<td>Absent</td>
</tr>
<tr>
<td>Monofilament</td>
<td>□ 0</td>
<td>□ 0.5</td>
<td>□ 1</td>
</tr>
</tbody>
</table>

Signature: ________________________________

Total Score _____________________________ /10

Modified: 27 January 2004
QUANTITATIVE SENSORY TESTING (QST)

- Cold Detection Threshold (CDT), Vibration Detection Threshold (VDT), and Heat Pain Responses

The threshold for cooling (small nerve fiber function) and vibration (large nerve fiber function) sensation will be obtained for all patients. CDT will be assessed on the dorsum of the foot, and the VDT on the dorsum of the great toe, using the CASE IV sensory testing device. The methodology, validity and reproducibility of psychophysical methods of quantitative sensory testing has been previously described (1-3). Measurements will be performed on the left side, unless physical abnormalities or other considerations require the measurement to be made on the right side. For each patient the cold detection threshold percentile will be converted to a standard normal deviate relative to an extensive group of age and sex matched normal subjects as previously described by Dr. Peter Dyck. This will allow for age-specific variance. Based on previous clinical studies a difference of 0.09 standard normal deviates for CDT or VDT correlates with a change of 2 points on the neuropathy impairment score (NIS) and is felt to be clinically significant (2).

References

COOLING DETECTION THRESHOLD (CDT)

Patient Indentification: 
Accession Number: 
Age: 
Sex: 
Date: 
Visit Number: 

Foot Examined: 
_____ Left 
_____ Right - only if left not available 
_____ Not Done 

Reminder: Use the left side for all QST assessments if possible
The same side must be used for all testings
The side used at Baseline must be used consistently throughout the study
To record a negative value for Normal Deviate, please enter a minus (-) sign in the first box

<table>
<thead>
<tr>
<th>Site</th>
<th>Test</th>
<th>JND</th>
<th>Normal Deviate</th>
<th>No Response (&gt;25 JND)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsum of Foot</td>
<td>Cooling Detection Threshold</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Technician’s Signature: ___________________________ Date: __________

Neurologist’s Signature: ___________________________ Date: __________
VIBRATION DETECTION THRESHOLD (VDT)

Patient Identification: ________
Accession Number: ________
Age: ________
Sex: ________
Date: ________
Visit Number: ________

Foot Examined:
_____ Left
_____ Right - only if left not available
_____ Not Done

Reminder: Use the left side for all QST assessments if possible
The same side must be used for all testings
The side used at Baseline must be used consistently throughout the study
To record a negative value for Normal Deviate, please enter a minus (-) sign in the first box

<table>
<thead>
<tr>
<th>Site</th>
<th>Test</th>
<th>JND</th>
<th>Normal Deviate</th>
<th>No Response (&gt;25 JND)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Great Toe</td>
<td>Vibration Detection Threshold</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Technician’s Signature: ______________________ Date: ____________
Neurologist’s Signature: ______________________ Date: ____________
NERVE CONDUCTION STUDIES (NCV’s)

*Reminder: Use the left side for all NCS assessments if at all possible. The same side must be used for all studies. The side used at Baseline must be used consistently throughout the study. If the test is not attempted, please indicate by checking the Not Done box for each test below.

Leg Examined: _____ Left _____ Right - only if left not available

Reminder: Leg temperature must be $\geq 31$ degrees C at start and completion of exam

Temperature of leg at start: _____ degrees C

Temperature of leg at completion: _____ degrees C

<table>
<thead>
<tr>
<th>Not Done</th>
<th>Site</th>
<th>Test</th>
<th>Results</th>
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<tbody>
<tr>
<td></td>
<td>Sural Sensory</td>
<td>Onset Distal Latency</td>
<td>milliseconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sural Sensory</td>
<td>NCV</td>
<td>meters/sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sural Sensory</td>
<td>Amplitude</td>
<td>microvolts</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peroneal Motor</td>
<td>Onset Distal Latency</td>
<td>milliseconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peroneal Motor</td>
<td>NCV</td>
<td>meters/sec</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peroneal Motor</td>
<td>Amplitude</td>
<td>millivolts</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tibial Motor</td>
<td>Onset Distal Latency</td>
<td>milliseconds</td>
<td></td>
</tr>
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<td>Tibial Motor</td>
<td>NCV</td>
<td>meters/sec</td>
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<td>Tibial Motor</td>
<td>Amplitude</td>
<td>millivolts</td>
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Technician’s Signature: _________________________ Date: ____________

Neurologist’s Signature: ______________________ Date: ____________
QUANTITATIVE SUDOMOTOR AXON REFLEX TESTING (QSART)

QSART is based upon the axon reflex sweat response mediated by the postganglionic sympathetic sudomotor axons activated by iontophoresis of acetylcholine. The stimulus is a constant electrical current and then the sweat response is recorded for a total of 10 min (1). Measurements are made on the foot, distal leg, proximal leg, and forearm. Measurements will be performed on the left side, unless physical abnormalities or other considerations require the measurement to be made on the right side. In peripheral neuropathy there is a decrease in the distal sweating response in the foot and distal leg, with preserved responses in the proximal leg and forearm. A reduced or absent sweat response indicates postganglionic sympathetic sudomotor failure. The QSART is sensitive and reproducible, and able to detect early neuropathy (1, 2).

The QSART equipment currently in use is provided by WR Electronics (Stillwater, MN) and consists of the following components:

1) Sudorometers
2) sweat capsules
3) constant current generator with a range of 0-3 mA
4) source of dried air
5) computer and data acquisition interface
6) acetylcholine
7) infrared lamps with controllers used for the resting sweating

The sweat capsules are applied to the following sites:

1) forearm
2) proximal leg
3) distal leg
4) foot

Normative data is available for each of these sites and is presented on the test form as 5th and 95th percentiles.

References


# QSART TEST REPORT

## Patient Information

<table>
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<th>Patient ID:</th>
<th>Test Information</th>
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<tbody>
<tr>
<td>Accession Number:</td>
<td>Test Type: Sweat Response</td>
</tr>
<tr>
<td>Gender:</td>
<td>Test Date:</td>
</tr>
<tr>
<td>Height:</td>
<td>Physician:</td>
</tr>
<tr>
<td>Weight:</td>
<td>Technician:</td>
</tr>
<tr>
<td>Date of Birth:</td>
<td></td>
</tr>
<tr>
<td>Visit Number:</td>
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<tr>
<td>Visit Remarks:</td>
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## Test Site

<table>
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<th>Baseline Rate</th>
<th>Response Latency</th>
<th>Total Volume</th>
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<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prox Leg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dist Leg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foot</td>
<td></td>
<td></td>
<td></td>
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Technician’s Signature: ______________________ Date: ________

Neurologist’s Signature: ______________________ Date: ________

## Male QSART responses: 5th, and 95th percentile values

<table>
<thead>
<tr>
<th>Sites</th>
<th>20 years</th>
<th>40 years</th>
<th>60 years</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>5%</td>
<td>95%</td>
<td>5%</td>
</tr>
<tr>
<td>Forearm</td>
<td>0.76</td>
<td>5.06</td>
<td>0.76</td>
</tr>
<tr>
<td>Proximal leg</td>
<td>1.27</td>
<td>4.54</td>
<td>0.93</td>
</tr>
<tr>
<td>Distal leg</td>
<td>1.37</td>
<td>5.27</td>
<td>0.98</td>
</tr>
<tr>
<td>Proximal foot</td>
<td>0.87</td>
<td>4.48</td>
<td>0.78</td>
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</table>

QSART, quantitative sudomotor axon reflex test.

## Female QSART responses: 5th, and 95th percentile values

<table>
<thead>
<tr>
<th>Sites</th>
<th>20 years</th>
<th>40 years</th>
<th>60 years</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5%</td>
<td>95%</td>
<td>5%</td>
</tr>
<tr>
<td>Forearm</td>
<td>0.20</td>
<td>2.78</td>
<td>0.20</td>
</tr>
<tr>
<td>Proximal leg</td>
<td>0.36</td>
<td>3.17</td>
<td>0.36</td>
</tr>
<tr>
<td>Distal leg</td>
<td>0.61</td>
<td>2.85</td>
<td>0.39</td>
</tr>
<tr>
<td>Proximal foot</td>
<td>0.23</td>
<td>3.07</td>
<td>0.18</td>
</tr>
</tbody>
</table>

QSART, quantitative sudomotor axon reflex test.


Modified: 27 January 2004
# MICHIGAN AUTONOMIC SYMPTOM SURVEY (MASS)

**Symptom/Health Problem**

<table>
<thead>
<tr>
<th></th>
<th>Q1a. Have you had any of the following health symptoms during the past 6 months?</th>
<th>Q1b. If you answered yes in Q1a, how much would you say the symptom bothers you?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 = Yes 0 = No</td>
<td>1 = Not At All 2 = A Little 3 = Some 4 = A Moderate Amount 5 = A Lot</td>
</tr>
<tr>
<td>1. Do you have lightheadedness?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>2. Do you have a dry mouth or dry eyes?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>3. Are your feet pale or blue?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>4. Are your feet colder than the rest of your body?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>5. Is sweating in your feet decreased compared to the rest of your body?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>6. Is sweating in your feet decreased or absent (for example after exercise or during hot weather)?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>7. Is sweating in your hands increased compared to the rest of your body?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>8. Do you have nausea, vomiting, or bloating after eating a small meal?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>9. Do you have persistent diarrhea (More than 3 loose bowel movements per day)?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>10. Do you have persistent constipation (less than 1 bowel movement every other day)?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>11. Do you have leaking of urine?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
<tr>
<td>12. Do you have difficulty obtaining an erection (men)?</td>
<td>1 0</td>
<td>1 2 3 4 5</td>
</tr>
</tbody>
</table>

**Number of Symptoms Reported:** __________ (sum of column A, 0 – 12 for men . 0 – 11 for women)

**Total Symptom Impact Score:** __________ (sum of column B, 0 – 60 for men and 0 – 55 for women)

Updated 5/7/2003

Modified: 27 January 2004
CARDIOVASCULAR REFLEX TESTING AND FUNCTION
**24 HOUR HEART RATE VARIABILITY (HRV) RECORDING**

24-h Holter monitoring. Ambulatory electrocardiograms of 24 h are recorded using a Syneflash digital holter recorder ELA Medical (Plymouth, MN). Data are stored on flash cards which are then downloaded to a computer for analysis. Each recording is divided into daytime and nighttime according to individually reported sleeping patterns and a heart rate trend curve. The typical heart rate pattern in the period from being awake to sleep and vice versa are included in daytime and nighttime periods.

**HEART RATE RANGE (HRR)**

Sinus Arrhythmia is the term used to describe the normal variability of the heart rate in response to respiration. The HRR measures the R-R ratio of the maximal to the minimal heart rate during respiration. The patient breathes in and out 6 times per minute and is instructed using one of the following methods: 1) the technician asks them to breathe in and out deeply so that one cycle of inspiration and expiration takes 10 sec., 2) the patient follows a sinusoidal computer generated wave or an oscillating bar. The five largest consecutive responses are averaged and a ratio of the R-R interval during inspiration compared to expiration is calculated. It takes about 30 sec. to reach stable values, so initial responses should not be used.

The HRR has proved to be a reliable test of autonomic function. It is usually not affected by the gender of the subject, time of day, or the amount of preceding rest in a relaxed subject. The rate of breathing has a profound effect on results, thus a standard rate of 6 breaths per minute is used. The HRR has an advantage compared to other methods in that the regularity of respiratory cycling has a negligible effect on the recorded result, compared to the E:I ratio and the mean circular resultant (MCR). Furthermore the time to reach a stable value is shorter (30 sec) compared to the MCR.

**VALSALVA RATIO (VR) AND TEST REPORT**

The Valsalva maneuver causes the heart rate to vary as a function of the changes in intrathoracic pressure and blood pressure. It consists of four phases, which can be helpful in assessing autonomic function. Phase one consists of a brief increase in blood pressure, without a change in heart rate. Phase two consists of falling blood pressure and an increase in heart rate as the strain continues. Phase three begins with the abrupt release of strain and results in a transient further dropping of blood pressure. Phase four consists of an increase in blood pressure and decrease of the heart rate below the baseline rate. Phases two and four are the most important for measuring reflex autonomic responses. The patient is instructed to blow into a mouthpiece (with an air leak to prevent closure of the glottis) connected to a pressure-monitoring device, and the patient maintains a pressure of 40 mm Hg for 10 to 15 seconds. This pressure is chosen because it produces the most reliable and reproducible responses. The heart rate is monitored for thirty seconds and the Valsalva ratio is calculated by comparing the highest heart rate to the lowest heart rate recorded during the fifteen to twenty seconds after the release of strain. The ratio is then expressed as a fraction comparing the lowest heart rate divided by the highest heart rate.
Peak heart rate is the maximum heart rate generated by the Valsalva Maneuver. Lowest rate is the lowest heart rate within 30 seconds of the peak heart rate.

Technician’s Signature: __________________________ Date: __________

Neurologist’s Signature: __________________________ Date: __________

Valsalva Ratio: 5th and 95th Percentiles

<table>
<thead>
<tr>
<th>Age Range</th>
<th>Male 2.5%</th>
<th>Male 97.5%</th>
<th>Female 2.5%</th>
<th>Female 97.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 - 39</td>
<td>1.5</td>
<td>3.0</td>
<td>1.41</td>
<td>3.0</td>
</tr>
<tr>
<td>40 - 59</td>
<td>1.4</td>
<td>2.6</td>
<td>1.47</td>
<td>2.9</td>
</tr>
<tr>
<td>60 - 80</td>
<td>1.2</td>
<td>2.23</td>
<td>1.36</td>
<td>2.7</td>
</tr>
</tbody>
</table>
24-hour time domain and spectral analysis

24-h time domain analysis. This is performed as previously reported (1). The 24-h ambulatory electrocardiograms are analyzed using commercially available software (ELA Medical). All periods with non-sinus beats or possible artifacts are omitted. Each QRS complex is detected and the RR intervals assessed. The mean RR interval and the difference between night and day heart rate are assessed in addition to: SDNN, standard deviation of all normal RR intervals; SDANN, standard deviation of the mean RR in all 5-min segments in a 24-h period; SDNN index, mean of SD's calculated on 5 min of RR intervals during a 24-h period; sNN6%, number of successive RR interval differences greater than 6%, standardized to 24 h; sNN50, number of successive RR interval differences greater than 50 ms, standardized to 24 h and HRV index, geometric index of total variability in a 24-h period.

24-h spectral analysis. The 24-h RR-interval file is divided into consecutive 5-min segments. RR intervals are time equidistantly sampled at 4 Hz using an IPFM (integral pulse frequency modulation) algorithm (2, 3) and linear interpolation is used for periods with invalid data. After editing, at least 70% of each 5-min segment should be available for HRV analysis to qualify. Files are down-sampled to 1 Hz, and high-pass filtered with a lower limit of 0.04 Hz because signals below this threshold are considered as noise (3). The 24-h power spectral analysis is based on a parametric, autoregressive method (3). Mean RR, distribution of power, and central frequency of LF and HF components are assessed, and coefficient of component variance for HFpower (CCVHF) and coefficient of component variance for LFpower (CCVLF) is calculated. Results were shown as 24-h, day and night values.

References:


DETERMINATION OF 24 HOUR AMBULATORY BLOOD PRESSURE AND LV FUNCTION

LV function:

Echocardiography: 2D echocardiography, color Doppler M-mode and measures of LV inflow propagation velocity (Vp) and pulsed tissue Doppler parameters reflecting (peri-)mitral annular velocity are utilized to classify the stages of diastolic function (1, 2). All recordings and measurements are obtained by the same observer according to the recommendations of the American Society of Echocardiography (3) and previously described protocols (1, 2, 4). LV systolic and diastolic dysfunction are evaluated using well-standardized diagnostic criteria (1, 2, 4-6). Nagueh and associates have reported that pulsed tissue Doppler measures of early diastolic mitral annular velocity (Em) behave as a relatively pre-load-independent index of LV relaxation (4) By using a ratio of transmural E velocity to Em, these workers were able to estimate mean pulmonary capillary wedge pressure (r=0.87 p<0.001).An E/Em ratio < 10 was associated with normal LV filling (left atrial or pulmonary capillary wedge) pressure. Furthermore, color Doppler M mode imaging has been used to record and measure LV inflow propagation velocity (Vp). This parameter is generally reduced (< 45 cm/sec.) in all stages of abnormal LV diastolic function, as is Em In a recent report, 67 patients with acute myocardial infarction had measurements of Em, Vp and transmural peak-E wave velocity measured post-myocardial infarction. Cox proportional hazards analyses identified E/ Vp >1.5 [relative risk, 12.4 (95% confidence interval, 4.1-37.30)], E/ Em > 10 [relative risk 11.5 (95% confidence interval, 3.8-34.7)], and Killip class > II [relative risk, 7.8 (1.6 - 40.4)], to be good predictors of the composite end-point of cardiac death and readmission because of heart failure (7). Of additional interest, the newer diastolic function measures, Em and Vp shared a good correlation (r=0.72, p<0.0001).

24 h ambulatory BP measuring:

24 h ambulatory BP measuring is performed with a portable oscillometric recorder (Spacelabs 90207, Redmond, WA) as previously described (8). BP is measured at 20-minute intervals from 6am until midnight and at 30-minute intervals from midnight to 6am. Each recording is divided into daytime and nighttime according to individually reported sleeping patterns and a heart rate trend curve. The mean values for all the systolic and diastolic readings of the daytime and nighttime periods are recorded.


AUTONOMIC FUNCTION TESTING
POSITRON EMISSION TOMOGRAPHY (PET) METHODOLOGY

a. PET Assessment of Cardiac Sympathetic Innervation with $^{11}$C]HED and PET Measurement of Myocardial Blood Flow with $^{13}$N]ammonia

Tracer Syntheses. $^{11}$C]meta-hydroxyephedrine ($^{11}$C]HED) is synthesized by direct N-methylation of (−)-metaraminol using $^{11}$C]methyltriflate as the labeling reagent (1, 2). The final product has radiochemical purity >98% and specific activity in the range 500-1,500 Ci/mmol. $^{13}$N]ammonia is rapidly synthesized in-target by proton irradiation of a water and ethanol mixture under hydrogen overpressure (3).

Data acquisition. The subject’s heart rate and blood pressure will be monitored continuously during the examination and recorded at 1 min intervals. An injection port will be established by introducing a 22-gauge intravenous cannula into the antecubital vein. A 2 mCi dose of $^{13}$N]ammonia will be injected to facilitate positioning of the patient’s heart in the field of view of the Siemens/ECAT Exact HR+ PET scanner (CTI, Knoxville, TN/Siemens Medical Systems, Inc., Hoffman Estates, IL). This instrument simultaneously acquires 63 cross-sectional images covering a 15.5 cm field of view. A germanium-68 transmission scan is acquired for attenuation correction during image reconstruction.

$^{13}$N]ammonia scan. The first PET scan will assess resting perfusion with $^{13}$N]ammonia. The resting perfusion scan will be started by initiating a 15 min dynamic PET acquisition sequence as 20 mCi of $^{13}$N]ammonia are intravenously injected. The dynamic acquisition sequence will consist of 20 image frames (frame rates 12×10 s, 6×30 s, 2×300 s). Following the resting perfusion study, a 50 min delay will be imposed to allow myocardial levels of $^{13}$N]ammonia to decay to <3% of its initial activity.

$^{11}$C]HED scan. A 60 min dynamic PET image acquisition sequence will be started as 20 mCi of $^{11}$C]HED are injected as a slow bolus over 30 s. The dynamic acquisition sequence will consist of 23 image frames (frame rates 12×10 s, 2×30 s, 2×60 s, 2×150 s, 2×300 s, 2×600 s, 1×1200 s).

Image reconstruction. After the studies, the emission data will be corrected for attenuation and reconstructed using iterative reconstruction (4 iterations, 8 subsets, 7 mm Gaussian filter). Siemens/CTI image analysis software will be used to reorient and reslice the acquired transaxial PET data into short-axis view data sets (slice thickness 0.8 cm) for subsequent quantitative analyses.

Data analysis.

Resting perfusion estimates from $^{13}$N]ammonia data. Regional myocardial blood flow will be estimated from the measured $^{13}$N]ammonia kinetics using a previously validated compartmental modeling approach (4, 5). Eight short-axis slices covering the left ventricle (LV) from apex to base will be used for quantitative analyses. For each short-axis slice, an automated contour detection algorithm will be used to divide the LV wall circumferentially into 12 angular regions (‘sectors’) using the final image frame of the dynamic data set. In order to allow the compartmental model to account for partial volume and spillover effects, the radial positions of these sectors are intentionally offset into the LV chamber to add signal from radioactivity in blood to the measurements (6). After applying corrections for patient motion during the scan, the concentrations of $^{13}$N]ammonia in each of the 96 sectors as a function of time (‘time-activity curve’) will be determined and stored for further analysis. The blood radioactivity
concentration as a function of time will be obtained directly from the PET images by placing a small region of interest (5 × 5 pixels) over the LV chamber in the most basal short-axis slice, and used as the input function for the compartmental model. A non-linear least-squares fitting algorithm will use the time-activity curves and input function to estimate myocardial blood flow (MBF) for each sector (4, 5). Regional mean MBF values will be determined for 9 LV regions (septal, inferior, lateral, and anterior segments for proximal and distal regions, plus one apical region). Proximal values will be obtained by averaging MBF values for the given segment (e.g., septal) from sectors in the 3 most basal short-axis slices. Distal values will be obtained using the MBF values from the next 3 short-axis slices, while the apical value will be obtained by averaging together the MBF values for all sectors values in the two most apical slices.

\[ ^{11}\text{C}]\text{HED retention.} \] Regional neuronal retention of \(^{11}\text{C}]\text{HED will be quantified using a ‘retention index’ (7). Eight short-axis slices encompassing the left ventricle from apex to base will be selected. For each short-axis slice, an automated contour detection algorithm will be used to divide the LV wall circumferentially into 60 angular regions (‘sectors’). The myocardial concentration of \(^{11}\text{C}]\text{HED in the final image frame will be determined for each of the 480 sectors (8 slices × 60 sectors/slice) and stored for further analysis. Blood radioactivity data as a function of time (\(C_p(t)\)) will be obtained directly from the PET images by placing a small region of interest (5 × 5 pixels) over the left ventricular chamber in the most basal short-axis slice. A ‘retention index’ (RI; mL blood/min/mL tissue) will be generated for each sector by normalizing the measured tissue concentration of \(^{11}\text{C}]\text{HED in the final image frame (\(C_{\text{tissue}}(T_1:T_2)\)) to the time integral of the arterial blood radioactivity curve:

\[
RI = \frac{C_{\text{tissue}}(T_1:T_2)}{\int_0^{T_2} C_p(t)dt}
\]

The calculated RI data will be displayed as polar coordinate maps (‘polar maps’), with the apex depicted at the center and the base as the outer-most ring. RI data will be analyzed in two ways. First, regional mean RI values will be determined for the same 9 left ventricular regions defined for the MBF data, as described above. Second, the heterogeneity of \(^{11}\text{C}]\text{HED retention in each subject will be assessed by regionally comparing RI values to a database of RI values from normal subjects using a z-score analysis (7). Sectors that have a z-score greater than 2.5 (i.e., the patient’s RI value is more than 2.5 standard deviations away from the mean RI value of the normal subjects for that sector) will be defined to be ‘abnormal’. An ‘extent’ measure of \(^{11}\text{C}]\text{HED retention heterogeneity will be expressed as the percentage of all sectors in the subject’s polar map that are abnormal (i.e., the fraction of sectors that had z-scores > 2.5).

References.


PET evaluation of myocardial blood flow detects perfusion abnormalities with high diagnostic accuracy (1, 2). The time course of tracer distribution in myocardium and blood was defined by dynamic image acquisition using a previously described method (2, 3). After the transmission scan, 20 mCi N-13 ammonia was administered into a peripheral arm vein over 30 s. Dynamic scan acquisition for this study was initiated with varying frame duration (12 X 10 s, 6 X 30 s, 2 X 300 s). After the baseline N-13 ammonia study, 50 min was allowed for decay of the N-13 ammonia to <3% of its initial activity. Intravenous adenosine was infused into a peripheral vein over 6 min at a dose of 140 µg/kg body weight per min to achieve maximal coronary vasodilation (3, 4). At 3 min, 20 mCi N-13 ammonia was injected over 30 s and imaged for 3 to 18 minutes using the same imaging sequence as the at rest study. Blood pressure and heart rate were measured at baseline, every minute during adenosine infusion, and then at 10 and 18 min. Pressure-rate product was calculated as heart rate times systolic blood pressure divided by 100.

Twelve myocardial regions per plane were defined in the eight planes in the last time frame of the dynamic study sequence. After correction for subject motion, the dynamic image set was sampled and 96 (8 planes X 12 regions) time-activity curves were stored for further analysis. Arterial input function was determined from circular regions at the two most basal planes corresponding to the large blood pool at the center of the largest left ventricle diameter of the resliced images. A previously validated tracer kinetic model for N-13 ammonia (3) was used to calculate myocardial blood flow in ml/g per min for the nine regions studied. This three compartment model represents vascular and extravascular N-13 ammonia as well as metabolically trapped N-13, which comprises glutamine (3). In this model, the delivery and extraction of N-13 ammonia (which is >90% (3)) is used as an estimate of myocardial blood flow, and the flow values generated are in close agreement with those obtained by invasive techniques (3).

References:

MANUAL OF OPERATIONS

CHRONOTROPIC RESPONSE TO ISOPROTERENOL (ISP) INFUSION

a. Study Protocol The chronotropic response to β-agonist stimulation is determined by measuring the heart rate response to bolus intravenous infusions of isoproterenol (ISP) administered 5 minutes following 2 mg of atropine. ISP is given at doses of 1, 2, 4, 8, 12, and 16 µg in consecutive order allowing time to return to baseline heart rate following each dose. The maximal ISP-mediated heart rate response allowed is 80% of the subject's predicted maximal heart rate (either from their exercise treadmill test prior to study entry or from an estimate of 220 - age). No higher ISP dose is given if this heart rate is achieved or if any significant arrhythmias develop. Heart rate is continuously monitored throughout the study.

b. Data Analysis and Interpretation The heart rate is monitored for 12 second intervals at 0.5, 1, 1.5, 2, 3, 4, and 5 minutes following the ISP injection. The peak heart rate is determined and the delta heart rate calculated from the preceding baseline heart rate prior to each ISP infusion. Chronotropic response to ISP is analyzed as the change in heart rate from baseline at each ISP dose to generate a dose-response relationship. The effect of ISP on delta heart rate is compared between groups using repeated measures ANOVA of these data. An alternative analysis is carried out to determine the I25, the dose of ISP required to produce a 25 beat per minute increase in heart rate above baseline, from each individual's dose-response relationship. This provides a single index of sensitivity to be utilized in the multiple regression analyses. We propose that sensitivity to ISP will be unchanged in healthy diabetic subjects, consistent with a lack of adrenergic supersensitivity.

EFFECT OF CLONIDINE ON THE RETENTION OF LV $[^{11}\text{C}]$HED

Study Protocol.

a. Clonidine infusion: Dynamic PET scans with $[^{13}\text{N}]$ammonia and $[^{11}\text{C}]$HED will be acquired before and after the administration of clonidine using the PET methodology described above. Clonidine will be administered via an intravenous infusion initially at a dose of 10µg/kg over 5 minutes, followed by 5 µg/kg/h for the following hour as previously reported (1).

Data Analysis and Interpretation:

Myocardial blood flow. Regional resting myocardial blood flow will be estimated from the $[^{13}\text{N}]$ammonia kinetics as described above. Quantitative regional assessment of resting myocardial perfusion will exclude changes in myocardial perfusion as the cause of altered $[^{11}\text{C}]$HED retention in these subjects. Sympathetic stimulation can result in vasoconstriction via the activation of postjunctional α1-and postjunctional α2 adrenoreceptors (2). Stimulation of prejunctional a2 adrenoreceptors inhibits NE release (3) Therefore clonidine is expected to reduce sympathetic cardiac tone (4) and attenuate the release if NE from presynaptic terminals (5) and reduce circulating NE and epinephrine levels (6) reduce heart rate, BP, heart rate variability, systemic vascular resistance, renin-angiotensin II vasoconstriction and aldosterone secretion (7, 8). In our healthy diabetic subjects with normal sympathetic tone, we expect the net response of clonidine to be depressor. The net effect of clonidine on myocardial perfusion (and hence delivery of $[^{11}\text{C}]$HED) is difficult to predict since clonidine has been reported to both vasodilate or constrict coronary blood vessels, dependent upon on mode of administration and the dose utilized (9). Clonidine has been reported to reduce myocardial contractility and increase vascular capacitance, which may increase subendocardial blood flow (9, 10). In any event, the retention of $[^{11}\text{C}]$HED will be corrected
for blood flow (a quantitative N-13 ammonia study is performed with each $^{11}$C[HED scan), and so any effects of clonidine on myocardial perfusion will be taken into account.

$^{11}$C[HED Retention. Regional retention of $^{11}$C[HED will be quantitatively assessed by calculation of a ‘Retention Index’ (RI) as described above. The RI value reflects the ability of sympathetic neurons to take up and store NE. We propose that a clonidine-mediated reduction of sympathetic outflow will decrease synaptic NE and be associated with enhanced retention of LV $^{11}$C[HED to levels in excess of untreated nondiabetic control values. As stated above $^{11}$C[HED retention will be corrected for changes in myocardial perfusion.

References:
SYMPATHETIC NERVOUS SYSTEM TONE ASSESSMENT
SYSTEMIC AND RENAL SNS \( ^3\)H-NE KINETICS

Protocol: Subjects will be fasted from midnight, abstain from caffeine and nicotine containing products and samples taken at 8 am the following day. Because of Dr. Supiano’s collaboration in this project, we have the advantage of being able to assess sympathetic activity using a well validated kinetic analysis method which allows calculation of norepinephrine release into the extravascular compartment. Since norepinephrine measurements in plasma provide only an indirect index of sympathetic activity, Dr. Supiano and his colleagues have used radioisotope dilution studies to permit the independent identification of the rates of NE appearance into and clearance from the circulation. To obtain a more comprehensive view of NE metabolism the methods of compartmental analysis using \( ^3\)H-NE infusion and decay data have been applied to estimate NE kinetics in an inaccessible extravascular compartment (1).

From these studies the rate of NE release into the extravascular compartment (NE\(_2\)) has been used as a more proximate index of the rate of NE release from sympathetic nerve terminals. In addition to an estimate of the rate of NE release into the extravascular compartment, the two-compartment model provides estimates of the volume of distribution of NE, NE mass in each compartment, flux rates for NE between compartments, and its metabolic clearance rate from plasma (1). Prior studies have demonstrated superiority of the kinetic method when alterations in norepinephrine clearance occur (2).

On the day of study, subjects will report to the angiography suite for placement of a renal vein catheter through the femoral vein. Upon completion of renal venous catheterization, subjects will be transported to the General Clinical Research Center. Upon arrival at the GCRC, a brachial arterial catheter will be placed. Whole body norepinephrine kinetics and renal venous norepinephrine spillover will then be determined.

\( ^3\)H-Norepinephrine will be infused for one hour at a rate of 0.35 \( \mu \text{Ci}/\text{min/m}^2 \). Arterial and renal venous plasma samples will be obtained after 40, 50 and 60 minutes of infusion. Arterial samples will also be obtained 1, 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 minutes after stopping the infusion. In order to determine renal blood flow, a priming dose of 8 mg/kg of 20% PAH will be given followed by a continuous infusion at a rate calculated to maintain plasma concentration at approximately 1.5 mg/dl. PAH and \( ^3\)H-Norepinephrine will be given concurrently. Arterial and renal venous plasma samples for measurement of PAH will be obtained after 20, 25 and 30 minutes for quantitation of renal blood flow. Renal venous norepinephrine spillover and norepinephrine release into the extracellular compartment will be calculated as described in section 6 (analytic methods).

i. Renal plasma flow: Plasma samples for determination of PAH will be immediately centrifuged at 3000 rpm for 10 minutes at 4°C. Plasma will be separated, placed on ice and stored at -70°C before assay. PAH will be measured spectrophotometrically as described by Brun (3, 4). Renal plasma flow (ml/min) will be calculated as:

\[
\text{PF} = \frac{\text{Infusion rate}}{\text{(PAHa - PAHv)}}
\]

where PF is renal plasma flow and PAHa and PAHv are arterial and venous steady state concentrations of PAH.

ii. Renal NE spillover: Blood samples will be centrifuged at 4°C to obtain plasma which is stored at –70°C until assay. Plasma NE will be determined by a single isotope derivative radioenzymatic assay; each subject’s samples will be measured in the same assay. The radioenzymatic assay technique is
described in the Chemistry Core application. 3H-NE concentrations will be determined following alumina extraction of plasma (recovery approximately 60%) within 24 h of the study. Liquid scintillation counting of the radiolabeled catecholamines is then performed to determine 3H–NE concentration. Calculation of renal NE spillover will be determined as described by Vaz et al.:

\[
\text{NE Spillover} = (\text{Nev} - \text{Nea}) + (\text{Nea} \times \text{NEex}) \times \text{PF}
\]

where Nea and Nev are the arterial and venous plasma concentrations of NE, NEex is the fractional extraction of tracer NE across the organ, and PF is the renal plasma flow. NEex is calculated as follows:

\[
\text{NEex} = \frac{1 - \text{Hepatic vein 3H-NE}}{\text{Arterial 3H-NE}}
\]

iii. NE kinetics: A comprehensive description of NE kinetics will be derived from the compartmental analysis approach using the two-compartment model as described by Linares et al. (1) Mathematical modeling will be performed on a VAX 11/730 computer (Digital Equipment Corporation, Maynard, MA) using the SAAM29 and CONSAM programs (5, 6). The modeling involves determining a simultaneous fit of all the plasma NE and 3H-NE data by solving differential equations for both the endogenous trace (plasma NE) and tracer (3H-NE) systems by the method of weighted non-linear least squares. Parameters which are estimated by SAAM29 are the fractional transfer rate coefficients, the volume of distribution of NE in compartment 1 (V₁, liters), and the steady-state input rate into compartment 2, (NE₂, nmol/min/m²). Additional measures which will be available include the mass of NE in each compartment, the flux rates of NE between compartments, and the metabolic clearance rate of NE from plasma. The primary outcome measure of renal SNS activity is the rate of NE release into the extravascular compartment (NE2). We anticipate that this measure of renal SNS activity will be unchanged in healthy diabetic subjects. Future studies will also quantifying cardiac NE mass transport, by measuring arterial and coronary sinus plasma levels of infused [3H]NE and endogenous NE.

To assess responsiveness to α-adrenergic stimulation platelet α-adrenergic receptor-adenylate cyclase studies are performed. Platelet membrane lysates are prepared from 150 to 200 ml whole venous blood as previously described (7, 8). An aliquot of the freshly prepared membrane lysate is used for adenyl cyclase assays. The remainder of the sample is quick frozen in liquid nitrogen and stored at -70°C; radioligand binding studies are performed within two weeks of preparation as previously described (8). Platelet membrane basal adenyl cyclase activity is determined using freshly prepared membranes in buffer as previously described (7, 8).

References


PLATELET $\alpha$-ADRENERGIC RECEPTOR-ADENYLATE CYCLASE STUDIES:

$[\text{methyl}^{3}H]$Yohimbine, a specific $\alpha$-adrenergic receptor antagonist, is used to determine platelet membrane total $\alpha$-2 (low + high affinity) receptor density ($B_{\text{max}}$ from Scatchard binding plot). An unweighted nonlinear least squares fit of the specific binding data is made to a hyperbolic binding curve, $Y= (A * X)/(B + X)$, where $Y$ is specific binding in fmol/mg protein, $X$ is free $^{3}H$yohimbine concentration in nM, $A$ is the maximum receptor density, $B_{\text{max}}$, and $B$ is the apparent dissociation constant, $K_{d}$ (InPlot 3.1, GraphPAD Software, San Diego, CA). $^{3}H$bromoxidine is used to determine platelet membrane $\alpha$-2-adrenergic receptor agonist binding properties ($B_{\text{max}}$, receptor density) and will determine the number of $\alpha$-2 receptors in the high affinity (coupled) state. The maximum receptor density and apparent dissociation constant for $^{3}H$bromoxidine are determined from the unweighted least squares fit of the specific binding data as described above for $^{3}H$yohimbine. A limitation of this approach is that peripheral blood cell receptors may or may not parallel vascular or other sites of $\alpha$-adrenergic receptor properties and function. However, this approach seems reasonable, since the aim of this application is to identify and validate readily accessible peripheral biomarkers for microvascular complications.

**Adenylyl cyclase assay:** The concentration of cAMP in the zero time basal activity condition is subtracted from the 15 min basal, NaF-stimulated, and EPI-inhibition conditions so that the values will reflect only the accumulation of cAMP over the 15 min incubation period. The extent of EPI-mediated inhibition at each EPI concentration is determined as the percentage decrease in cAMP accumulation in the presence of EPI and NaF from the NaF-stimulated activity without EPI. Potentially, the identification of augmented NE responses in response to cold pressor testing (see Preliminary Studies) could be secondary to dysinhibition of the SNS secondary to ganglionic NO depletion (1), a reduction of peripheral $\alpha$-2 adrenergic receptor density, or a decreased number of $\alpha$-2 adrenergic receptors in the high affinity (coupled) state. If resting SNSa is decreased (but augmented response i.e. NE release- to sympathetic stimuli) one might predict an upregulation in $\alpha$-2 adrenergic receptor responsiveness. This has been demonstrated in vivo in response to treatment with guanadrel (resulting in 50% decrease in SNSa) for venous (2) and arterial (3) responses. A decreased response in platelet $\alpha$-2 adrenergic receptors may not be identified by a decrease in total receptor number (as assessed by yohimbine), but should be identified by a decrease in coupled, high affinity receptors (as assessed by bromoxidine) as well as enhanced EPI-mediated inhibition of adenylyl cyclase. We anticipate that the total $\alpha$-2 adrenergic receptor density will be unchanged in early CAN, but that adrenergic sensitivity will be increased reflecting NO depletion and early subclinical sympathetic denervation.

**References:**
